DNA DAMAGE AND DNA BASE EXCISION REPAIR IN AGING RAT CORTICAL NEURONS

A THESIS SUBMITTED FOR DEGREE OF

DOCTOR OF PHILOSOPHY

BY

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CERTIFICATE

This is to certify that the thesis entitled "DNA DAMAGE AND DNA BASE EXCISION REPAIR IN AGING RAT CORTICAL NEURONS" submitted by Mr. Umakanta Swain to University of Hyderabad is based on the studies carried out by him under my guidance and supervision. This thesis or any part of this thesis has not been submitted elsewhere for any other degree.

Prof. Kalluri Subba Rao
Supervisor

Prof. M. Ramanadham
Co- Supervisor

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Dean, School of life sciences

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DECLARATION

I hereby declare that the work presented in this thesis entitled "DNA DAMAGE AND DNA BASE EXCISION REPAIR IN AGING RAT CORTICAL NEURONS" is entirely original work and was carried out by me in the department of Biochemistry, University of Hyderabad, Hyderabad, under the supervision of Prof. Kalluri Subba Rao. I further declare that to the best of my knowledge this work has not formed the basis for the award of any degree or diploma of any university or institution.

Date:

Umakanta Swain
Enrollment no: 03LBPH02

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Finally, I thank the Almighty for all the blessings in my life.

Umakanta.

Figure 1

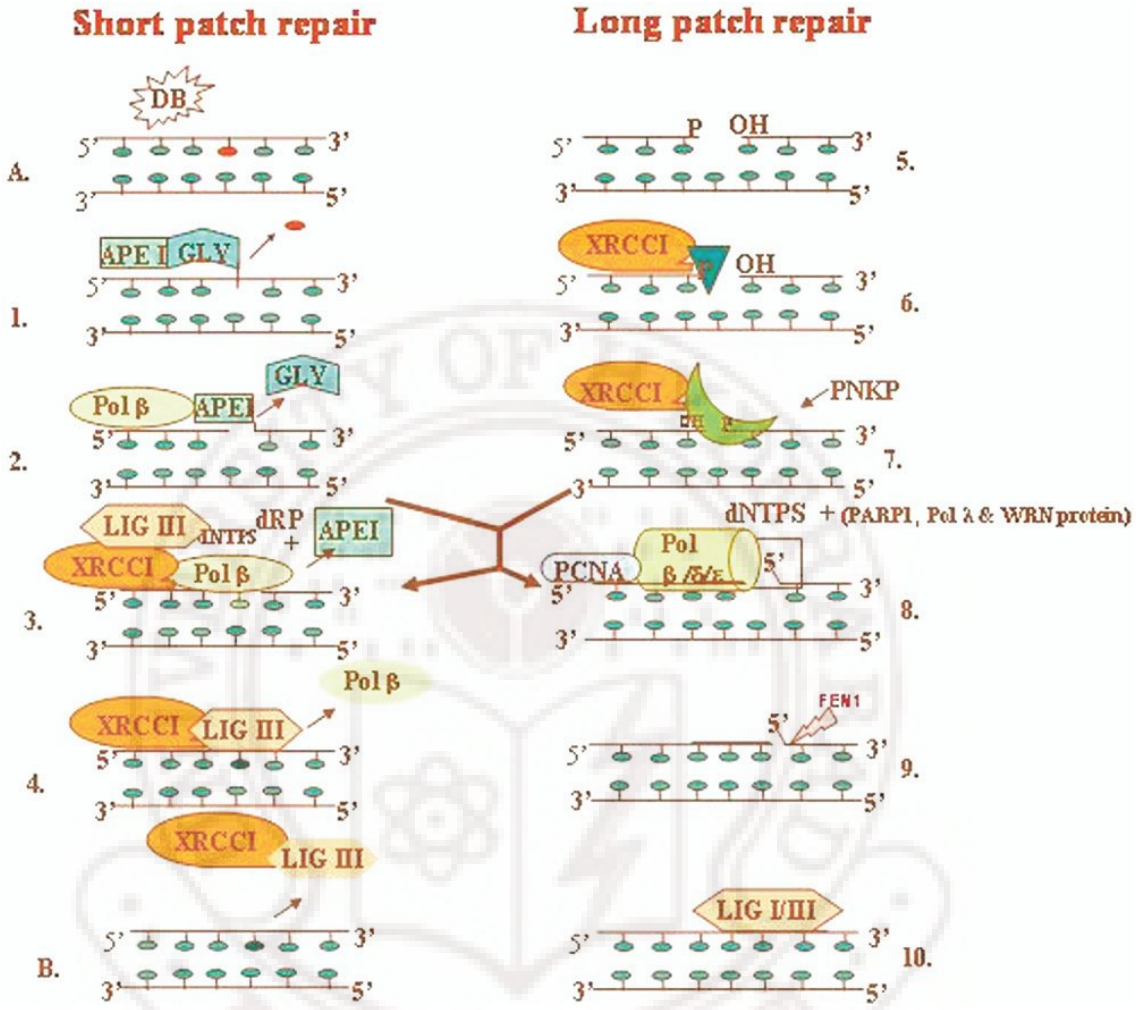
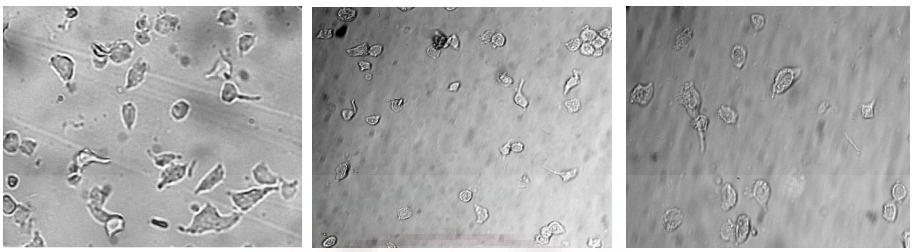

