TABLES AND FIGURES
Table 1. Production of hydroxyl radicals by Fe$^{2+}$- ascorbate (2-deoxyribose degradation method)

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Absorbance at 532 nm (No. of observation = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Deoxyribose alone</td>
<td>0.015 ± 0.002</td>
</tr>
<tr>
<td>2-Deoxyribose + Ascorbate + Fe$^{2+}$</td>
<td>0.227 ± 0.010*</td>
</tr>
<tr>
<td>2-Deoxyribose + Ascorbate + Fe$^{2+}$ + Catalase</td>
<td>0.094 ± 0.008*</td>
</tr>
<tr>
<td>2-Deoxyribose + Ascorbate + Fe$^{2+}$ + Mannitol</td>
<td>0.077 ± 0.012*</td>
</tr>
<tr>
<td>2-Deoxyribose + Ascorbate + Fe$^{2+}$ + DMSO</td>
<td>0.061 ± 0.007*</td>
</tr>
<tr>
<td>2-Deoxyribose + Fe$^{2+}$ + H$_2$O$_2$</td>
<td>0.142 ± 0.006*</td>
</tr>
<tr>
<td>2-Deoxyribose + Fe$^{2+}$ + H$_2$O$_2$ + Catalase</td>
<td>0.054 ± 0.012*</td>
</tr>
<tr>
<td>2-Deoxyribose + Fe$^{2+}$ + H$_2$O$_2$ + Mannitol</td>
<td>0.065 ± 0.012*</td>
</tr>
<tr>
<td>2-Deoxyribose + Fe$^{2+}$ + H$_2$O$_2$ + DMSO</td>
<td>0.061 ± 0.012*</td>
</tr>
<tr>
<td>2-Deoxyribose + H$_2$O$_2$</td>
<td>0.040 ± 0.012*</td>
</tr>
</tbody>
</table>

Reactions were carried out with incubation mixtures containing 2-deoxyribose (1mM) with or without Fe$^{2+}$ (50μM) plus ascorbate (2mM) or Fe$^{2+}$ (50μM) plus H$_2$O$_2$ (500μM) or H$_2$O$_2$ (500μM) at 37°C in the presence or absence of other additions like, catalase (50μg/ml) or mannitol (20mM) or DMSO (20mM) as described in the text.

* Statistically significant, p<0.001 vs. control. ‡ Significantly different, p<0.001 vs. mitochondria + Fe$^{2+}$ + ascorbate. £ Significantly different, p<0.001 vs. mitochondria + Fe$^{2+}$ + H$_2$O$_2$, Student’s ‘t’ test, paired.

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FIGURES, TABLES AND LEGENDS
### Table 2. Hydroxyl radical formation from incubated Fe²⁺-ascorbate (Benzoate hydroxylation assay)

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Absorbance at 532 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoate alone (control)</td>
<td>294</td>
</tr>
<tr>
<td>Benzoate + Fe²⁺ + Ascorbate</td>
<td>1349</td>
</tr>
<tr>
<td>Benzoate + Fe²⁺ + Ascorbate + Catalase</td>
<td>224</td>
</tr>
<tr>
<td>Benzoate + Fe²⁺ + Ascorbate + Mannitol</td>
<td>404</td>
</tr>
<tr>
<td>Benzoate + Fe²⁺ + Ascorbate + DMSO</td>
<td>174</td>
</tr>
</tbody>
</table>

Benzoate (1mg/ml) was incubated for 1 h with or without Fe²⁺ (50μM) and ascorbate (2mM) at 37°C in the presence or absence of catalase (50μg/ml) or mannitol (20mM) or DMSO (20mM). Fluorescence of the reaction mixture was measured immediately after incubation ($\lambda_{ex}$ 305nm, $\lambda_{em}$ 407nm). The results are from one representative experiment repeated thrice.
Table 3. Lipid peroxidation in mitochondrial fraction exposed to Fe\textsuperscript{2+}-ascorbate

