Functional proteomics of adenosine triphosphatase system in the rat striatum during aging☆

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Abstract
The maximum rates of adenosine triphosphatase (ATPase) systems related to energy consumption were systematically evaluated in synaptic plasma membranes isolated from the striata of male Wistar rats aged 2, 6, 12, 18, and 24 months, because of their key role in presynaptic nerve ending homeostasis. The following enzyme activities were evaluated: sodium-potassium-magnesium adenosine triphosphatase (Na⁺, K⁺, Mg²⁺-ATPase); ouabain-insensitive magnesium adenosine triphosphatase (Mg²⁺-ATPase); sodium-potassium adenosine triphosphatase (Na⁺, K⁺-ATPase); direct magnesium adenosine triphosphatase (Mg²⁺-ATPase); calcium-magnesium adenosine triphosphatase (Ca²⁺, Mg²⁺-ATPase); and acetylcholinesterase. The results showed that Na⁺, K⁺-ATPase decreased at 18 and 24 months, Ca²⁺, Mg²⁺-ATPase and acetylcholinesterase decreased from 6 months, while Mg²⁺-ATPase was unmodified. Therefore, ATPases vary independently during aging, suggesting that the ATPase enzyme systems are of neuropathological and pharmacological importance. This could be considered as an experimental model to study regeneration processes, because of the age-dependent modifications of specific synaptic plasma membranes. ATPases cause selective changes in some cerebral functions, especially bioenergetic systems. This could be of physiopathological significance, particularly in many central nervous system diseases, where, during regenerative processes, energy availability is essential.

Key Words: ATPase; synaptic plasma membranes; aging; striatum; functional proteomics

INTRODUCTION
Age-related changes in brain energy metabolism have a fundamental role in determining the possibility of recovery of cerebral tissue after physiopathological and regeneration conditions in terms of producing and consuming energy-rich compounds[1-4]. Substantial evidence indicates that bioenergetic dysfunction and mitochondrial impairment contribute directly to the pathogenesis and recovery of numerous neurodegenerative disorders. Therapeutic intervention aimed at ameliorating this cellular energy deficit and/or improving mitochondrial function may be useful[1-2].

The striatum is particularly susceptible to the aging process, as highlighted by Parkinson’s disease (PD), an age-related neurodegenerative disorder marked by the neuronal loss and/or dysfunction of dopaminergic nigro-striatal neurons. PD ultimately leads to suppressed activation and/or function of neurons in the motor cortex[9].

Mitochondrial dysfunction and oxidative stress are believed to be central to the pathogenesis of PD[10-12]. A number of studies have indicated that deficiencies exist within complex I of the mitochondrial electron transport chain[10-12] and aging seems to produce specific changes in oxidative metabolism enzymes that may be causative of neurological disorders in selective brain areas.

Thus, we decided to study these changes in oxidative metabolism enzymes in the striatum, and not in a whole brain tissue preparation. Age-induced modifications in energy-consuming systems of adenosine triphosphatase (ATPases) during aging at systematic time intervals (2, 6, 12, 18 and 24 months) were assessed by evaluating the catalytic properties of enzyme systems directly in their in vivo subcellular locations. These enzymes are responsible for adenosine triphosphate (ATP) utilization and their correct functioning is of physiopathological significance[10, 14] because of their modulation of presynaptic nerve ending homeostasis[15-16]. Sodium-potassium adenosine triphosphatase (Na⁺, K⁺-ATPase) is located in synaptic plasma membranes. It functions as an electrogenic Na⁺/K⁺-pump to maintain low intracellular Na⁺ concentration and Na⁺ and K⁺ gradients, which modulate the resting transmembrane potential, some
postsynaptic activities, and transmitter turnover\textsuperscript{[16]}.

Calcium-magnesium adenosine triphosphatase (Ca\textsuperscript{2+}-
Mg\textsuperscript{2+}-ATPase) helps to maintain the homeostasis of the
intracellular Ca\textsuperscript{2+} that initiates the interaction between
ATP and ATPases to promote the attachment of
secretory granules to plasmalemma and the secretion of
their neurotransmitters at nerve terminals\textsuperscript{[16]}.

