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# Activation of c-Jun-N-terminal kinase and decline of mitochondrial pyruvate dehydrogenase activity during brain aging

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ABSTRACT

**Mitochondrial dysfunction is often associated with aging and neurodegeneration. c-Jun-N-terminal kinase (JNK) phosphorylation and its translocation to mitochondria increased as a function of age in rat brain. This was associated with a decrease of pyruvate dehydrogenase (PDH) activity upon phosphorylation of the $E_{1\alpha}$ subunit of PDH. Phosphorylation of PDH is likely mediated by PDH kinase, the protein levels and activity of which increased with age. ATP levels were diminished, whereas lactic acid levels increased, thus indicating a shift toward anaerobic glycolysis. The energy transduction deficit due to impairment of PDH activity during aging may be associated with JNK signaling.**


## 1. Introduction

Aging is marked by a general decline of physiological functions, including a pronounced effect on brain activities, such as neuromuscular coordination, cognitive performance, and environmental awareness [1]. The decrease in these neurological activities during normal brain aging has been found to be related to oxidative stress [2], mitochondrial dysfunction [1] (mitochondria have become a focal point of the free radical theory of aging [3]), and dysregulation of cell redox signaling [4–6].

Within these notions, c-Jun-N-terminal kinase (JNK) – a stress-activated protein kinase (SAPK) [7–10] and a member of the mitogen-activated protein kinase (MAPK) subfamily – is considered to be a central signal transducer in neuronal death in the mammalian brain [11] and, among others, functions as a signal transducer that conveys cytosolic oxidative stress signals to mitochondria [12]. Oxidative stress-induced JNK activation entails the phosphorylation of its threonine and tyrosine residues at specific positions by upstream JNK kinases (MAP kinase kinases, MKK). Three major isoforms of JNK have been identified: JNK1 and JNK2 are expressed ubiquitously, whereas the expression of JNK3 appears to be limited to the brain, heart, and testis [13,14]. These three JNK isoforms exhibit differences in specificity toward substrates and binding proteins and in their regulation by upstream kinases and scaffold proteins [13,15]. The activation (phosphorylation) of JNK leads to its translocation to the outer mitochondrial membrane, from where it triggers a phosphorylation cascade affecting different mitochondrial targets [12,16]. Activation of JNK pathways was also shown to enhance neuronal cell death in cultured primary neurons and, conversely, JNK knockout mouse models show protection against excitotoxicity, MPTP, and hypoxia [17–20]. The activity of JNK is significantly increased in the brains of patients with Parkinson's or Alzheimer's disease [21,22].

Mitochondria play a key role in brain aging, as these organelles are (a) the sites of energy transduction, (b) major cellular sources of oxidants, (c) targets for radical damaging effects, and (d) sources of 'redox' signaling molecules and pro-apoptotic factors [23]. Pyruvate dehydrogenase plays a fundamental role in mitochondrial bioenergetics, for this enzyme complex bridges the anaerobic and aerobic brain energy metabolism, and it is the entry point of carbohydrates into the tricarboxylic acid cycle in the form of acetyl-CoA units. The activity of the pyruvate dehydrogenase complex is regulated at different levels, one of them being phosphorylation/dephosphorylation: phosphorylation by specific pyruvate dehydrogenase kinases (PDK) leads to inactivation of the complex, where

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dephosphorylation (catalyzed by a specific phosphatase) to its reactivation. Previous work from this laboratory showed that activation of JNK by $H_2O_2$ or anisomycin in primary cortical neurons led to its translocation to mitochondria [12] and that the mitochondrion-associated active JNK induced phosphorylation of the pyruvate dehydrogenase and, thereby, its inhibition.

The goals of this study work are to assess (i) JNK activation in brain as a function of age, (ii) the ensuing translocation to mitochondria, (iii) JNK-mediated modulation of mitochondrial bioenergetics, and (iv) the physiological consequences inherent in these processes.

## 2. Materials and methods

### 2.1. Materials

Antibodies against JNK1, JNK2, PDK-2 and COX were purchased from Santa Cruz Biotech (Santa Cruz, CA). Antibodies against JNK3 and pJNK were from Upstate Biotechnology (Waltham, MA). Antibody against the PDH-$E_{1\alpha}$ was from Mitoscience (Eugene, OR). All other chemicals or reagents were obtained from Sigma–Aldrich (St. Louis, MO).

### 2.2. Animals

Male Fisher 344 rats of different ages (6, 14, and 24 months) were from the National Institute on Aging (Baltimore, MD). Each rat was individually caged in the animal facility under standard conditions (12 h light/12 h dark cycle, humidity at 50 ± 15%, 22 ± 2 °C, and 12 air changes/h) for 3 days to recover from the shipment stress.

### 2.3. Isolation of rat brain mitochondria

Whole brain mitochondria were isolated from adult male Fisher rats by differential centrifugation followed by discontinuous Percoll density-gradient centrifugation [24]. Brains were excised, rinsed, and homogenized using a Dounce homogenizer in isolation buffer (250 mM sucrose, 20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, protease inhibitor (100 μl per brain), 0.5% bovine serum albumin (BSA), pH 7.4). The homogenate was centrifuged at 1330×*g* (5 min) to remove nuclei and cell debris and the resulting supernatant was centrifuged at 21 200×*g* (10 min). The pellet was resuspended in 15% Percoll and was centrifuged 21 000×*g* for 10 min. The resulting loose pellet was layered onto a preformed discontinuous Percoll gradient and centrifuged at 31 000×*g* for 10 min. Mitochondrial fractions were collected and washed twice with isolation buffer followed by washing in BSA-free isolation buffer.