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Lipid peroxides (nmole MDA/ mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(No. of observation = 5)</td>
</tr>
<tr>
<td>Mitochondria alone (control)</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>Mitochondria + Fe\textsuperscript{2+} + Asc</td>
<td>25.25 ± 0.34*</td>
</tr>
<tr>
<td>Mitochondria + Fe\textsuperscript{2+} + Asc + BHT</td>
<td>1.94 ± 0.50v</td>
</tr>
<tr>
<td>Mitochondria + Asc</td>
<td>13.40 ± 0.24*</td>
</tr>
<tr>
<td>Mitochondria + Fe\textsuperscript{2+}</td>
<td>0.82 ± 0.20</td>
</tr>
<tr>
<td>Mitochondria + Fe\textsuperscript{2+} + H\textsubscript{2}O\textsubscript{2}</td>
<td>0.20 ± 0.30</td>
</tr>
<tr>
<td>Mitochondria + H\textsubscript{2}O\textsubscript{2}</td>
<td>0.25 ± 0.34</td>
</tr>
</tbody>
</table>

Incubation of mitochondria was performed in vitro without (control) or with Fe\textsuperscript{2+} (50µM) plus ascorbate (Asc, 2mM) or Fe\textsuperscript{2+} (50µM) plus H\textsubscript{2}O\textsubscript{2} (500µM) or H\textsubscript{2}O\textsubscript{2} (500µM) or ascorbate (Asc, 2mM) or Fe\textsuperscript{2+} (50µM) with or without addition of BHT (0.2mM) followed by the estimation of malonaldehyde (MDA) as described in the Materials and Methods. The values presented are the means ± SEM.

* Statistically significant, p<0.001 vs. control. v Significantly different, p<0.001 vs. mitochondria + Fe\textsuperscript{2+} + ascorbate, Student’s ‘t’ test, paired.
Fig 1. Immuno-detection of mitochondrial protein carbonyls after treatment with Fe$^{3+}$- ascorbate

Rat brain mitochondria were incubated with or without Fe$^{3+}$ (50μM) and ascorbate (2mM) in vitro for 1h at 37$^\circ$ in the presence or absence of BHT (0.2mM) followed by the detection of protein carbonyls by DNPH derivatization and immunoblotting using anti-DNP antibodies as described in the Materials and Methods.

Lane a: mitochondria incubated alone;
Lane b: mitochondria+Fe$^{3+}$+ascorbate+BHT;
Lane c: mitochondria+Fe$^{3+}$+ ascorbate.
Fig 2. Immuno-detection of mitochondrial protein carbonyls after oxidative stress

Rat brain mitochondria were incubated in vitro without (control) or with the presence of Fe^{2+} (50μM) plus ascorbate (Asc, 2mM) or ascorbate (Asc, 2mM) or Fe^{2+} (50μM) plus H_{2}O_{2} (500μM) or H_{2}O_{2} (500μM) with or without addition of BHT (0.2mM) for 1h at 37° followed by the detection of protein carbonyls by DNPH-derivatisation and immunoblotting using anti-DNP antibodies as described in the Materials and Methods.

Lane a: mitochondria incubated alone; Lane b: mitochondria + Fe^{2+} + H_{2}O_{2}; Lane c: mitochondria + H_{2}O_{2}; Lane d: mitochondria + Fe^{2+} + ascorbate + BHT; Lane e: mitochondria + ascorbate; Lane f: mitochondria + Fe^{2+} + ascorbate.
Fig 3. Cardiolipin loss in rat brain mitochondria during in vitro oxidative stress.

Incubation of mitochondria was performed in vitro without (control) or with Fe$^{2+}$ (50μM) and ascorbate (Asc, 2mM) in the absence or presence of other additions like of BHT (0.2mM) or DMSO (20mM) or mannitol (Man, 20mM) or benzoate (Bz, 20mM) or catalase (Cat, 50μg/ml) followed by the estimation of cardiolipin as described in the text. BHT was added from an alcoholic stock solution and at this concentration the added alcohol did not have any effect on mitochondrial cardiolipin content (data not shown). The values presented are the means ± SEM. * Statistically significant, p<0.001 vs. control. v Significantly different, p<0.001 vs. mitochondria + Fe$^{2+}$+ascorbate, Student’s ‘t’ test, paired.
Table 4. Cardiolipin content in rat brain mitochondria

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cardiolipin content (Relative fluorescence unit/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact mitochondria</td>
<td>0.182 ± 0.013</td>
</tr>
<tr>
<td>Intact mitochondria + 2mM KCN</td>
<td>0.184 ± 0.015</td>
</tr>
<tr>
<td>Lysed mitochondria</td>
<td>0.189 ± 0.019</td>
</tr>
</tbody>
</table>