The ectoenzyme, magnesium adenosine triphosphatase
(Mg\textsuperscript{2+}-ATPase) has externally oriented active sites on
synaptic plasma membranes and is involved in the
hydrolysis of ATP to adenosine. In contrast, the enzyme
located in synaptic vesicles is involved in the turnover of
different transmitters\textsuperscript{[16]}.

These enzyme activities were assayed in a purified
synaptic plasma membranes subcellular fraction, to take
into consideration the actual in vivo localization of the
ATPase systems.

RESULTS

Quantitative analysis of experimental animals

The number of 2-, 6-, 12-, 18- and 24-month-old male
Wistar rats are shown in Table 1, and there was no rat
dropping out during the experimentation.

Enzyme activities in the rat striatum

The activities of Na\textsuperscript{+}, K\textsuperscript{+}, Mg\textsuperscript{2+}-ATPase,
ouabain-insensitive Mg\textsuperscript{2+}-ATPase, “direct” Mg\textsuperscript{2+}-ATPase,
Na\textsuperscript{+}, K\textsuperscript{+}-ATPase, Ca\textsuperscript{2+}, Mg\textsuperscript{2+}-ATPase and
acetylcholinesterase (for details see Materials and
Methods section) were assayed spectrophotometrically
on synaptic plasma membranes purified from the striata
of male Wistar rats aged 2, 6, 12, 18 and 24 months
(Table 1).

The value of enzyme activities from rats aged 2 months
were chosen as the reference value to evaluate the effect
of aging per se, and the significances reported in Table 1
refer to this “control” value.

During aging, the Na\textsuperscript{+}, K\textsuperscript{+}, Mg\textsuperscript{2+}-ATPase significantly
decreased at 18 and 24 months (P < 0.05); while the
Mg\textsuperscript{2+}-ATPase ouabain-insensitive activity significantly
diminished from 12 months of age (P < 0.05) and
remained lower at 18 and 24 months compared with
control values.

Similarly to the Na\textsuperscript{+}, K\textsuperscript{+}, Mg\textsuperscript{2+}-ATPase, the Na\textsuperscript{+},
K\textsuperscript{+}-ATPase activity also decreased, starting at 18 and
24 months of age, but showing a more significant
decrease (P < 0.01).

In contrast to the Mg\textsuperscript{2+}-ATPase ouabain-insensitive
activity, the “direct” Mg\textsuperscript{2+}-ATPase activity did not show a
different significance at any age compared with control
values. It should be noted that the “indirect”
ouabain-insensitive and the “direct” activities show very
similar values, because the two assay methods
evaluated the activity of the same enzyme system.

Ca\textsuperscript{2+}, Mg\textsuperscript{2+}-ATPase activity showed a biphasic trend,
decreasing at 6 months (P < 0.001) and increasing at
12 months of age, although still lower than that at
2 month-value (P < 0.01). The activity decreased again
at 18 and 24 months (P < 0.001).

Acetylcholinesterase activity was diminished at all the
considered ages, with respect to the 2 month-value. The
values reported in Table 1 show that the activity primarily
diminished at 6 and 12 months of age (by 35%);
thereafter, the activity diminished further at 18 and
24 months of age, by about 57%.

Finally, protein concentration and content increased
during aging. Protein concentration reached its highest
value at 18 months of age, showing an increase of 251%;
at the same age, protein content was also at its highest
level, increasing by 248% with respect to the
2 month-value, these two parameters being tightly
connected.

DISCUSSION

The characteristic decline of basal metabolic rate and
performance in energy-requiring homeostatic processes
during aging suggests a fundamental role of energy
metabolism in aging; therefore, much attention has been
paid to mitochondria, because these organelles produce
the chemical energy required to meet neuronal functional
requirements\textsuperscript{[17]}.