The purity of the mitochondrial fraction was assessed as previously described [12] by measuring markers of microsomal (NADPH-cytochrome P450 reductase) and cytosolic (lactic dehydrogenase, β-actin) contamination. Activities of NADPH-cytochrome P450 reductase and lactic dehydrogenase were negligible when compared to those in the crude homogenate. β-Actin was absent in the mitochondrial fraction when assessed by immunoblot analysis (shown in Fig. 2).

### 2.4. SDS–PAGE gel and immunoblot analysis

Mitochondria or total brain homogenate were lysed in RIPA buffer containing Tris–HCl (50 mM), NP-40 (1%), sodium deoxycholate (0.25%), NaCl (150 mM), EDTA (1 mM), pH 7.4. Samples (50 μg/well) were denatured at 95 °C for 5 min; separated on 12% SDS–PAGE gels and electro-transferred to a PVDF membrane (Millipore, Billerica, MA). Membranes were blocked with casein (Pierce, Rockford, IL) and then incubated with specific antibodies at concentrations indicated by the manufacturers. Chemiluminescence detection was used to visualize protein bands. The bands of interest were quantified by Scion Image beta 4.0.2.

### 2.5. Pyruvate dehydrogenase (PDH) activity assay

For PDH activity measurements, mitochondria were sonicated (30 s, setting of 3.0, 100% pulse rate) in a buffer containing 35 mM $KH_2PO_4$, 5.0 mM $MgCl_2$, 2.0 mM NaCN, 0.5 mM EDTA, 0.25% Triton X-100, and phosphatase inhibitor at pH 7.25. PDH activity was assayed at 37 °C by measuring the reduction of $NAD^+$ at 340 nm upon supplementation of 50 μg mitochondrial protein/ml with 0.5 mM $NAD^+$ in the presence of 200 μM TPP, 40 μM coenzyme A, and 4.0 mM pyruvate. The assay was carried out in the presence of 2.5 μM rotenone to prevent NADH consumption by complex **I**.

### 2.6. Pyruvate dehydrogenase kinase (PDK) activity assay

PDK activity was measured by a two-step immunocapture plus spectrophotometric assay.

#### 2.6.1. Immnuocapture of PDK-2

α-PDK-2 (5 μg) was attached onto each well of Protein-G coated 96-well immunoprecipitation plate (Pierce, Rockford, IL) by 1 h at room temperature. Brain mitochondrial lysate (50 μg) from rats of different ages (6, 14, and 24 months) was then incubated in the well for 2 h at room temperature to facilitate PDK-2 immunocapture onto the well.

#### 2.6.2. In-well PDK-induced PDH phosphorylation

Mitochondrial lysate (100 μg) from brain of 6-months old rats was diluted into phosphorylation buffer (100 μl) containing 30 mM HEPES, 1.5 mM $MgCl_2$, 0.05% Triton X-100, 0.1 mM EDTA, 5 mM DTT, 0.5 mM ATP, proteases inhibitor and phosphatases inhibitor. The lysate was then incubated in the α-PDK-coated well for 5 min at 30 °C. Aliquots (25 μl) were removed from the plate and PDH activity was measured as mentioned above. PDK activity was expressed as percentage of inhibition of PDH activity.

### 2.7. 2D gel and LC/MS/MS

#### 2.7.1. 2D gel

Mitochondrial protein was separated by isoelectric point (pI) on precast gel strips (17 cm) with a pH gradient of 3–10 (Bio-Rad, Hercules, CA) by using the Bio-Rad Protean IEF System [12]. The gels were fixed overnight and stained with Pro-Q® Diamond phosphoprotein gel stain (Molecular Probes, Eugene, OR) then imaged using a VersaDoc™ imaging system (Bio-Rad). The same gels were then stained with SYPRO™ Ruby protein gel stain (Molecular Probes) and imaged again using a VersaDoc™ imaging system. Density of protein spots were quantified by Scion Image beta 4.0.2. Proteins of interest (candidate spots of $E_{1\alpha}$-PDH: based on the molecular weight, pi value, and phosphorylation signals) were excised from the 2D gel using biopsy punches (Acuderm, Fort Lauderdale, FL) and subjected to LC/MS/MS (USC Proteomics Core Facility).

#### 2.7.2. LC/MS/MS

Samples were reduced with DTT and then alkylated with iodoacetamide prior to in-gel tryptic digestion using trypsin that was reductively methylated to reduce autolysis (Promega, Madison, WI). The digestion product were extracted twice from the gel with 5% formic acid/5% acetonitrile solution and once with

acetonitrile followed by evaporation using an ADP SpeedVac (Thermo Savant, Watham, MA). Tryptic peptides were analyzed by tandem mass spectrometry. Protein identification was carried out with the MS/MS search software Mascot 1.9 (Matrix Science).

### 2.8. Lactic acid and ATP concentration measurements

Total brain homogenates were lysed in an equal volume of perchloric acid (2 M) and centrifuged for 10 min at 12 000×*g*. Supernatants were neutralized with $KHCO_3$ (3 M) and recentrifuged at 12 000×*g*. Extracts (50 μl) were added to 500 μl of reaction buffer and the concentration of lactic acid was measured using a lactic acid assay kit (r-Biopharm, Germany). Samples for ATP measurements were prepared as described above; ATP levels were determined using an ATP determination kit (Molecular Probes).

### 2.9. Statistical analysis

Data are expressed as the means ± S.E. of at least three independent experiments. Statistical comparisons were performed by one way ANOVA. Differences were considered significant when $P < 0.05$.

