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ENERGY METABOLISM OF RAT CEREBRAL CORTEX, HYPOTHALAMUS AND HYPOPHYSIS DURING AGEING

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Abstract—Ageing is one of the main risk factors for brain disorders. According to the neuroendocrine theory, ageing modifies the sensitivity of hypothalamus–pituitary–adrenal axis to homoeostatic signals coming from the cerebral cortex. The relationships between the energy metabolism of these areas have not been considered yet, in particular with respect to ageing. For these reasons, this study was undertaken to systematically investigate in female Sprague–Dawley rats aged 4, 6, 12, 18, 24, 28 months and in 4-month-old male ones, the catalytic properties of energy-linked enzymes of the Krebs' cycle, electron transport chain, glutamate and related amino acids on different mitochondrial subpopulations, i.e. non-synaptic perikaryal and intra-synaptic (two types) mitochondria. The biochemical enzymatic pattern of these mitochondria shows different expression of the above-mentioned enzymatic activities in the investigated brain areas, including frontal cerebral cortex, hippocampus, striatum, hypothalamus and hypophysis. The study shows that: (i) the energy metabolism of the frontal cerebral cortex is poorly affected by physiological ageing; (ii) the biochemical machinery of non-synaptic perikaryal mitochondria is differently expressed in the considered brain areas; (iii) at 4–6 months, hypothalamus and hypophysis possess lower oxidative metabolism with respect to the frontal cerebral cortex while (iv), during ageing, the opposite situation occurs. We hypothesised that these metabolic modifications likely try to grant HPA functionality in response to the incoming external stress *stimuli* increased during ageing. It is particularly notable that age-related changes in brain bioenergetics and in mitochondrial functionality may be considered as remarkable factors during physiological ageing and should play important roles in predisposing the brain to physiopathological events, tightly related to molecular mechanisms evoked for pharmacological treatments.

Key words: hypothalamus, hypophysis, cerebral cortex, ageing, mitochondria, bioenergetics.

INTRODUCTION

Energy availability and exploitation are of fundamental importance to grant neuronal functions and survival, because any alterations in energy metabolism affect proper physiology in a negative way (Hertz and Dienel, 2002). According to the second principle of thermodynamics, in a closed system like the brain, entropy increases when energy transduction is compromised or reduced and the cerebral biomass spontaneously evolves towards an increased disorganisation, i.e. ageing (Villa and Gorini, 1991a).

In physiopathology, ageing has been recognised as the main risk factor for acute (e.g. cerebral ischaemia) (Hirtz et al., 2007; Villa et al., 2009) and chronic (neurodegenerative) brain disorders, such as dementias (Moretti et al., 2011), Alzheimer's (Reitz et al., 2011) and Parkinson's diseases (Schapira, 2009). Because many studies indicate that changes in brain energy metabolism are causative of development/progression of several diseases, only subsequently implying morphological modifications (Villa et al., 2002; Nicholls, 2004; Villa et al., 2009; Small et al., 2011; Saxena, 2012), it was of interest to investigate the bioenergetics of functionally interconnected areas, i.e. frontal cerebral cortex, hippocampus, striatum, hypothalamus and hypophysis.

During ageing, the cerebral cortex undergoes biochemical, functional and morphological changes (Villa et al., 2006; Nyberg et al., 2010) also in intellectually preserved elderly people (Brody, 1955). The neuroendocrine theory of ageing underlines the key role of mechanisms changing the sensitivity to homoeostatic signals of hypothalamus–pituitary–adrenal axis (HPA) (Dilman, 1971) and to glucocorticoids. These mechanisms have effects on memory (Hibberd et al., 2000; Wolf, 2003) and on neuroendocrine responses to stress (DeKloet et al., 1996) in the aged brain. In rats and primates, a high density of glucocorticoid receptors in frontal cortex supports that this area plays a crucial linking role in HPA regulation (Kern et al., 2008), likely

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Abbreviations: CCRS, cytochrome *c* reductase rotenone-sensitive; CCRT, total cytochrome *c* reductase; COX, cytochrome oxidase; CS, citrate synthase; CTX, cortex; EDTA, ethylenediaminetetraacetic acid; ETC, electron transport chain; GlDH, glutamate dehydrogenase; GOT, glutamate–oxaloacetate transaminase; GPT, glutamate–pyruvate transaminase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIP, hippocampus; HPA, hypothalamus–pituitary–adrenal axis; HPT, hypothalamus; HYPH, hypophysis; IM, isolation medium; MDH, malate dehydrogenase; S.P.M., synaptic plasma membranes; S.V., synaptic vesicles; SD, Sprague–Dawley; SDH, succinate dehydrogenase; STR, striatum.