Table 1 Specific enzymatic activities (µmoles substrate transformed/min/mg of synaptic plasma membranes protein) (acetyl-
cholinesterase) and (µmoles P released/h/mg of synaptic plasma membranes protein) (ATPases) of indicated enzymes assayed
on synaptic plasma membranes from striatum of rats aged 2, 6, 12, 18, and 24 months

| Enzyme                                      | 2 months (n = 7-9) | 6 months (n = 8-14) | 12 months (n = 8-12) | 18 months (n = 7-8) | 24 months (n = 9) |
|---------------------------------------------|-------------------|---------------------|---------------------|---------------------|-------------------|
| Na\textsuperscript{+}, K\textsuperscript{+}, Mg\textsuperscript{2+}-ATPase | 14.46±1.10        | 10.79±1.25          | 9.57±0.51           | 7.67±0.58\textsuperscript{a} | 7.76±1.09\textsuperscript{a} |
| Mg\textsuperscript{2+}-ATPase ouabain insensitive | 4.78±0.47         | 3.45±0.22           | 3.14±0.37\textsuperscript{b} | 2.75±0.26\textsuperscript{b} | 3.11±0.36\textsuperscript{b} |
| Na\textsuperscript{+}, K\textsuperscript{+}-ATPase | 8.78±0.92         | 7.00±0.44           | 8.59±0.45           | 5.12±0.43\textsuperscript{c} | 4.67±0.43\textsuperscript{c} |
| Mg\textsuperscript{2+}-ATPase “direct” | 4.14±0.66         | 3.43±0.41           | 3.11±0.31           | 3.10±0.27          | 3.28±0.39          |
| Ca\textsuperscript{2+}, Mg\textsuperscript{2+}-ATPase | 8.90±1.31         | 7.32±0.31           | 4.08±0.44\textsuperscript{d} | 2.88±0.43\textsuperscript{d} | 2.43±0.46\textsuperscript{d} |
| Acetylcholinesterase | 0.200±0.011       | 0.121±0.009\textsuperscript{e} | 0.129±0.006\textsuperscript{e} | 0.086±0.004\textsuperscript{e} | 0.087±0.005\textsuperscript{e} |
| Protein concentration | 0.55±0.04         | 0.85±0.04           | 0.98±0.07\textsuperscript{f} | 1.95±0.08\textsuperscript{f} | 1.76±0.10\textsuperscript{f} |
| Protein content | 0.166±0.013       | 0.247±0.011\textsuperscript{e} | 0.300±0.020\textsuperscript{e} | 0.578±0.023\textsuperscript{e} | 0.535±0.030\textsuperscript{e} |

Protein concentration (expressed as mg/mL) and content (multiplying for final volume of the sample, expressed as mg) are indicated. Data are expressed as mean ± SEM, and compared using two-way analysis of variance, followed by Bartlett’s test and Tukey’s or Dunnett’s tests. \( ^{a} P < 0.05, ^{b} P < 0.01, ^{c} P < 0.001, \) vs. 2 month-old rats. ATPase: Adenosine triphosphatase.
A complete discussion of the complex relationships among catalytic enzyme activities up to 24 months of age is an intriguing problem. For the sake of simplicity, in our series of previous studies\[1-3\], we pointed out that the theoretical implications concerning energy yield and ATP synthesis suggest that specific biochemical situations take place at each single age, requiring a continuous energy supply to be maintained at a level compatible with life and that this observation is of pharmacological significance\[1-3\].

Interestingly, to date, systematic information about age-related modifications of the ATPase enzyme systems are quite scarce, in spite of the fact that these enzymes are involved in many important cellular processes. In particular, they link energy metabolism with electric activity and neuromodulation\[14, 18\]; enzyme catalytic activities of ATPases are related to energy utilization, and are used for neuronal ionic homeostasis and neurotransmitter release.