## 3. Results

### 3.1. Increased pJNK association with mitochondria as a function of age

The basal levels of JNK1, JNK2, JNK3, and phosphorylated JNK (pJNK, reflecting activation) in brain homogenate from rats of different ages (6, 14, and 24 months) are shown in Fig. 1 ($n \geqslant 5$). The expression level of JNK1, JNK2, and JNK3 in the brain homogenates did not change significantly with age (Fig. 1A–C). JNK activation, determined by antibodies against pJNK (dual phosphorylation of JNK is essential for kinase activity), increased with age (Fig. 1D). The increased pJNK level reflects an increase in the activation of all three JNK isoforms and is an effect consistent with previous findings showing that JNK activity was constitutively high and significantly increased in older rats [25].

Activation (phosphorylation) of JNK under defined stress conditions results in its partial translocation to mitochondria [12]. Immunoblot analyses showed that protein levels of JNK2 and JNK3 associated with rat brain mitochondria did not change significantly as a function of age (6-, 14-, and 24-months old rats) (Fig. 2B and C; ($n \geqslant 5$)), whereas those of JNK1 and phosphorylated JNK (pJNK) associated with mitochondria increased as a function of age (Fig. 2A and D; ($P < 0.05$, $n \geqslant 5$)).

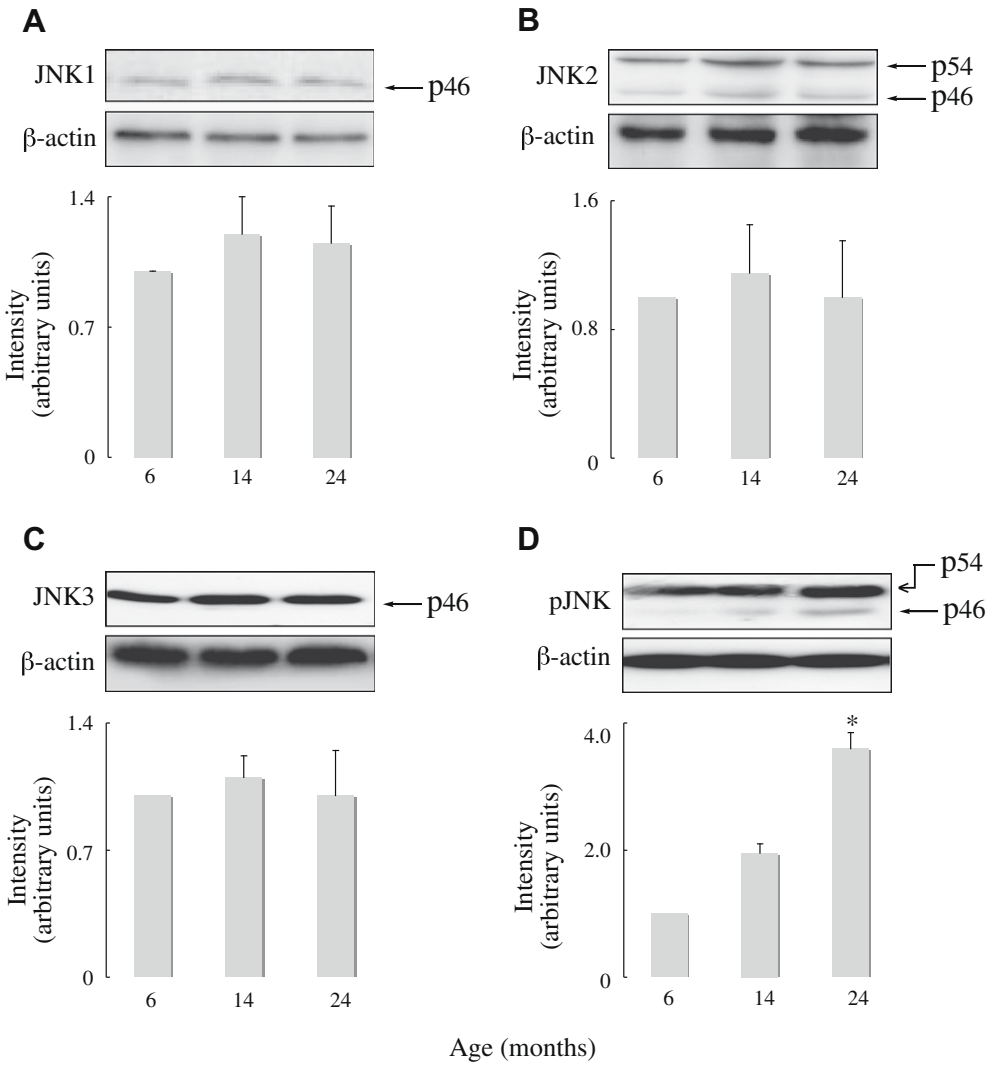



**Fig. 1.** JNK protein levels and activation (pJNK) in rat brain during aging. (A) JNK1, (B) JNK2, and (C) JNK3 protein levels were detected by immunoblot using different antibodies in brain homogenates from rats of different ages (6, 14, and 24 months). (D) Activity (dual phosphorylation) was assessed with an antibodies against pJNK. β-Actin levels shown as a control of equal loading (*$P < 0.05$).