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for its functions in higher processing for ascending and descending neuronal network projections.

Up to date, age-related relationships between the energy metabolism of the cerebral cortex and HPA have not been considered yet. Thus, this research is a systematic investigation on energy-transducing pathways of cerebral areas to study: (i) the bioenergetic properties of the so-called pacemaker areas (hypothalamus and hypophysis) with respect to other significant areas, i.e. cerebral cortex, hippocampus and striatum, (ii) the connections between their metabolic flux during brain ageing, (iii) the assessment of a model to evaluate the pharmacological actions of drugs on brain energy metabolism. Age-related changes in mitochondrial functionality should in fact be considered as relevant factors for therapy, possibly influencing diversified responses to pharmacological treatments affecting the metabolism of perikaryal mitochondria with respect to intra-synaptic ones (Villa and Gorini, 1991b, 1997; Gorini et al., 1998).

The ages of rats, 4–12–18–24 months (cortex [CTX]) and 6–12–28 months (hypothalamus–hypophysis [HPT–HYPH]), were chosen to cover the entire life-span and considered to pinpoint the metabolism in ''adult'', ''mature'' and ''aged'' animals (Benzi et al., 1980), the 28-month-old rats representing the ''survivals''. Energy-related enzyme systems were studied in some instances also on male rats, to avoid any influence by hormones (Morrison et al., 2006), known to interfere with HPT oxidative activity (Moguilevsky and Malinow, 1964).

Thus, the catalytic properties of energy-linked enzymes were studied in their actual *in vivo* localisation, i.e. on non-synaptic mitochondria located in the perikaryon of neurons, especially because the Gibbs free energy ($\Delta G^{o\prime}$) of these mitochondria is directly related to protein synthesis (Villa et al., 1989b), and on intra-synaptic mitochondria, located *in vivo* in synapses, whose $\Delta G^{o\prime}$ is mainly related to ions homoeostasis and neurotransmission (Benzi et al., 1993, 1994; Gorini et al., 2002; Villa et al., 2002; Gilmer et al., 2010; Villa et al., 2011).

EXPERIMENTAL PROCEDURES

Care and ages of the animals

The experiments were performed on 4, 6, 12, 18, 24, and 28-month-old female Sprague–Dawley (SD) rats and 4-month-old male SD rats (Cobs–Charles River, Calco, Lecco, Italy), depending on the area and subfractions tested. The animals were kept from birth under standard cycling and housing conditions (temperature: 22 ± 1 °C; relative humidity 60 ± 3%; lighting cycle: 12 h light and 12 h darkness; low-noise disturbances), fed with a standard diet in pellets with water *ad libitum*. The selection of the animals was established by permutation tables and, to avoid any circadian changes of enzyme activities, the animals were sacrificed by a lethal dose of urethane ($1.4\ g \times kg^{-1}$, i.p.) at 09:00 a.m., under anaesthesia by ether.

Animal maintenance and research approval for this study has been given by ad hoc authorities of the University of Pavia and in accordance with the guidelines of the Italian Ministry of Health. All efforts were made to minimise the number of animals used and their suffering.

Preparation of synaptosomal fraction and of non-synaptic mitochondria

The synaptosomal fraction and non-synaptic mitochondria were prepared according to the methods of Lai et al. (1977), as modified for analytical evaluations for brain areas of single animal by Villa et al. (Villa et al., 1989a; Villa and Gorini, 1991a,b).