The present results agree with those in our previous studies on different functional areas of the rat brain\[19-21\], suggesting that the modifications of ATPases during aging exert important physiopathological changes on the responsiveness of the nerve endings to noxious stimuli. The Na$^+$, K$^+$-ATPase is considered to be part of the Na$^+$, K$^+$, Mg$^{2+}$-ATPase complex\[22\], as confirmed by the present results: ouabain-insensitive Mg$^{2+}$-ATPase activity is assayed by blocking the Na$^+$, K$^+$-ATPase portion of the complex, and the difference between Na$^+$, K$^+$, Mg$^{2+}$-ATPase and ouabain-insensitive Mg$^{2+}$-ATPase activities is very similar to the values obtained for Na$^+$, K$^+$-ATPase activity assayed with the “direct” method (Table 1). On the other hand, the Mg$^{2+}$-ATPase ouabain-insensitive activity is very similar to the “direct” Mg$^{2+}$-ATPase activity, implying that these are actually the same enzymatic system.

Na$^+$, K$^+$-ATPase, the so-called “sodium pump”, is an integral membrane enzyme responsible for the pumping function that is essential in restoring ion-gradients across plasma membranes in electrically excitable tissues and for the maintenance of neurotransmitter turnover.

Therefore, the assessment of this enzyme activity has been proposed as a potential indicator for membrane structure and function\[23\]. The marked effect of aging on this enzyme activity could be related to the gender of the utilized animals. As shown by Fraser et al\[24-26\], the activity of Na$^+$, K$^+$-ATPase in rat brain synaptosomes is greater in male than in female rats, and these differences are even more significant in aging rats (12- and 19-month-old), probably because of the lack of reproductive hormones in aged female animals. Interestingly, the Na$^+$, K$^+$-ATPase is activated in age-related physiopathological processes, confirming that this enzyme retains its functionality in 2-year-old rats. In a study on ATPase enzyme activities of hippocampal synaptic plasma membranes\[25\], after 15 minutes of complete cerebral ischemia and different reperfusion times (1, 24, 48, 72 and 96 hours), Na$^+$, K$^+$-ATPase activity was significantly increased. However, these results reflect the fact that in the post-ischemic period oxygen and glucose are again available for the previously ischemic brain, which tries to re-establish the transmembrane ion gradient and therefore the electrochemical potential\[4\].

In fact, Benzi et al\[16\] reported that Na$^+$, K$^+$-ATPase activity is significantly decreased during chronic intermittent mild hypoxia, and Torlinska et al\[29\] found that this enzyme is markedly affected by hyperglycemia in synaptosomes of 18 month-old rats. In addition, during Parkinson’s-like syndrome induced by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) administration, Na$^+$, K$^+$-ATPase activity increased in “large” synaptosomes isolated from the cerebellum of Macaca Fascicularis, whose efferent pathways are connected, among others, to the extra-pyramidal system\[7\].

The present results indicate that “direct” Mg$^{2+}$-ATPase enzyme activity is preserved during aging in the striatum, contrary to what has been reported for the hippocampus and cortex\[18, 20\]. Murali et al\[27\] showed that Mg$^{2+}$-ATPase activity decreases during aging in the striatum; nevertheless, these data should be regarded with caution because (1) they were obtained on mitochondria and not on synaptic plasma membranes, the actual in vivo localization of this ecto-ATPase, and (2) they evaluated the Mg$^{2+}$-ATPase of Ca$^{2+}$, Mg$^{2+}$-ATPase, a different enzyme system from “direct” Mg$^{2+}$-ATPase\[14\]. It is clear that the control of the extracellular ATP concentration exerted by Mg$^{2+}$-ATPase located on synaptic plasma membranes could mediate many processes, such as membrane permeability changes and neurotransmission regulation\[28\], which are preserved during aging in the striatum.

