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Developmental changes of the adenine nucleotide translocation in rat brain

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Abstract

The perinatal development of the adenine nucleotide translocation in isolated rat brain mitochondria was studied. For that purpose the content of the adenine nucleotide translocase (ANT), the activity of adenine nucleotide translocation and the control of the ANT protein over State 3 respiration were estimated. From the newborn to the adult state there was a 4-fold increase in State 3 respiration which was paralleled by a 3-fold increase in the respiratory control ratio. The capacity of uncoupled respiration exceeded that of State 3 respiration in all developmental stages indicating that the activity of oxidative phosphorylation is influenced by that of ANT and/or ATP synthase. The content of the ANT protein, measured as bound pmoles of [^{3}H]atractyloside per mg mitochondrial protein, increased more than 2-fold from birth to adultness in the first three postnatal weeks. The size of the exchangeable matrix (ATP + ADP)-pool was only sligthly expanded during the same period. The translocation activity increased 2-fold from the newborn to the adult state and was a linear function of the ANT protein. Control of the ANT protein over State 3 respiration (quantified as flux control coefficient, C^{Jo}_{ANT}), was remarkable in brain mitochondria from newborn rats ($C^{Jo}_{ANT} = 0.45 \pm 0.15$), but declined during further development ($C^{Jo}_{ANT} = 0.11 \pm 0.03$, at the 20th day). The obtained results suggest that the postnatal enrichment of the ANT protein in rat brain mitochondria is an essential factor for the development of oxidative phosphorylation capacity in the early postnatal period.

Keywords: Adenine nucleotide translocase; Respiration; Mitochondrion; Postnatal development; Flux control coefficient; (Brain)

1. Introduction

Rat brain is functional and energetically immature at birth [1,2]. Mitochondria isolated from the brain of newborn rats have a poor capacity of oxidative phosphorylation as indicated by the low rate of State 3 respiration [1]. Since the developing brain has a high energy demand (myelination, de novo protein synthesis, neurotransmission, maintenance of ionic gradients), postnatal acquisition of fully differentiated mitochondria is vital for the functional maturation of brain. Studies of brain mitochondria have been mainly concerned on the postnatal development of the hydrogen delivering reactions (e.g., pyruvate dehydrogenase complex and the citrate synthase) and the complexes of the respiratory chain [1–5]. However, less attention has been focused on the role of the adenine nucleotide translocation across the inner mitochondrial membrane and the ATP synthase reaction during perinatal development.

Based on studies with isolated liver mitochondria, the concept was formulated that the onset of the respiratory control is mainly accomplished by two events: the accumulation of adenine nucleotides in the mitochondrial matrix and the enrichment of proteins involved in the energy transduction (for review, see [6,7]). Among these proteins, especially the role of F_1-ATP synthase and of adenine nucleotide translocase (ANT) during the development of State 3 respiration has been studied [8–11]. In line with this concept are the observations: (i) that the low content of mitochondrial adenine nucleotides found in the late fetal liver increases after birth within few hours to a level even higher than found in adults [6,8,9] and (ii) that the content of F_1-ATP synthase and ANT protein increases 2-fold during the first day of extrauterine life [8,10,11]. The low

Abbreviations: ANT, Adenine nucleotide translocase; ATR, atractyloside; CAT, carboxyatractyloside; RCR, respiratory control ratio; C^{Jo}_{ANT}, flux control coefficient ; FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone.

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content of adenine nucleotides in fetal mitochondria has been considered to limit the activities of F_1-ATP synthase and of ANT [6].

The capacity of aerobic ATP-formation develops slowly in brain mitochondria after birth [1,2]. Thus, in contrast to mitochondria from rat liver [6] and kidney [12], where the capacity of oxidative phosphorylation reaches the adult level during the first day of the extrauterine life, the same process requires in rat brain mitochondria 6 weeks after birth [1]. Therefore the question arose whether the maturation of oxidative phosphorylation in rapidly and slowly developing mitochondria is controlled by the same mitochondrial events. Since the ANT protein has been found to exert a major control on aerobic ATP formation in mitochondria of various tissues [13–16], we studied in the present paper the development of the adenine nucleotide translocation in mitochondria from rat brain prepared at various developmental stages. The content of ANT protein, the activity of adenine nucleotide translocation and the flux control coefficient of the ANT over State 3 respiration were measured. The obtained results suggest that the enrichment of ANT protein in rat brain mitochondria is an essential factor in the development of oxidative phosphorylation in rat brain during the first postnatal week.