After the sacrifice, in a refrigerated box at 0–4 °C, the brain was isolated (< 20 s) and frontal cerebral cortex, hippocampus, striatum, hypothalamus and hypophysis were carefully dissected and placed in the isolation medium (IM: 0.32 M sucrose, Merck, Darmstadt, FR, Germany, 1.0 mM EDTA–K^+, Sigma, St. Louis, MO, USA, 10 mM Tris–HCl, Merck, Darmstadt, FR, Germany, pH 7.4). The homogenate was obtained by a Teflon-glass homogeniser (Braun S, Melsungen, AG, Germany) by five up and down strokes of the pestle (total clearance 0.1 mm) rotating at 800 r.p.m., with electronic control of the speed. The homogenate was diluted (7–10% w/v) and the nuclear fraction was removed by centrifugation at $5.5 \times 10^3 g$ min ($3.9 \times 10^7\ \omega^2 t$) in a Sorvall RC-5B Supercentrifuge, rotor SS-34, DuPont Instruments, Newtown, CT, USA, repeating this procedure twice. The combined supernatants were centrifuged at $300 \times 10^3 g$ min ($213 \times 10^7\ \omega^2 t$), to yield the ''crude'' mitochondrial pellet, washed in IM, applied on a Ficoll-sucrose gradient (Ficoll 7.5% and 12% (w/w), Pharmacia Biotech AB, Uppsala, Sweden, in 0.32 M sucrose, 50 μM EDTA–K^+, 10 mM Tris–HCl, pH 7.4) centrifuged at $140 \times 10^4 g$ min ($988 \times 10^7\ \omega^2 t$) in a OTD-65B Sorvall Ultracentrifuge (AH-650 type rotor), DuPont Instruments, Newtown, CT, USA. After centrifugation, the myelin fraction was sucked off and the synaptosomal fraction was collected at the interface of 7.5–12% Ficoll-sucrose. The pellet of purified non-synaptic mitochondria (FM) was centrifuged at $162.4 \times 10^3 g$ min ($106.7 \times 10^7\ \omega^2 t$) and the washed pellet was resuspended in 0.32 M sucrose (pH 7.4) for the assay of the catalytic activity of enzymes.

The band of synaptosomes, previously collected by aspiration at the interface of 7.5–12% Ficoll-sucrose gradient was diluted in IM and centrifuged at $375 \times 10^3 g$ min ($266 \times 10^7\ \omega^2 t$), rotor SS-34. The synaptosomal pellet was osmotically lysed in 6 mM Tris–HCl, pH 8.1, for 1.5 h, at 0–4 °C. From this lysate, intra-synaptic mitochondria were purified from frontal cerebral cortex of 4, 12, 18 and 24-month-old female rats (see Preparation of intra-synaptic light and heavy mitochondria of frontal cerebral cortex) and synaptic plasma membranes (S.P.M.) and synaptic vesicles (S.V.) were isolated from the hypothalamus of 4-month-old male rats (see Preparation of synaptic plasma membranes and synaptic vesicles of the hypothalamus).

Preparation of intra-synaptic light and heavy mitochondria of frontal cerebral cortex

After osmotic shock (see above), the lysate was centrifuged at $399 \times 10^3 g$ min ($262.1 \times 10^7\ \omega^2 t$) and the pellet was resuspended and centrifuged at $192.6 \times 10^3 g$ min ($126.5 \times 10^7\ \omega^2 t$); the pellet was resuspended in 3% w/w Ficoll, 0.12 M mannitol, Merck, Darmstadt, FR, Germany, 30 mM sucrose, 25 mM EDTA–K^+, 5 mM Tris–HCl, pH 7.4 and was layered on a Ficoll gradient consisting of 4.5% w/w Ficoll in 0.24 M mannitol, 60 mM sucrose, 50 mM EDTA–K^+, 10 mM Tris–HCl, pH 7.4 and 6% w/w Ficoll, at the bottom. This gradient was centrifuged at $280.2 \times 10^3 g$ min ($197.3 \times 10^7\ \omega^2 t$): after centrifugation, the layer between 4.5–6% w/w of Ficoll solution, that is the ''light'' intra-synaptic mitochondrial fraction (LM), was sucked off and pelletted at $166.5 \times 10^3 g$ min ($109.4 \times 10^7\ \omega^2 t$); the pellet of this centrifugation and that at the bottom of gradient, the ''heavy'' intra-synaptic mitochondrial fraction (HM), were separately resuspended in 0.32 M