Cardiolipin content were measured in freshly isolated intact mitochondria (mitochondria in isotonic buffer) incubated with pyruvate plus succinate (10mM each), with or without 2mM KCN and in lysed mitochondria in hypotonic buffer (10mM phosphate buffer, pH 7.4) without any substrate as described in the text. Values of nonyl acridine orange binding were expressed as relative fluorescence unit normalized to 1mg protein and as the means ± SEM of 5 observations.
Table 5. Effect of in vitro oxidative stress on brain mitochondrial complex I and complex IV activity

<table>
<thead>
<tr>
<th>Incubation protocol</th>
<th>Complex I activity (nmoles NADH oxidized/min/mg protein)</th>
<th>Complex IV activity (nmoles cytochrome c oxidized/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria incubated alone (control)</td>
<td>1509 ± 95</td>
<td>458 ± 25</td>
</tr>
<tr>
<td>Mitochondria + Fe^{2+} + Ascorbate</td>
<td>1440 ± 62</td>
<td>380 ± 22*</td>
</tr>
<tr>
<td>Mitochondria + Fe^{2+} + H$_2$O$_2$</td>
<td>1505 ± 90</td>
<td>470 ± 30</td>
</tr>
<tr>
<td>Mitochondria + H$_2$O$_2$</td>
<td>1544 ± 80</td>
<td>429 ± 23</td>
</tr>
</tbody>
</table>

Mitochondria were incubated in vitro without (control) or Fe$^{2+}$ (50μM) plus ascorbate (Asc, 2mM) or Fe$^{2+}$ (50μM) plus H$_2$O$_2$ (500μM) or H$_2$O$_2$ (500μM) for 1h at 37°, followed by the estimation of complex I and complex IV activities as described in the Materials and Methods. The values presented are the means ± SEM of 6 observations. * Statistically significant, p<0.001 vs. control, Student’s ‘t’ test, paired.
Table 6. Effect of radical scavengers and catalase on complex IV activity in mitochondria exposed to Fe²⁺-ascorbate

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Complex IV activity (nmoles cytochrome c oxidized/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria incubated alone (control)</td>
<td>480 ± 25</td>
</tr>
<tr>
<td>Mitochondria + Fe²⁺ + Asc</td>
<td>390 ± 26*</td>
</tr>
<tr>
<td>Mitochondria + Fe²⁺ + Asc + Catalase</td>
<td>405 ± 27*</td>
</tr>
<tr>
<td>Mitochondria + Fe²⁺ + Asc + Mannitol</td>
<td>381 ± 22*</td>
</tr>
<tr>
<td>Mitochondria + Fe²⁺ + Asc + DMSO</td>
<td>394 ± 31*</td>
</tr>
<tr>
<td>Mitochondria + Fe²⁺ + Asc + BHT</td>
<td>385 ± 30*</td>
</tr>
</tbody>
</table>

Incubation of rat brain mitochondria was performed in vitro without (control) or with Fe²⁺ (50μM) plus ascorbate (Asc, 2mM) in the presence or absence of BHT (0.2mM) or DMSO (20mM) or mannitol (Man, 20mM) or catalase (50μg/ml) followed by the estimation of complex IV activity as described in the Materials and Methods. The values presented are the means ± SEM of six observations.

* Statistically significant, p<0.001 vs. control, Student's 't' test, paired.
MTT-reduction by intact mitochondria (unincubated control and incubated control) was measured in the presence of NAD⁺-linked substrates (e.g. α-ketoglutarate, 10mM or pyruvate, 10mM) or FAD-linked substrate (e.g. succinate, 10mM). Values from unincubated controls (data not shown) were taken as 100%. Mitochondria were kept at 0°C (unincubated control) or incubated at 37°C in isotonic buffer (incubated control).

* Statistically different, p<0.001 vs. unincubated control, Student’s ‘t’ test, paired.
Rat brain mitochondria were incubated at 37°C in isotonic buffer in the absence (incubated control) or presence of Fe²⁺ (50μM) plus ascorbate (Asc, 2mM) or Fe²⁺ (50μM) plus H₂O₂ (500μM) or H₂O₂ (500μM) with or without other additions like BHT (0.2mM) or DMSO (20mM) or mannitol (Man, 20mM) followed by the measurement of MTT reduction in the presence of 10mM succinate as described in the Methodology. Values from incubated controls (data not shown) were taken as 100%. Each value expressed as percentage of control represents mean ± SEM of 6 observations. ■ Statistically significant, p< 0.001 vs. incubated control.
Fig 6. Confocal microscopic determination of mitochondrial membrane potential after oxidative stress with Fe\textsuperscript{2+}-ascorbate