2. Materials and methods

2.1. Preparation of homogenates and mitochondria

Wistar rats were fed with standard laboratory chow and water ad libitum. The animals were maintained on a 12 h light/dark cycle. Females with a mean weight of 180 g were caged with males overnight. Fetuses were obtained from pregnant anaesthetized rats by Cesarean section. The body weight of the fetuses was used to control their age [17]. Fetuses and puppies used in these studies were of mixed sex. After decapitation the brain was immediately dissected and homogenized in the 5-fold amount of ice-cold 250 mM sucrose medium by an Ultra Turrax homogenizer (twice for 20 seconds). The homogenate was used for preparing mitochondria or directly used in the [^{3}H]ATR binding assay. Synaptosome-free mitochondria were isolated from the homogenate by using the Ficoll gradient method essentially as described in [18]. The protein content in the mitochondrial stock suspension and in the homogenate was determined by the biuret method or with bicinchoninic acid (BCA) assay of Pierce (Rockford, IL) in the presence of 0.2% sodium dodecyl sulfate.

2.2. Measurement of respiration and flux control coefficient

State 3 respiration of rat brain mitochondria was measured polarographically in a water-jacketed chamber maintained at 30°C. Mitochondria (0.5–1.0 mg/ml) were incubated in 2 ml medium containing 110 mM mannitol, 60 mM KCl, 60 mM Tris, 10 mM potassium phosphate and 0.5 mM Na_2EDTA (pH 7.4). The respiratory control ratio (RCR) was calculated from rates of oxygen consumption in presence of ADP and after phosphorylation of added ADP. The flux control coefficient of the adenine nucleotide translocase on State 3 respiration (C^{Jo}_{ANT}) was estimated by inhibitor titration with CAT as described in [13] by measuring the effects of small additions of CAT on the mitochondrial oxygen uptake using a rate-meter.

2.3. Determination of the adenine nucleotide translocator protein

The ANT protein content in isolated mitochondria and in homogenate samples was measured using an assay based on the high-affinity binding of tritium-labelled atractyloside to the ANT as recently described [11]. Briefly, aliquots of 0.1 ml of the mitochondrial stock suspension (0.1–0.3 mg protein) or of the homogenate (1–3 mg protein) were added to 0.9 ml 250 mM sucrose medium containing 2 μM [^{3}H]ATR (348 dpm/pmol). The tubes were centrifuged, the supernatant was aspirated and the pellet washed three times with 0.5 ml sucrose medium. Finally, the pellet was dissolved in 200 μl 2% sodium dodecyl sulfate and 1 ml of a scintillation cocktail (Quicksafe A; Zinsser Analytic) was added. In order to correct the data for adherent [^{3}H]ATR in the pellet (blank radioactivity), each incubation was repeated in the presence of 10 μM CAT. The blank was subtracted from the total radioactivity measured in the absence of CAT.

2.4. Adenine nucleotide translocase activity

The translocator activity was measured at 0°C using the carboxyatractyloside-inhibitor stop technique [19]. Mitochondria (0.1–0.4 mg) were incubated in microcentrifuge tubes in 0.3 ml incubation medium. The medium was the same as used for the measurements of respiration. The forward exchange reaction was initiated by addition of 100 μl 0.38 mM [^{3}H]ATP (specific activity 1170 dpm/nmol; Du Pont) and terminated after 10 or 120 s by quick addition of 0.2 ml 150 μM CAT. Tubes were centrifuged for 5 min at $10000 \times g$, the pellets were washed with 0.5 ml incubation medium and finally dissolved in 200 μl 2% sodium dodecyl sulfate. After mixing the dissolved pellet with 1 ml scintillation cocktail, the entrapped radioactivity was measured. Nonspecific binding of [^{3}H]ATP to mitochondria was estimated by incubation of mitochondrial samples with 100 μM CAT prior addition of [^{3}H]ATP. This blank was subtracted from the measured radioactivities. The activity of the adenine nucleotide translocase was expressed as nanomoles [^{3}H]ATP per minute per milligram protein or when normalized to the [^{3}H]ATR-bind-

