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BBA Report

Cytochrome oxidase content of rat brain during development

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The cytochrome oxidase concentration and content of rat brain during development was measured using a simple new assay for cytochrome *a*. The cytochrome oxidase concentration increased from 1.2 nmol/g wet wt. of brain at birth to about 5.5 nmol/g in the adult, most of the change occurring between 5 and 25 days after birth.

Cytochrome oxidase is the terminal enzyme of the mitochondrial respiratory chain and is a vital component of cellular energy transduction, responsible for virtually all oxygen consumption in mammals [1]. The cellular concentration of cytochrome oxidase has been used extensively as an indicator of relative energy use and electrical activity by different cells and areas in the brain [2]. This technique makes the assumption that oxidase levels correlate directly with rates of ATP turnover and thus indirectly with electrical activity. Since the energy use and electrical activity of the whole brain changes during development, it is of interest to know how the cytochrome oxidase level of brain changes with age. In the present paper we report the changes during development of cytochrome oxidase levels measured in rat brain homogenates, using a simple new assay for cytochrome *a*. Cytochrome *a* is an integral component protein of the cytochrome oxidase complex, there being one cytochrome *a* per cytochrome oxidase [3].

The technique of near infrared spectroscopy (NIRS) has been developed for monitoring the redox state of cytochrome oxidase non-invasively in the brain [4]. Recently, the changes in cytochrome oxidase redox state monitored by NIRS have been quantitated [5], but the technique still can not quantitate the absolute level of oxidase in the brain, and this absolute level is important for interpreting results from NIRS. We therefore set out to measure the absolute level of cytochrome oxidase in rat brain using the visible spectral changes.

Whole brains were removed from Sprague-Dawley rats ranging in age from 3 days before the expected time of birth to 12 months after birth. Brains from several rats of the same age were pooled to give roughly 2–3 g wet weight of brain; this required between 2 (for adult rats) and 16 (for prenatal rats). A small amount of ice-cold buffer (100 mM potassium phosphate, 2 mM EDTA (pH 7.1)) was added to the minced brains to give between 0.3 and 0.5 g wet weight of brain per ml final volume of homogenate. The suspension was homogenised with an Ultraturrax (Polytron), followed by a small very tight hand-held Dounce homogenizer, and then stored on ice and assayed within 3 h of preparation.

To assay cytochrome *a*, 1 ml of homogenate was added to 1 ml of 20% w/w Triton X-100 and 1.5 ml of buffer, and whirlymixed. The detergent was added to decrease light scattering, which increases the pathlength of the spectrophotometer beam. 3 ml of the suspension was filtered through muslin into a cuvette. The cuvette was placed in a Hitachi (model 356) dual beam spectrophotometer recording at 605–625 nm. Cytochrome *a* was reduced by adding 30 μl of a mixture containing 0.1 M sodium cyanide, 0.1 M potassium ascorbate and 5 mg *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) per ml (pH 7.1). The cyanide inhibits the oxidase at the oxygen binding site, while the ascorbate reduces the oxidase via the lipid soluble electron mediator TMPD. The difference in absorbance at 605/625 nm (before and immediately after addition of the reducing mixture) was used as a measure of the concentration of cytochrome *a* in the homogenates. Addition of the reducing mixture to buffer or buffer

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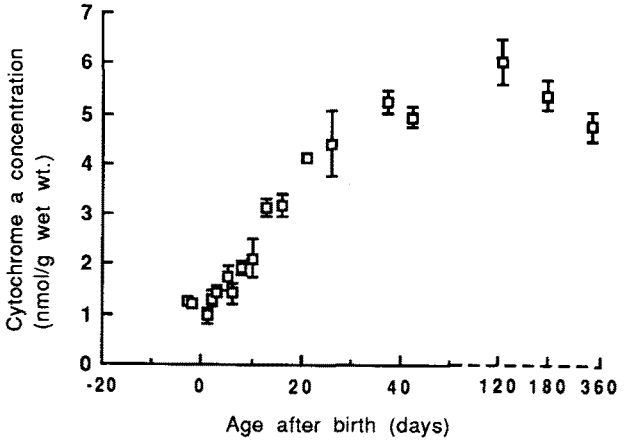

Fig. 1. Change in rat brain cytochrome *a* concentration with age. Points represent the mean and one standard deviation on either side of the mean of at least three spectral determinations on one brain homogenate, derived from the pooled brains of from 2 to 16 rats. Ages of rats of about 120, 180 and 360 days of age are not shown to scale.

plus purified haemoglobin gave no significant absorbance change. The difference spectrum before and after the addition of the reducing mixture to a brain homogenate showed the normal mitochondrial redox difference spectrum with peaks at 550 nm (for cytochrome *c*) and 603 nm (for cytochrome *a*) and a shoulder at 560 nm (for cytochrome *b*). There was no sign of haemoglobin reduction in the spectrum. Cytochrome *a* appeared to be fully oxidised before addition of the reducing mixture, as addition of rotenone (to inhibit mitochondrial complex I) or antimycin (to inhibit mitochondrial complex III) caused no detectable change in absorbance.