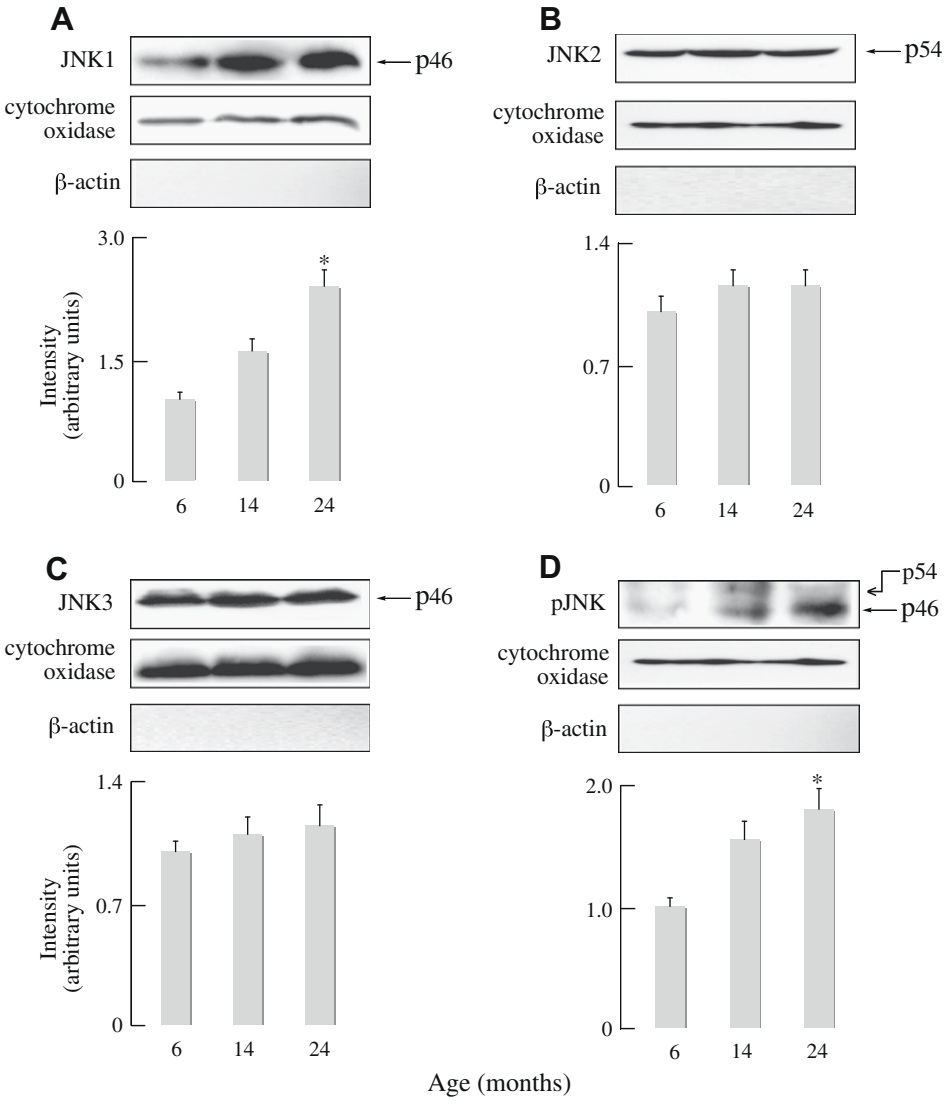



**Fig. 2.** Association of JNK with mitochondria and its activity in rat brain during aging. Brain mitochondria (50 μg) isolated from rats of different ages (6, 14, and 24 months) were subjected to immunoblotting. Levels of (A) JNK1, (B) JNK2, (C) JNK3, and (D) pJNK were detected in mitochondrial proteins by immunoblot using different antibodies. COX levels are shown as a control for equal loading. Experimental conditions as described in Section 2 ($^{*}P < 0.05$).

### *3.2. Increased pJNK association with mitochondria and inhibition of pyruvate dehydrogenase (PDH) activity: role of pyruvate dehydrogenase kinase-2 (PDK-2)*

As previously reported [12], pJNK associated with the outer mitochondrial membrane and triggered a phosphorylation cascade that resulted in the inhibition (phosphorylation) of mitochondrial matrix pyruvate dehydrogenase (PDH) activity. Accordingly, PDH activity in brain mitochondria decreased significantly as a function of age: decreases in activity of ~25% and ~45% were found in mitochondria from 14- and 24-months old rats, respectively (Fig. 3A, ($P < 0.01$, $n \geqslant 5$)).

PDH activity is, in part, controlled by phosphorylation/dephosphorylation, where a specific pyruvate dehydrogenase kinase (PDK) phosphorylates three serine residues of PDH-$E_{1\alpha}$, thereby decreasing the oxidative decarboxylation of pyruvate to acetyl-CoA. Because pJNK associates with the outer mitochondrial membrane (without crossing into the mitochondrial matrix), it may be surmised that PDK is the effector that ultimately conveys the inhibitory signal from pJNK to PDH. Protein levels of PDK-2, the most abundant PDK isoenzyme in brain, increased during aging with significantly higher levels at 14- and 26-months as compared to 6 months (Fig. 3B). Furthermore, PDK activity (Fig. 3C) correlated with its protein levels: the PDK-2-dependent inhibition of PDH activity amounting to ~20%, 43%, and 49% at 6-, 14-, and 24-months of age, respectively. Taken together, it may be inferred that increased phosphorylated JNK association to mitochondria may up-regulate PDK activity, thus causing increased phosphorylation (and inhibition) of PDH.