Rat brain mitochondria were incubated alone (control) or with KCN (1mM) or with Fe\textsuperscript{2+} (50μM) and ascorbate (2mM) in the presence or absence of BHT (0.2mM) for 1h at 37°C. At the end of the incubation mitochondrial membrane potential was monitored by confocal microscopy using the cationic 'Mitosensor dye' as described in the Materials and Methods. a: control mitochondria; b: mitochondria+ Fe\textsuperscript{2+}-ascorbate; c: mitochondria+ Fe\textsuperscript{2+} ascorbate + BHT; d: mitochondria+ KCN.
Fig 7. Spectrofluorometric measurement of mitochondrial membrane potential after in vitro oxidative stress with Fe\(^{2+}\) ascorbate.

The incubation mixtures containing mitochondria in isotonic buffer A in the absence (control) or with KCN (1mM) or with Fe\(^{2+}\) (50\(\mu\)M) plus ascorbate (Asc, 2mM) with or without BHT (0.2mM) were kept at 37\(^\circ\)C for 1h. Mitochondrial membrane potential was measured spectrofluorometrically using JC-1 as described in the text. Values (relative fluorescence intensity in percentage), normalized to 1 mg protein are the means ± SEM of 6 observations. * Statistically significant, pc0.001 vs. control, ■ Statistically different, pc0.001 vs. iron + ascorbate, Student’s ‘t’ test, paired.
Rat brain genomic DNA was incubated without (control) or with Fe²⁺ (50µM) and ascorbate (Asc, 2mM) for various periods of time (5, 15, 30 and 60 min.) in the absence or presence of other additions like sodium benzoate (20mM) or mannitol (20mM) or catalase (50µg/ml) for 1h at 37°, followed by agarose gel electrophoresis as described in the text.

Lane a: control DNA; lanes b-e: DNA + Fe²⁺+ascorbate incubated for 5 min (b), 15 min (c), 30 min (d), 60 min (e); lanes f-h: DNA + Fe²⁺+ascorbate with mannitol (f) or sodium benzoate (g) or catalase (h).
Rat brain genomic DNA was incubated with Fe$^{2+}$ (50μM) and ascorbate (2mM) for varying periods of time up to 60min. in the presence or absence of mannitol (20mM) or catalase (50μg/ml) at 37°. DNA samples were subsequently processed and used for FADU assay as described in the Materials and Methods to assess the degree of DNA strand breakage. * Statistically significant p<0.001 vs. control.

# Significantly different, p<0.001 vs. DNA + Fe$^{2+}$ + ascorbate, Student’s 't' test, paired.

a: Time Course of DNA damage.

b: Effects of antioxidants on iron-ascorbate induced DNA strand breaks.
Genomic DNA isolated from rat brain was used to amplify a 923bp β-actin gene fragment by PCR as described in the Materials and Methods.

A. 15-60ng of template DNA was utilized to amplify the β-actin gene fragment and the product quantified by densitometric scanning and analysis by Biorad Quantity 1 software (Fig 10 a.).

Lane a: marker DNA (100bp DNA ladder), lanes b-d: 923bp β-actin gene fragment amplified from 15 (d), 30 (c) and 60 (b) ng of genomic DNA.
Rat brain genomic DNA was incubated without (control) or with Fe$^{2+}$ (50μM) and ascorbate (2mM) for varying periods of time up to 60min. in the presence or absence of mannitol (20mM) or catalase (50μg/ml) or sodium benzoate (20mM) and the DNA samples were processed and used for PCR amplification of beta-actin gene fragment as described in the Materials and Methods.

A. Time course of PCR inhibition

Lane a: marker DNA; lane b: control DNA; lane c-f: DNA + Fe$^{2+}$+ascorbate incubated for 5min. (c), 15min. (d), 30min. (e), 60min. (f).
B. Effects of antioxidants on PCR impairment by oxidative stress in vitro

Lane a: marker DNA; lane b: control DNA; lane c: DNA+ Fe$^{2+}$+ascorbate; lane d-f: DNA+ Fe$^{2+}$+ascorbate with mannitol (d) or benzoate (e) or catalase (f).

C. Band intensity of the PCR product (experiment B) in each lane was calculated and analyzed by BioRad Quantity 1 software and expressed as percentage of the control.