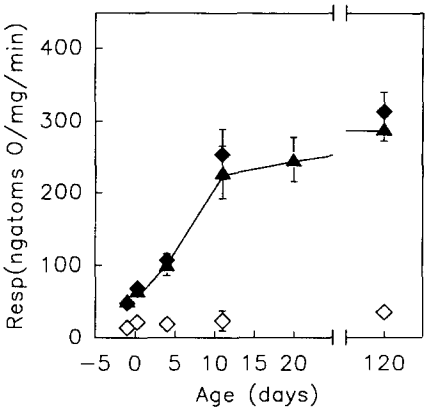

Fig. 1. Developmental change of respiration and respiratory control in brain mitochondria. Mitochondria of various perinatal ages were incubated as described in Section 2 in presence of 5 mM pyruvate plus 5 mM malate. Mitochondria from adult rat brain are designated as mitochondria from rats of 120 days throughout the figures. First ADP (0.1–0.3 mM) was added and after complete phosphorylation uncoupled respiration was measured by stepwise addition of FCCP up to the maximal stimulation (0.2–1.4 μM). Shown are the rates of respiration under State 3 (▲), State 4 (◇) and uncoupled conditions (◆). Values are given as mean ± S.D. of 4–5 experiments obtained with separate mitochondrial preparations. Where no error bars are shown, the S.D. falls within the size of the symbols.

ing sites as nanomoles [^{3}H]ATP per minute per nmol [^{3}H]ATR.

3. Results

3.1. Development of State 3 respiration and respiratory control

Fig. 1 shows perinatal development of State 3, State 4 and FCCP-uncoupled respiration in rat brain mitochondria. Mitochondria of 1-day before birth had a low rate of State 3 respiration (Fig. 1). Between the late fetal (1-day before birth) and the newborn state there is only a slight increase in both, State 3 respiration as well as respiratory control (Table 1). During development from birth to adultness there was a 4–5-fold enhancement in State 3 rate of respiration, paralleled by a 3-fold increase in respiratory control. The steepest enhancement of the State 3 respiration occurred in the first two weeks of extrauterine life. State 4 rate of respiration increased 2-fold from birth to adultness. Moreover, in each incubation experiment (with exception of fetal age) the maximally achievable rate of respiration obtained by stepwise addition of FCCP at State 4 was above State 3 respiration (Table 1).

Table 1
Respiratory control ratios and FCCP-uncoupled respiration of rat brain mitochondria of various ages

Age (days)	RCR	Uncoupled respiration (in % of State 3)
−1	2.9 ± 1.1	93 ± 10
0	3.0 ± 0.3	108 ± 7
4	4.8 ± 1.2	110 ± 6
11	6.6 ± 1.3	111 ± 8
adult	7.8 ± 1.9	107 ± 5

Incubation conditions were identical to those described in Fig. 1. Values shown are means ± S.D. of 4–5 separate mitochondrial preparations.