Ca$^{2+}$, Mg$^{2+}$-ATPase activity has the physiological role of regulating intraneuronal calcium ion concentrations, within the range of 0.1–1.0 M\[29\], together with other cellular mechanisms\[30\]. In particular, the low $K_m$ for calcium ($K_m$ = 0.23 M) of the Ca$^{2+}$, Mg$^{2+}$-ATPase\[29\] signifies that this enzyme is activated at very low calcium concentrations; therefore, it is primarily involved in calcium homeostasis regulation. The results of the present study suggest that the homeostatic capacity for calcium is particularly affected during aging, because the Ca$^{2+}$, Mg$^{2+}$-ATPase activity significantly decreases, starting as early as 6 months of age, compared with the 2-month values. Several studies confirm the altered striatal Ca$^{2+}$ homeostasis during aging\[31-32\]. An electrophysiological analysis conducted by Dunia et al\[31\] showed a significant age-related decrease in the duration of Ca$^{2+}$-mediated plateau potentials that could not be explained by alterations in the activation or inactivation properties of the plateau potential itself\[31\]. Interestingly, reduced complex I activity, central to the pathogenesis of the failure of dopaminergic system in PD\[33\], predisposes to excitotoxicity by altering ATP levels and by impairing Ca$^{2+}$ homeostasis\[32\]. Reduced ATP
levels, also induced during Parkinson’s-like syndrome by MPTP administration\cite{34}, decrease the activity of plasma membrane Na⁺, K⁺-ATPase. This results in partial neuronal depolarization, which decreases the voltage-dependent Mg²⁺ blockade of the N-methyl-D-aspartate glutamate receptor and results in elevation of intracellular Ca²⁺\cite{35}, to which striatal mitochondria are more sensitive than cortical ones\cite{36}. This also indicates an increased propensity towards activation of the mitochondrial permeability transition\cite{37}. The intrasympathetic mitochondria isolated from rat striata show a decreased NADH-cytochrome c reductase activity (Complex I-III) during aging, in particular in the heavy mitochondrial fraction, suggesting that also in physiological aging, this step of oxidative metabolism is partially impaired\cite{38}.

With regard to acetylcholine catabolism, acetylcholinesterase activity has been proven to decrease with aging in various cerebral areas\cite{39}. In addition to the numerically prevailing population of gamma-aminobutyric acid-ergic neurons, the striatum also contains a small percentage of interneurons, which provide this area with one of the highest acetylcholine levels in the brain\cite{40}. Although the increased striatal acetylcholine level in PD has long been attributed to the removal of tonic inhibitory control by D₂ receptors on cholinergic interneurons\cite{41}, this might also occur because the neurotransmitter is not readily hydrolyzed by acetylcholinesterase in the striatum\cite{42}, as the present data suggests.

The detected modifications in ATPases and acetylcholinesterase activities can therefore predispose the tissue to the physiopathological alterations typical of PD. Although gross abnormalities of metabolic and neurotransmitter function are unlikely to be compatible with survival, even in unpaired, but aged animals\cite{43}, subtle changes may accompany the aging process, with biochemical modifications occurring before the lesion becomes apparent\cite{7}.

The study of the enzymes localized on synaptic plasma membranes also allows us to assess if the membranes are markedly modified by the aging process, as the catalytic properties of acetylcholinesterase\cite{44}, as well as Na⁺, K⁺-ATPase, are significantly influenced by the membrane composition of phospholipids.

Acetylcholinesterase is less deeply imbedded in the membrane double phospholipid bilayer than the Na⁺, K⁺-ATPase\cite{45} and it is therefore less susceptible to changes in membranes phospholipids composition\cite{46}. The present data show that Na⁺, K⁺-ATPase activity is decreased, starting from 18 months of age, with respect to acetylcholinesterase, suggesting that the micro-environment of the synaptic plasma membranes is preserved during aging in the rat striatum and confirming that the observed changes in enzymes activities are due to actual modifications of the ATPase catalytic properties. Decrease in enzymatic activities may not indicate reduction in protein (enzyme) expression. Reduced enzymatic activities during aging may result from deficiencies in co-factors for the enzymes. Actually, protein concentration increased in association with aging (Table 1) and questions may then arise as to why (by what mechanism(s)) the activities declined. It is important to note that the results show that both protein concentration and content increase during aging. This increase probably relates to the increased number of glial cells that is known to occur during aging\cite{47,48}. In fact, an age-dependent increased protein concentration was previously observed in a bulk synaptic plasma membranes preparation from the frontal cerebral cortex of 4-, 8-, 12-, 16-, 20-, and 24-month-old rats\cite{49}.