# Statistically significant, $p<0.001$ vs. control. * Significantly different, $p<0.001$ vs. DNA + Fe$^{2+}$ + ascorbate, Student's $t$ test, paired.

Fig 12. Effect of in vitro oxidative stress on PCR amplification of p53 gene fragment from rat brain genomic DNA

Rat brain genomic DNA was incubated without (control) or with Fe$^{2+}$ (50μM) and ascorbate (2mM) for varying periods of time up to 60min. in the presence or absence of mannitol (20mM) or catalase (50μg/ml) or benzoate (20mM) and the DNA samples were processed and used for PCR application of p53 gene fragment as described in the Materials and Methods. Lane a: marker DNA; lane b: control DNA; lane c: DNA+ Fe$^{2+}$+ascorbate; lane d-f: DNA+ Fe$^{2+}$+ascorbate with mannitol (d) or benzoate (e) or catalase (f).
Mitochondria isolated from young (4 to 6 months) and aged (20 to 24 months) rat brain were used for the detection of protein carbonyls by DNPH-derivatization and immunoblotting using anti-DNP antibodies as described in the Materials and Methods.
The total and organic solvent soluble fractions of fluorescent lipid peroxidation products of young and aged rat brain mitochondria were measured by the methods described in the text. The results are means ± SEM of 5 different observations.

* implies statistically significant difference with respect to young, p<0.001. Student’s ‘t’ test (unpaired).
Cardiolipin content in freshly isolated intact mitochondria (mitochondria in isotonic buffer) containing pyruvate plus succinate (10mM each), Cardiolipin were measured spectrofluorometrically using nonyl-acridine orange in brain mitochondria from young and aged rats of described in the text. The values are the means ± SEM of 6 observations. Statistical significance was calculated by Student’s ‘t’ test, unpaired. * p < 0.001 vs. young.
Table 7. Cardiolipin content in lysed preparation of young and aged rat brain mitochondria

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cardiolipin content (Relative fluorescence unit/ mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>0.192 ± 0.015</td>
</tr>
<tr>
<td>Aged</td>
<td>0.148 ± 0.015*</td>
</tr>
</tbody>
</table>

Cardiolipin was measured spectrofluorometrically using nonyl-acridine orange in lysed preparation of mitochondria isolated from young and aged rat brain in the absence of respiratory substrate as described in the text. The values are the means ± SEM of 6 observations. Statistical significance was calculated by Student’s ‘t’ test, unpaired. * p < 0.001 vs. young.
Fig 16. Reactive oxygen species generation in mitochondria isolated from young and aged rat brain

Reactive oxygen species generation was measured spectrofluorometrically using the dye H$_2$DCF-DA in brain mitochondria from young (4-6 months) and aged (20-24 months) rats as described in the text. The values are the means ± SEM of 6 observations.

Statistical significance was calculated by Student's 't' test, unpaired.

*p < 0.001 vs. young.
Table 8. Mitochondrial ETC activity in young and aged rat brain

<table>
<thead>
<tr>
<th>Sample</th>
<th>Complex I activity (nmole NADH oxidized/min/mg protein)</th>
<th>Complex IV activity (nmole cytochrome c oxidized/min/mg protein)</th>
<th>MTT reduction (A_{550} - A_{420} normalized to 0.1mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>1.742 ± 0.145 (9)</td>
<td>416 ± 22.5 (6)</td>
<td>0.86 ± 0.102 (6)</td>
</tr>
<tr>
<td>Aged</td>
<td>1.736 ± 0.150 (9)</td>
<td>363 ± 14.2* (6)</td>
<td>0.99 ± 0.107 (6)</td>
</tr>
</tbody>
</table>

Brain mitochondria were isolated from young and aged rats followed by the measurement of complex I and complex IV activities as well as MTT reduction ability as described in the Materials and Methods. Values are expressed as the means ± SEM of the number of observations given in parenthesis.

* Statistically significant, p<0.05 vs. young, Student's 't' test, unpaired.
Transmembrane potential of mitochondria from aged and young rat brain was monitored by confocal microscopy using the cationic 'Mitosensor dye' as described in the Materials and Methods. The experiment shown was representative of six experiments which gave similar results.
Mitochondrial potential was measured spectrofluorometrically in freshly isolated brain mitochondria from young and aged rats using the cationic dye JC-1 as described in Materials and Methods. The values are the means ± SEM of 6 observations.