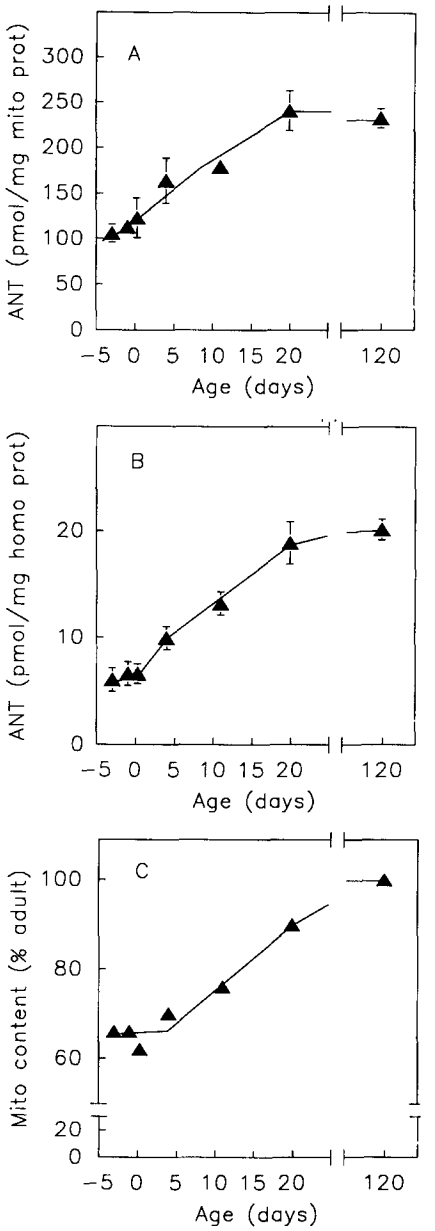

Fig. 2. Developmental change of [^{3}H]ATR binding sites in mitochondria and in tissue homogenates. Samples of mitochondrial stock suspension (A) and of tissue homogenate (B) from rat brain tissue were treated as described in Section 2. Proliferation of mitochondria in brain tissue (C) was calculated by dividing ATR binding sites per g homogenate protein by the ATR binding sites per g mitochondrial protein. Values are given as mean ± S.D. of 5–7 separate mitochondrial preparations.

3.2. Developmental change of the adenine nucleotide translocase protein

Next we studied the developmental change of the ANT protein by measuring the binding of [^{3}H]ATR to brain mitochondria. From the 3rd day before birth to the newborn state there was only a slight increase in the ANT content in mitochondria, whereas the ANT content developed rapidly after birth (Fig. 2A). From birth to adultness the content of ANT increased more than 2-fold in brain mitochondria within the first three weeks of postnatal life. In brain tissue the content of the ANT protein increased 4-fold in the same period (Fig. 2B), suggesting that it was caused by an enrichment of the ANT protein in mitochondria and an increase of the number of mitochondria per cell. On the basis of the measured specific binding sites of ATR in mitochondria and the homogenate, the mitochondrial proliferation was estimated. Fig. 2C shows that proliferation of mitochondria in rat brain tissue starts significantly at the end of the first week of extrauterine life.

3.3. Activity of adenine nucleotide translocation and the exchangeable adenine nucleotide pool

The effect of the ANT protein enrichment on the activity of the adenine nucleotide translocation was studied by measuring the rate of [^{3}H]ATP uptake. Fig. 3 shows the time-course of the [^{3}H]ATP uptake by mitochondria from 4-days old, 11-days old and adult rat brain. Due to the low yield of mitochondria obtained from newborn rat brain it was not possible to measure the time-course of [^{3}H]ATP uptake by mitochondria isolated immediately after birth. Since the uptake of [^{3}H]ATP was proofed to be linear up to 10 s in all ages, values at 10 s were used to calculate the activity of adenine nucleotide translocase. After an incubation period of 120 s the exchange of intramitochondrial adenine nucleotides by [^{3}H]ATP was approximately complete. From this value therefore the size of the exchangeable adenine nucleotide pool was estimated. The translocation activity in the adult state was twice compared with that in the newborn state (Table 2). This increase in the translocation activity was caused by the enrichment of ANT protein in mitochondria as indicated when the ANT activity was referred to the ATR-binding sites. Moreover, the exchangeable (ATP + ADP)-pool in rat brain mitochondria increased only slightly from the newborn to the adult state. For comparison, the content and the translocation activity of the ANT was also measured in rat heart mitochondria (Table 2). Despite the fact that in rat heart mitochondria the content of ANT is 3-fold higher than in rat brain mitochondria, both types of mitochondria exhibit the same translocation activity when normalized on the ANT protein content.

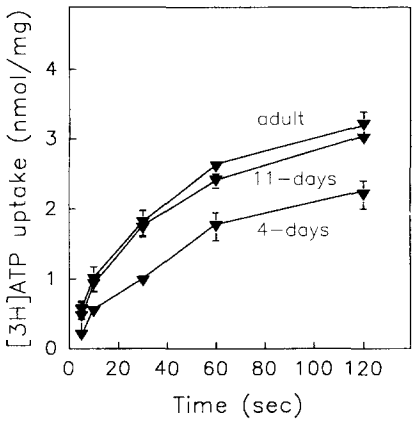

Fig. 3. Time-course of the [^{3}H]ATP uptake by brain mitochondria. Measurement of [^{3}H]ATP uptake by mitochondria was done as described in Section 2. Each data point is the mean ± S.D. of four separate incubations.