Furthermore, defective noncatalytic proteins, by increasing nonspecific protein content in a study on the proteomics of synaptic proteins, accumulate during aging on synaptosomal membranes\cite{50}, as well as in other subcellular structures, such as mitochondria\cite{1,49,50}.

Protein accumulation could be regarded as another predisposing factor to subsequent age-related pathologies: in PD, McNaught et al\cite{51} hypothesized that failure of the ubiquitin-proteasome system to adequately clear unwanted proteins may underlie the vulnerability and degeneration of the substantia nigra pars compacta\cite{8}.

In summary, this study presents a systematic analysis on the energy-consuming ATPase enzyme systems during aging in male rat striata and shows that aging has a selective effect on each type of ATPase, that vary independently of the other ATPase activities. From a physiopathological point of view, these considerations are of particular importance, because the modifications in catalytic enzyme activities in the striatum are consistent with those detected in the Parkinson’s-like syndrome induced by MPTP administration, suggesting that these biochemical changes predispose the tissue to age-related diseases and are likely to prevent recovery processes. Therefore, this study on ATPase systems of the rat striatum could be seen as an experimental model for assessing the efficacy of drug treatments during aging and in related pathologies. Finally, the recovery capacity (and possibly regeneration) and the responsiveness to pharmacological treatment of the cerebral tissue during aging are tightly related, not only to ATP availability, but also to its proper utilization through the ATPase enzyme systems\cite{7,14,28-29,52-53}.

From the present data, it is clear that the regenerative potential of a specific cerebral area could be dependent on the actual catalytic activity of enzymes, as an index of the bioenergetics systems that are involved, noting that these systems decrease during aging. Interestingly, this starts at 18 months of age for most of these systems.

**MATERIALS AND METHODS**

**Design**

A wide spectrum analysis of functional proteomics.
Time and setting
The samples of striata were selected from our brain areas bank (frozen in liquid nitrogen and stored at -20°C) collected in almost 20 years of the “Experimental Project of Aging” research in our laboratory. The experimental material was used from the end of 2009 until the end of 2010.

Materials
The experiments were performed on 2-, 6-, 12-, 18- and 24-months-old male Wistar rats, weighing 250–300 g at 2 months to 700–800 g at 24 months. Animal maintenance and research was conducted both in accordance with the Guidelines of the National Institutes of Health Committee on Laboratory Animal Resources, and Institutional Animal Care and Use Committee Approval for animal use and care. The animals were selected according to randomized experimental procedures and kept from birth under standard cycling and housing conditions (temperature: 22 ± 1°C, relative humidity 60 ± 3%, 12-hour light/dark cycle; low noise disturbances), allowing free access to a standard diet in pellets with water, and housed three and, subsequently, two per cage. At the set time, the animals were anaesthetized by ether and sacrificed by a lethal dose of urethane (1.4 mg/kg) via intraperitoneal injection at 9:00–9:30 a.m., to avoid any circadian changes of enzyme activities.

Methods
Preparation of purified synaptic plasma membranes
Purified synaptic plasma membranes were obtained from the striatum of a single animal according to the methods of Cotman and Matthews[54], as modified by Gurd et al.[55] and described in detail by Gorini et al.[48], to avoid microsomal contamination and to improve the purity of this subcellular fraction.