Statistically significant, *p < 0.001 vs. young, Student’s ‘t’ test, unpaired.
Fig 19. Fluorescence-detected alkaline DNA unwinding (FADU) assay of genomic DNA from young and aged rat brains

Rat brain genomic DNA was isolated from young (4-6 months) and aged (20-24 months) rats and used for FADU assay to detect the degree of DNA strand breaks as described in the Materials and Methods. The values are the means ± SEM of 5 observations. Statistically significant, *p < 0.001 vs. young, Student’s 't' test, unpaired.
Fig 20. Amplification of β-actin and p53 gene fragments from DNA isolated from young and aged rat brain

A. B.

Rat brain genomic DNA was isolated from young (4-6 months) and aged (20-24 months) rats and used for PCR amplification of 923bp fragment of β-actin or 1644bp fragment of p53 gene as described in the Materials and Methods.

A. β-actin gene amplification

Lane a: Intact genomic DNA of young rat
b: Intact genomic DNA of aged rat
c: Marker DNA
d: β-actin gene fragment (aged rat)
e: β-actin gene fragment (young rat)
f: Reference DNA (PCR amplified)

B. p53 gene amplification

Lane a: Intact genomic DNA of young rat
b: Intact genomic DNA of aged rat
c: Marker DNA
d: p53 gene fragment (aged rat)
e: p53 gene fragment (young rat)
f: Reference DNA (PCR amplified)
Table 9. Amplification levels of β-actin and p53 gene from young and aged rat brain genomic DNA

<table>
<thead>
<tr>
<th>Sample</th>
<th>β-actin gene fragment amplification (% of reference DNA)</th>
<th>p53 gene fragment amplification (% of reference DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>53 ± 5.2</td>
<td>52 ± 5.5</td>
</tr>
<tr>
<td>Aged</td>
<td>32 ± 2.6*</td>
<td>30 ± 2.9*</td>
</tr>
</tbody>
</table>

Rat brain genomic DNA was isolated from young (4-6 months) and aged (20-24 months) rat brain and used for PCR amplification of 923bp fragment of β-actin or 1644bp fragment of p53 gene as described in the Materials and Methods. Band intensities of PCR-products were measured and analyzed by BioRad Quantity-1 software. Amplification levels of β-actin or p-53 gene fragments were calculated as percentage of reference DNA sequence amplified under identical conditions. Six aged and young animals were used for the experiments. Values are expressed as the means ± SEM.

Statistical significance was calculated by Student’s ‘t’ test, unpaired. *p < 0.001 vs. young.
Fig 21. Activity of Caspase 3 and 9 in young and aged rat brain

Caspase activity was measured in the extract obtained from young and aged rat brains using the fluorogenic substrates as described in the text. Values are expressed as the means ± SEM of 5 animals in each group.

Fig A: caspase 3 activity;
Fig B: Caspase 9 activity.
Paraffin-embedded sections from young and aged rat brain were processed for Tunel stained by Hematoxilin & Eosine or by Tunel method as described in Materials and Methods.

a. Hematoxilin and Eosine stained section from rat brain (cerebellum).

b. Negative control (biotinylated nucluotide omitted; cerebellum) – No Tunel staining. (Magnification 51×)

c. Positive control (sections from young rat brain treated with DNase I; cerebellum)
- Dark brown apoptotic nuclei visible. (Magnification 51×)

d-g. Tunel staining for apoptotic cells rat brain sections from young and aged rats,

  d. Young (cerebellum), e. Young (cerebral cortex), f. Aged (cerebellum), g. Aged (cerebral cortex) - No tunel positive nuclei visible. (Magnification 51×)
Parafin-embedded sections from young and aged rat brain were used for immunostaining of bcl-2 as described in Materials and Methods.

**a.** Positive control (reactive lymphoid tissue follicle in appendix): Dark brown perinuclear staining for bcl-2. (Magnification 51×)

**b.** Negative control (reactive lymphoid tissue follicle in appendix): Immunostaining staining for bcl-2 (primary antibody omitted). (Magnification 51×)

**c-f.** Immuno-staining for bcl-2 in sections from young and aged rat brain,

c. Young (cerebral cortex), d. Young (cerebellum), e. Aged (cerebral cortex) f. Aged (cerebellum), -No perinuclear staining for bcl-2 visible. (Magnification 51×)