3.4. Control of adenine nucleotide translocator on State 3 respiration

The effect of the ANT enrichment on the control of the ANT over State 3 respiration was studied by titrating the

Table 2
Effect of postnatal mitochondrial maturation on exchangeable AdN pool size, ANT content and ANT activity

Age (days)	(ATP-ADP)-pool size (nmol/mg protein)	ANT content (nmol/mg protein)	ANT activity (nmol/min per mg protein)	normalized ANT activity (nmol/min per nmol ATR)
RBM				
0	2.70 ± 0.68 [a] (n = 5)	0.123 ± 0.022 [b] (n = 4)	3.88 ± 1.43 [b] (n = 5)	32
4	2.92 ± 0.87 (n = 5)	0.164 ± 0.025 [b] (n = 6)	5.00 ± 1.40 [a] (n = 5)	30
11	3.49 ± 0.59 (n = 3)	0.179 ± 0.028 [b] (n = 5)	6.95 ± 0.64 (n = 3)	39
adult	3.56 ± 0.51 (n = 5)	0.233 ± 0.011 (n = 4)	7.04 ± 0.81 (n = 5)	30
RHM(adult)	10.7 ± 1.33 (n = 5)	0.609 ± 0.047 (n = 5)	16.4 ± 1.09 (n = 4)	27

The experiments were performed as described in Section 2. Mitochondria from rat heart (RHM) were prepared as described in Ref. [20]. Values shown are means ± S.D. of n mitochondrial preparations. Values significantly different with respect to adult rat brain mitochondria: [a] $p = 0.05$; [b] $p = 0.01$.

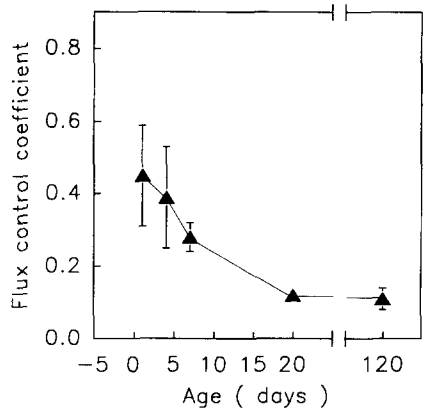

Fig. 4. Developmental change of the flux control coefficient of the ANT. Stationary rates of State 3 respiration weres adjusted by addition of 5 mM ADP. Incremental amounts of CAT were added and changes in the respiration were recorded by means of a rate-meter. Values of the flux control coefficient were estimated graphically from the titration curves [13]. The values given are the mean ± S.D. of three separate mitochondrial preparations.

State 3 respiration with the irreversible ANT inhibitor carboxyatractyloside. From these data the flux control coefficients of the ANT were estimated [13] and were plotted vs. the postnatal age of rat brain mitochondria (Fig. 4). The ANT had a greater control on State 3 respiration in mitochondria isolated from 1- and 4-day old rat brain than in mitochondria from 3-weeks old and adult rat brain. This indicates that the control of ANT over State 3 respiration declined during postnatal development. The value of flux control coefficient of ANT found in mitochondria from adult brain was lower (0.11) than in liver [13,14], but similar to heart and skeletal muscle [15,16].

4. Discussion

Despite of the fundamental role of the adenine nucleotide translocation in the cellular energy metabolism, its postnatal development in the brain was not studied. The results obtained in the present study indicate that the expression of ANT in rat brain plays a similar role for the development of oxidative phosphorylation as previously demonstrated for liver [10]. In contrast to rat liver [10,13,14], ANT exerts in the adult rat brain a lower control over respiration. In rat liver mitochondria State 3 respiration is fully developed after the first day of extrauterine life [8,21,22] and the expression of the adult level of ANT requires about the same time [11]. However, in rat brain mitochondria the full expression of ANT demands 3 weeks after birth, whereas the development of the maximal capacity of oxidative phosphorylation requires about 6 weeks [1]. This indicates that in rat brain mitochondria other proteins limit the respiration during later postnatal period. A late-developing protein within the system of oxidative phosphorylation in rat brain mitochondria is the pyruvate dehydrogenase [1,2]. The full achievement of the pyruvate dehydrogenase activity coincides with the development of the maximal State 3 respiration [1].