### *3.3. 2D gel – LC/MS/MS analyses of pyruvate dehydrogenase subunit $E_{1\alpha}$*

Inhibition of PDH activity upon translocation of pJNK to mitochondria was reported to be a consequence of phosphorylation of the $E_{1\alpha}$ subunit of the pyruvate dehydrogenase complex [12]. The decrease of PDH activity as a function of age is likely to involve a similar inactivation by phosphorylation; to validate this notion, phosphorylated and non-phosphorylated forms of PDH-$E_{1\alpha}$ were identified on 2D gels by staining mitochondrial proteins with Sypro-ruby stain (Fig. 4A) and Pro-Q® Diamond stain (Fig. 4B), respectively. Spots *a*, *b*, and *c* (Fig. 4A and B) were subjected to

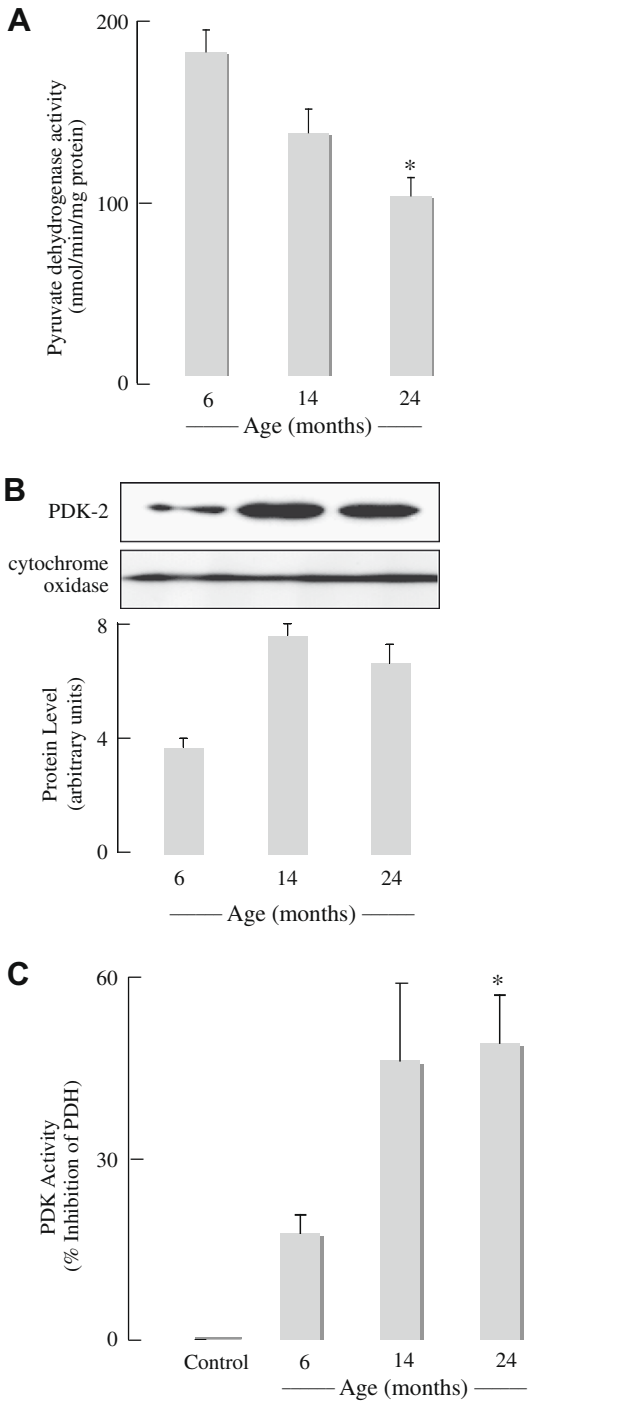



**Fig. 3.** PDH activity declines whereas PDK level and activity increases with age in rat brain. (A) PDH activity. (B) PDK-2 protein levels; COX levels are shown as a control for equal loading. (C) PDK activity expressed as percentage of inhibition of PDH activity. Experimental conditions as described in Section 2 ($^{*}P < 0.01$).

LC/MS/MS analyses in order to identify the proteins present. In each case the major species identified was pyruvate dehydrogenase subunit $E_{1\alpha}$. In the three analyses, 8-, 5-, and 6-peptides were identified with high confidence (Mascot score with $P < 0.5$) and corresponded to 25%, 18%, and 12% coverage of the protein, respectively. A representative tryptic peptide that was observed in each LC/MS/MS analysis was LPCIFICENNR, corresponding to amino acids 218–228 (Fig. 4C). The theoretical mass for the double charged peptide with two carbamidomethyl cysteine modifications (*i.e.*, alkylation by iodoacetamide) was 718.3. Masses of 718.2, 718.0, and 718.9 were observed in the three runs for peptides identified as LPCIFICENNR. The collision induced dissociation ($m/z$) spectrum is presented for the mass of 718.9 in Fig. 4D. This result was consistent with our previous report [12] in which we found that proteins spots *b* and *c* were the two major phosphorylated mitochondrial proteins and were identified as PDH-$E_{1\alpha}$.

### *3.4. Phosphorylation of pyruvate dehydrogenase subunit $E_{1\alpha}$ as a function of age*

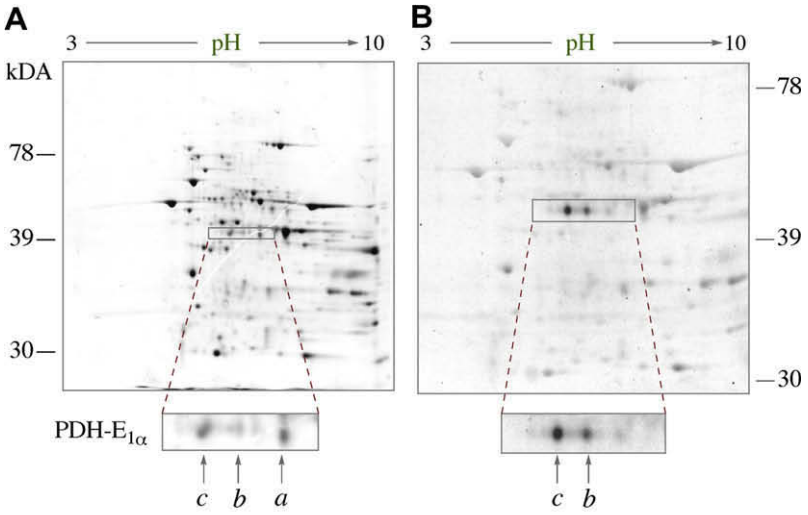
The percentage of phosphorylated PDH-$E_{1\alpha}$ was quantified based on the protein amount on 2D gels and was increased significantly during aging from 14- to 24-months; the percentage of the non phosphorylated form -the actual functional form- decreased during aging (Fig. 5, ($n \geqslant 3$, $P < 0.05$)). The total amount of PDH-$E_{1\alpha}$ did not change during aging (data not shown). Therefore, it can be concluded that the amount of the non-phosphorylated form of PDH-$E_{1\alpha}$ (the active form) decreases during aging (Fig. 5), thus accounting for the decrease in PDH activity as a function of age (Fig. 3A).