The subsequent procedures were performed at 0–4°C. The brain was isolated (within 20 seconds) in a refrigerated box at 0–4°C and immediately placed in an isolation medium (0.32 M sucrose; Merck, Darmstadt, Germany; 1.0 mM EDTA-K; Sigma Chemicals Company, St. Louis, MO, USA; 10 mM Tris-HCl; Merck; pH 7.4). The striatum was carefully dissected, isolated, and immediately placed in the isolation medium. A homogenate was obtained using a Teflon-glass homogenizer (Braun S Homogenizer) by five up and down strokes of the pestle (total clearance: 0.2 mm) rotating at 800 r/min, with electronic control of the pestle speed. The homogenate (usually 5 mL final volume) was centrifuged at 800 × g for 9 minutes, at 0–4°C. After osmotic shock, the lysate was centrifuged at 1 100 × g for 15 minutes, and the pellet obtained from lysed synaptosomes was resuspended in a 0.32 M sucrose solution (pH 7.4), and applied on a discontinuous sucrose gradient consisting of successive 1 mL layers of 25, 32.5, 35, and 38% (w/v), pH 7.4. The gradient was centrifuged at 81 000 × g for 90 minutes. At the end of this centrifugation, the band at the interface of the 25-32.5% sucrose was collected by aspiration, diluted with the isolation medium, and pelleted at 115 000 × g for 30 minutes. This is the fraction that contains the richest quantity of synaptic plasma membranes, as previously described by Gorini et al.[48] and by Gurd et al.[55]. The pellet of synaptic plasma membranes thus obtained was resuspended in a small volume of 0.32 M sucrose buffered solution (pH 7.4) for the assay of the catalytic activity of enzymes.

Enzyme assays
On synaptic plasma membranes, the maximum rate (V_{max}) of the following enzyme activities was evaluated: Na⁺,K⁺-ATPase[56], Mg²⁺-ATPase ouabain-insensitive[56]; Na⁺,K⁺-ATPase[56]; “direct” Mg²⁺-ATPase[56]; Ca²⁺, Mg²⁺-ATPase[57]. acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7)[58] for acetylcholine catabolism.

On samples, ATPase activities were determined by measuring the inorganic phosphorous (P_i) released from the hydrolysis of ATP in the presence of different ions, according to Le Bel et al.[59], as subsequently specified. The protein concentration of the tested sub-fraction was determined using crystalline bovine serum albumin (Merck) as a standard, according to Lowry et al.[60].

Enzyme activity calculation
ATPase activities were expressed as specific activities in µmoles of P_i released/h/mg of protein of the sub-fraction tested.

Enzyme activity of acetylcholinesterase was measured by graphic recordings for at least 3 minutes in double beam recorder spectrophotometers (Perkin-Elmer 554 or 551s; Perkin-Elmer Italia, Monza, Milan, Italy) and each value was calculated from two blind determinations on the same sample. Enzyme activities were expressed as
specific activities in µmoles of substrate transformed/min/mg of protein of the sub-fraction tested. The specific enzyme activity is an extensive quantity and the quantities derived from it include "specific catalytic activity" and "catalytic activity"; hence, the values calculated may be: (1) enzyme specific activity, if expressed as µmoles of substrate transformed/min/mg of protein (acetylcholinesterase); (2) µmoles of P_i released/h/mg of protein, if assayed as "end point" methods for ATPases; also (3) enzyme catalytic activity, if expressed as nanokatal, nmol of substrate transformed/s/mL (data not shown). It is possible to interconvert the enzymatic activities.

**Statistical analysis**

The two-way analysis of variance test was used to evaluate the comparisons of enzyme activity between the different cellular sub-fractions, according to the various ages of the animals. The analysis of variance test for multiple comparisons was used to evaluate the interactions between the values of different groups of animals at various ages of each individual enzyme activity and of each biochemical parameter tested. The homogeneity of variance was checked by Bartlett's test and post hoc tests were used to compare the differences between individual groups, controlling statistical evaluation by the Tukey's and Dunnett's tests.

**Author contributions:** Roberto Federico Villa had full access to the study design and wrote the manuscript. Antonella Gorini and Federica Ferrari performed research and analysed data.

**Conflicts of interest:** None declared.

**Ethical approval:** Approval for this study has been given by ad hoc authorities of the University of Pavia and of the Italian Ministry of Health.

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