The increase of ANT content in rat brain results from the simultaneous increase of the number of mitochondria in the tissue and the ANT enrichment in mitochondria. It has been demonstrated that the Ficoll gradient method allows to isolate mitochondria which are nearly free from cytosolic proteins (e.g., lactate dehydrogenase) and non-mitochondrial membrane proteins (e.g., acetylcholine-esterase), irrespective of the postnatal age [1]. The present paper shows that there is a twofold increase of the ANT content in rat brain mitochondria (Fig. 2). In the adult rat, the content of ANT protein in brain mitochondria (233 ± 11 pmol/mg protein) is lower than in heart mitochondria (610 ± 47 pmol/mg protein), but higher than in liver mitochondria (125 ± 15 pmol/mg protein [11]). The enrichment of the ANT in brain mitochondria is paralleled by a twofold increase of the ANT translocase activity.

Recently, a postnatal increase of the activities of respiratory chain complexes I–IV has been described [5,7]. In the first three weeks of extrauterine life, the activities of the complexes I–IV increased three- to fourfold. After that stage, there was a further increase in the activities of the complexes II–IV, whereas that of complex I declined slightly. On the other hand, the finding that the capacity of uncoupled respiration exceeds that of State 3 respiration as we have found in all postnatal ages (Table 1) is generally considered to indicate that ANT and ATP synthase limit the electron transport rate in State 3 [8,12]. An enhancement of the activity of ATP synthase during the maturation of rat brain mitochondria has been reported [5,7]. However, the available data give no clear picture to what extent its activity increased during the postnatal period. According to a previous study [7] the activity of ATP synthase increased from birth to adultness by 20%, but in a very recent study a 2-fold increase until day 10 of postnatal life has been reported [5].

From rat liver mitochondria was reported that the total content of mitochondrial adenine nucleotides increased within two hours after birth from 3 to 11 nmol/mg protein [8]. From this and other observations was postulated that in addition to the enrichment of mitochondrial proteins the postnatal accumulation of adenine nucleotides in the matrix compartment plays an essential role in the development of oxidative phosphorylation [6,7]. The importance of mitochondrial adenine nucleotides for the achievement of the maximal capacity of oxidative phosphorylation is supported by two additional findings, namely that the depletion of adenine nucleotides in adult rat liver mitochondria diminishes the State 3 respiration and that a diminution of the exchangeable (ATP + ADP)-pool by the conversion of ATP into AMP slows down the translocation activity [19,23–25]. The data obtained in the present study show

that in rat brain mitochondria the size of the exchangeable (ATP + ADP)-pool increased only slightly between birth and adultness. The small exchangeable (ATP + ADP)-pool seems to be a feature of rat brain mitochondria since a much larger exchangeable adenine nucleotide pool was estimated when the same procedure was applied to heart mitochondria (Table 2). In addition, a minor role of adenine nucleotides in the development of adenine nucleotide translocation activity is also supported by the finding that normalized translocation activity obtained by referring the translocation activity to values irrespectively of the postnatal age. The postnatal increase of the translocation activity reported here can be explained by a sole increase of the ANT content in the inner mitochondrial membrane. Therefore, it is not very likely that an accumulation of adenine nucleotides from the cytosol into the matrix contributes significantly to the postnatal increase of State 3 respiration in rat brain mitochondria. Moreover, in liver mitochondria from newborn rabbits the adenine nucleotide content increased in the first two postnatal hours twofold, but no increase of the State 3 respiration was seen during the same period [26]. This observation has been explained by a threshold function of the mitochondrial adenine nucleotide content for the achievement of maximal State 3 respiration.

The flux control theory developed by Kacser and Burns, and Heinrich and Rapoport, is a suitable tool to gain deeper insight into the relative importance of a protein during the postnatal development of metabolic processes [27,28]. According to the 'control theory' all the proteins involved in oxidative phosphorylation also contribute to the control and the sum of all individual control coefficient is one (flux summation property). A flux control coefficient of about 0.5 at the newborn stage indicates a high contribution of the ANT to the control of State 3 respiration (Fig. 4). It shows that 50% of the total control over State 3 respiration has the ANT, whereas all other proteins share the other 50%. When the process of mitochondrial maturation proceeds the control of the ANT over respiration declined. Interestingly, a similar observation was found in developing liver mitochondria [10]. In the adult rat brain, the control exerted by the ANT is low and comparable with that reported for heart and skeletal muscle mitochondria [15,16].

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