### *3.5. Decreased PDH activity and levels of ATP and lactic acid*

PDH activity links glycolysis to the tricarboxylic acid cycle where reducing equivalents in the form of NADH and $FADH_2$ are generated. Accordingly, an adequate PDH activity is particularly important for tissues to maintain a reducing environment and high ATP production. Inhibition or a decrease activity of PDH may lead to an increase in the anaerobic reduction of pyruvate to lactate via lactic dehydrogenase. To assess these metabolic effects related to PDH inhibition, levels of ATP and lactic acid were measured in brain homogenates from rats of different ages. A decrease in ATP levels along with a significant increase of lactic acid concentration was observed in rat brain homogenates as a function of age (Fig. 6; $n \geqslant 4$, $P < 0.05$). These metabolic patterns may be ascribed to the aforementioned inhibition of the PDH complex during aging.

## 4. Discussion

The age-dependent decrease and increase in ATP production and lactate accumulation, respectively, in brain tissue (Fig. 6) appear to represent a shift from aerobic glycolysis (mitochondrial pyruvate dehydrogenase-dependent) to anaerobic glycolysis (cytosolic lactate dehydrogenase dependent). The mechanistic implications of this shift are primarily based on the inactivation by phosphorylation of the $E_{1\alpha}$ subunit of mitochondrial matrix pyruvate dehydrogenase with the consequent diminished metabolism of acetyl-CoA. Within the context of this study, the impairment of pyruvate dehydrogenase activity may be associated with JNK signaling: endogenous JNK activation, especially at a low level, may reflect a chronic and cumulative stress process that contributes to mitochondrial dysfunction during brain aging. These data showed that JNK1 activation and its translocation to and association with mitochondria were significantly enhanced during aging, thus suggesting that the regulation of mitochondrial function by JNK is more potent in the aging brain. Of note, the ex vivo approach in this study (mitochondria isolated from brains of rats of different ages) shows a clear correlation – but not causality – between increase active JNK and PDK activities and decrease PDH activity over age; other experimental approaches, such as specific radioactive signals ($\gamma$-$^{32}$[P]ATP) in the mitochondrial proteome (2D IEF/SDS–PAGE) upon incubation of brain mitochondria

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| AA | Sequence | Mass 1 | Mass 2 | Mass 3 | Score 1 | Score 2 | Score 3 |
|---|---|---|---|---|---|---|---|
| 48 - 60 | LEEGPPVTTVLTR | NO | NO | 706.9<br>707.0<br>707.2 | NO | NO | 42<br>42<br>59 |
| 135 - 143 | AILAELTGR | 471.7 | NO | NO | 63 | NO | NO |
| 161 - 184 | NFYGGNGIVGAQVPLGAGIALACK | 1174.4 | 783.0 | NO | 43 | 67 | NO |
| 218 - 228 | LPCIFICENNR | 718.2 | 718.0 | 718.9 | 56 | 33 | 65 |
| 247 - 255 | RGDFIPGLR | NO | 515.8 | NO | NO | 31 | NO |
| 248 - 255 | GDFIPGLR | 437.5 | NO | NO | 39 | NO | NO |
| 256 - 265 | VDGMDILCVR | 596.8 | 597.2 | NO | 74 | 59 | NO |
| 280 - 290 | GPILMELQTYR | NO | NO | 669.6 | NO | NO | 48 |
| 305 - 313 | TREEIQEVR | 580.2 | NO | NO | 41 | NO | NO |
| 307 - 313 | EEIQEVR | 451.6 | NO | NO | 32 | NO | NO |
| 316 - 323 | SDPIMLLK | 466.5 | NO | NO | 42 | NO | NO |
| 326 - 338 | MVNSNLASVEELK | NO | NO | 725.8 | NO | NO | 54 |
| 326 - 345 | MVNSNLASVEELKEIDVEVR | 764.0 | NO | NO | 59 | NO | NO |