Cytochrome *a* concentration was calculated using a (reduced–oxidised) extinction coefficient at 605–625 nm of 20 $mM^{-1}\,cm^{-1}$ (note that cytochrome a_3 is reduced only very slowly in the presence of cyanide) [3].

Fig. 1 gives the measured concentration of cytochrome *a* in rat brain with age, showing that the cytochrome concentration rose progressively from about 1.2 nmol/g wet weight of brain (i.e., about 1.2 μM) around birth to about 5.5 nmol/g in the adult; most of the increase occurring between the ages of 5 and 25 days after birth. Fig. 2 shows the change with age in the total brain cytochrome *a* content, that is, the concentration multiplied by the brain weight. Cytochrome *a* content per brain rose from 0.15 nmol at 2–3 days before birth to 11 nmol 4–6 months after birth.

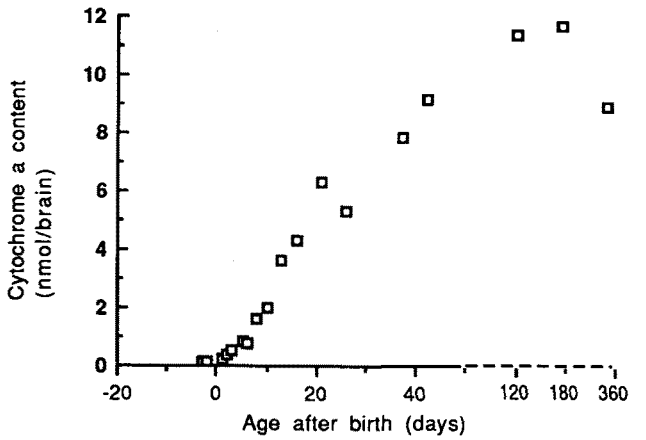

Fig. 2. Changes in total rat brain cytochrome *a* content with age. The points are derived from the concentrations of Fig. 1 multiplied by the mean brain weight.

There have, to our knowledge, been no previous measurements of the absolute concentration of cytochrome *a* in brain. Chepelinsky and Arnaiz [6] measured cytochrome levels in mitochondria isolated from rat brain of various ages and found an increase of cytochrome *a* from 0.1 nmol/mg of mitochondrial protein at 5 days to 0.22 nmol/mg at 90 days, most of the change occurring between 10 and 25 days. Dahl and Samson [7] measured the changes in rat brain mitochondrial protein with age, and found an increase from 11 mg mitochondrial protein per g wet weight of brain at 1 day old to 27 mg/g at 50 days old, most of this change occurring between 5 and 20 days after birth. If we put these two sets of data together we can calculate a change in cytochrome *a* concentration from 1.2 nmol/g at 5 days to 5.9 nmol/g at 50 days, most of the change occurring between 5 and 25 days. These calculated concentrations are very similar to the concentrations measured here, and demonstrate that the increase in cytochrome a concentration between neonate and adult is due to both a doubling of mitochondrial protein and a doubling of cytochrome *a* per mg of mitochondria. However, the values calculated from the above two references are not reliable as they depend on the assumption that both sets of authors used the same purity of mitochondrial preparation. Cytochromes *b* and *c* and ubiquinone show similar increases with age in concentration per mg of brain mitochondrial protein [6]. Mitochondrial number and surface area also increase over a similar time course during rat brain development [8]. The relative concentrations of cerebral phosphocreatine and ATP have been shown to change with development, and again most of the changes occurred between postnatal days 5 and 27 in the rat [9]. Cytochrome oxidase activity measured in rat brain homogenates also increases roughly 5-fold from 5 to 25 days after birth [10] and declines during senescence [11]. The developmental changes in cytochrome oxidase content and activity in the brain presumably reflect the concurrent changes in energy requirement for growth and synaptic activity [2,8].

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