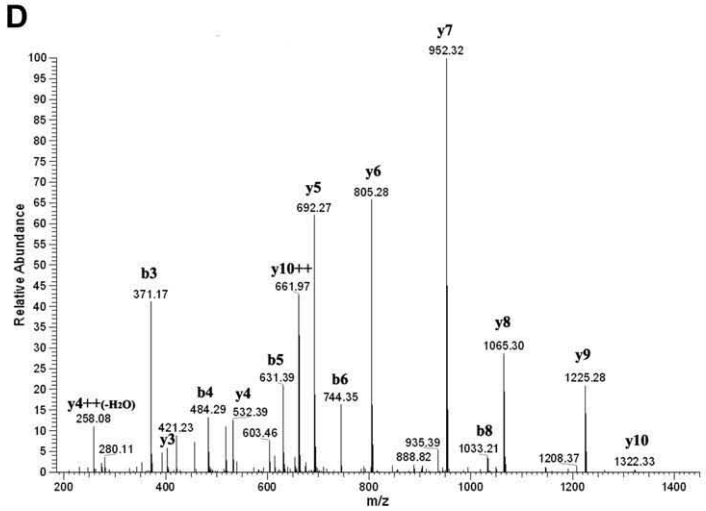


**Fig. 4.** Identification and localization of phosphorylated or non-phosphorylated forms of PDH-$E_{1\alpha}$ subunit. Mitochondrial proteins were separated on a 2D gel and subjected to (A) Sypro-ruby stain and (B) Pro-Q Diamond stain. Spots *a*, *b* and *c* were all identified as PDH-$E_{1\alpha}$ subunit by LC/MS/MS. Spots *b* and *c* showed the highest levels of phosphorylation. (C) Representative CID for pyruvate dehydrogenase peptide 218–228, LPCIFICENNR, which was observed in each of three analyses and contributed to the identification of the enzyme. (D) Peptides observed in three analyses, each of which identified pyruvate dehydrogenase based on multiple observations of tryptic peptides via LC/MS/MS. NO stands for not observed. Note that NFYGGNGIVGA QVPLGAGIALACK (161–184) was observed and identified as a double charged and triple charged peptide in analyses 1 and 2, respectively. Cysteines are all modified with iodoacetamide and mass values reflect an increased mass of 57 Daltons. Methionines are oxidized and have an increased mass of 16 Daltons.

with active recombinant JNK [16] strengthen the notion that JNK mediates PDH phosphorylation [12] as well as mitochondrial Bcl-2 and Bc1-$x_L$ [16].

Mitochondrial oxidative stress, JNK activation, and alterations of mitochondrial bioenergetics seem to be intimately linked: (a) mitochondrial $H_2O_2$ production increases with aging [26,27], (b)

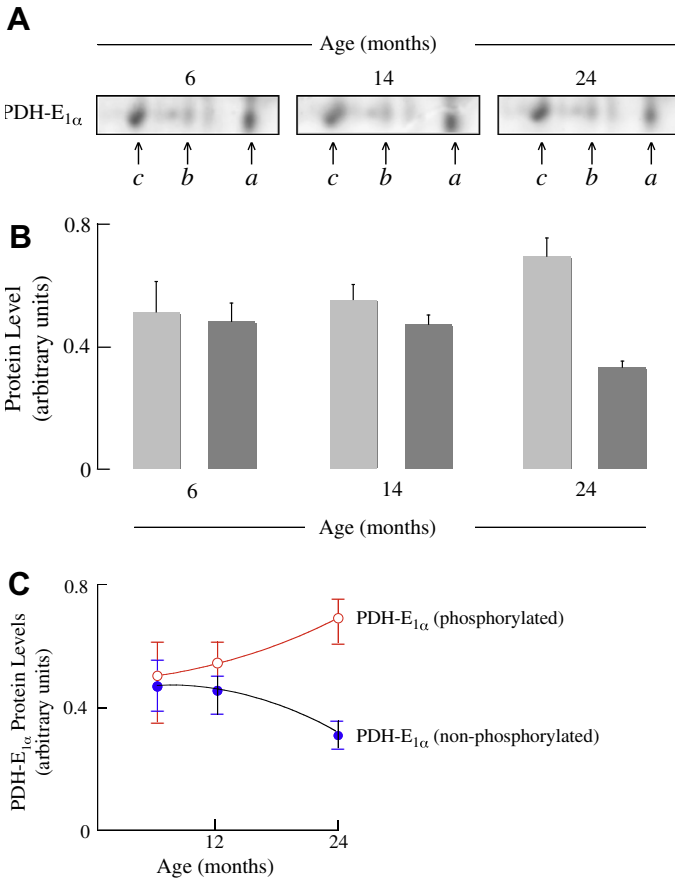


**Fig. 5.** Age-dependent changes in the ratio of phosphorylated and non-phosphorylated forms of PDH-$E_{1\alpha}$ subunit in rat brain. Proteins from brain mitochondria from rats of different ages (6, 14, and 24 months) were separated in 2D gels and stained with Sypro-ruby. (A) Spot *a* represents the non-phosphorylated form and spots *b* and *c* represent the phosphorylated forms of PDH-$E_{1\alpha}$ subunit. (B) The protein amounts of non-phosphorylated or phosphorylated forms of PDH-$E_{1\alpha}$ subunit were quantified based on the intensity of Sypro-ruby staining.

mitochondrially-released $H_2O_2$ leads to the activation of JNK [28] (by mechanisms likely entailing dissociation of JNK from glutathione transferase [15] or suppression of phosphatases involved in JNK inhibition [29,30]), (c) activation (phosphorylation) of JNK results in its translocation to the outer mitochondrial membrane [12], and (d) association of JNK with the outer mitochondrial membrane triggers phosphorylation cascades that affect mitochondrial bioenergetics and mitochondrion-driven apoptotic pathways [12,16] as shown by specific radioactive signals ($\gamma$-$^{32}$[P]ATP) on the mitochondrial proteome upon incubation of brain mitochondria with active recombinant JNK [16] and identification of Bcl-$x_L$ and Bcl-2 [16] and pyruvate dehydrogenase [12] as phosphorylation targets.

The above-mentioned effects represent a feedback loop that entails coordination of cytosolic and mitochondrial responses and a delicate balance that may be impaired during brain aging. It could be argued that JNK activation, especially at a low level, may reflect a chronic and cumulative stress that contributes to mitochondrial dysfunction during brain aging. Of note, the decline in pyruvate dehydrogenase activity in brain during aging is expected to be linked to decreased mitochondrial NADH levels and – via nucleotide transhydrogenase – to decreased NADPH reducing power, thereby diminishing $H_2O_2$ removal by the glutathione system.

The precise mechanism by which JNK leads to inactivation of PDH is not known: the pyruvate dehydrogenase complex is regulated by reversible phosphorylation of the $E_{1\alpha}$ subunit; pyruvate dehydrogenase kinase (PDK consists of four isozymes PDK1, PDK2, PDK3, and PDK4; among the four isoenzymes, PDK2 is the predominant form in brain [31]) is responsible for the phosphorylation (inactivation) [32], whereas a $Ca^{2+}$-sensitive phosphatase is responsible for the dephosphorylation (activation) [33,34]. Thus, at least two mechanism by which JNK may be involved in enhanced PDH phosphorylation can be envisaged: first, JNK upregulates PDK protein and enzymic activity and, second, JNK interacts with the $Ca^{2+}$ channels on mitochondria causing a reduction of $Ca^{2+}$ influx and deactivation of the $Ca^{2+}$-sensitive phosphatase. Although the former mechanism is somewhat suggested by the data in the present study, the underlying mechanism on how JNK may be modulating PDK remains unclear. Other signaling pathways may also contribute to increase PDK2 expression as a function of age.

Alterations of mitochondrial bioenergetics by MAPKs has been reported: in renal cells, the oxidant-induced activation of ERK1/2 decreases complex **I** activity, thereby diminishing mitochondrial respiration and energy transduction (ATP synthesis); pyruvate dehydrogenase activity was not affected [35]. Of note, ERK1/2 is present in mitochondria and exposure of renal cells to a tertiary hydroperoxide leads to activation (phosphorylation) of ERK1/2, which was not due to translocation of active ERK1/2 from cytosol to mitochondria [35]. ERK1/2, JNK, and p38 are present in mitochondria from different cell types: activation of mitochondrial ERK1/2 during rat brain development is apparently under the

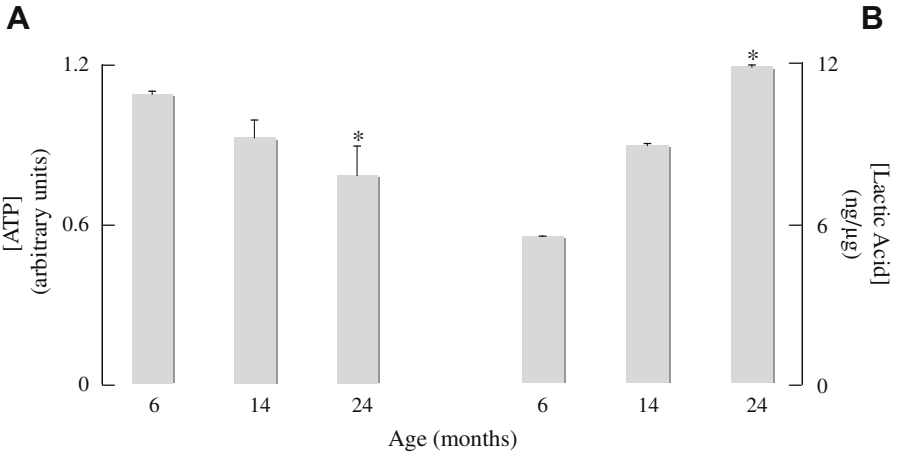


**Fig. 6.** Levels of ATP and lactic acid in rat brain as a function of age. Levels of (A) ATP and (B) lactic acid were measured as described in Section 2 in brain homogenates (50 μl) from rats of different ages (6, 14, and 24 months). Assay conditions as described in Section 2 ($^{*}P < 0.05$).

control of mitochondrial $H_2O_2$ levels [36]; mitochondrial protein kinase C $\varepsilon$ (PKC$\varepsilon$) forms signaling modules with ERK1/2, JNK, and p38 in murine heart with implications for cardioprotection [37]; studies of Lewy body disease neurons provided evidence for active ERK1/2 in mitochondria (colocalizing with Mn-superoxide dismutase) [38]. MAPKs are also involved in the regulation of the mitochondrion-driven apoptotic pathway by mechanisms entailing phosphorylation of Bcl-2 family members either cytosolic (and further translocation to mitochondria) or constitutive of the outer mitochondrial membrane (Bcl-$x_L$ and Bcl-2) [39,40].

The shift from aerobic glycolysis (mitochondrial pyruvate dehydrogenase-dependent) to anaerobic glycolysis (cytosolic lactic dehydrogenase-dependent) in brain during aging acquires further significance on two accounts: first, glucose is a primary energy source for mammalian brain and anaerobic glycolysis is an inefficient energy source and, second, pyruvate dehydrogenase was reported to be deficient in the brain of Alzheimer's disease patients [41–44]. This deficiency occurs not only in regions of brain that are neuropathologically damaged in Alzheimer's disease, but also in regions that are histopathologically normal, which suggests that the decreased PDH activity occurs in early stages of the disease [41–44]. Therefore, rescue of PDH activity in an early stage of Alzheimer's disease can be a therapeutic strategy: in this context, supplementation with lipoic acid – a naturally-occurring disulfide compound – may rescue the decreased PDH activity caused by the phosphorylation of $E_{1\alpha}$ – PDH. Acetyl-L-carnitine has also been purported as an agent for the treatment of early stages of Alzheimer's disease [45–47]: the presence of an acetylcarnitine-CoA transferase in the brain allows the entry of acetyl units from acetyl-L-carnitine into the TCA cycle [48]. This strategy should be viewed as an energy source other than that from the oxidative decarboxylation of pyruvate.

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