Short patch repair
Long patch repair
DB
A.
APE I
GLY
1.
Pol β
APE I
2.
LIG III
dNTPS
dRP
+
APE I
XRCCI
Pol β
3.
Pol β
XRCCI
LIG III
4.
XRCCI
LIG III
B.
P
OH
5.
XRCCI
OH
6.
XRCCI
PNKP
7.
dNTPS + (PARP1, Pol λ & WRN protein)
PCNA
Pol
β /δ/ε
8.
FEN1
9.
LIG I/III
10.

Figure 2

(A)



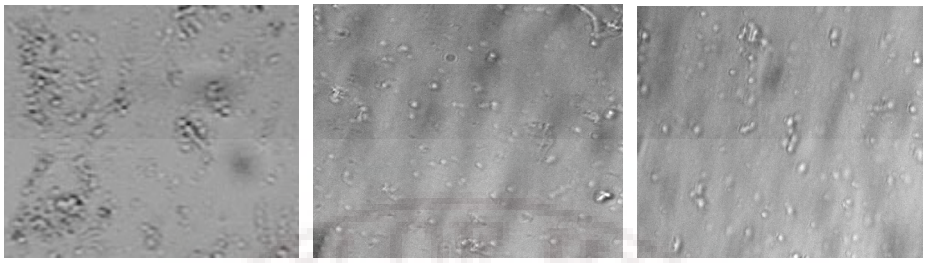
Young Adult Old

(B)

Young

Figure 3

(A)



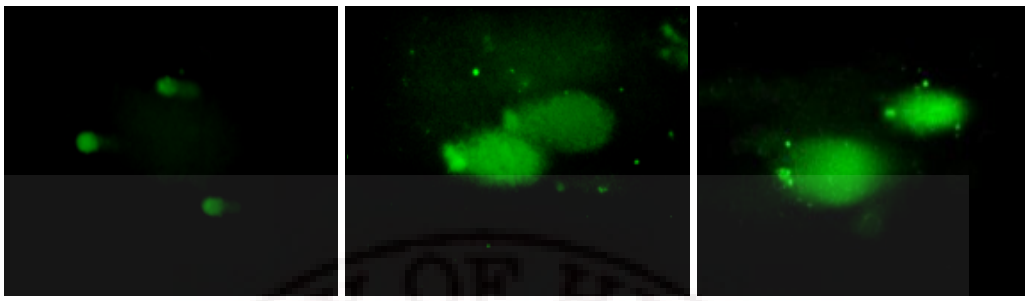
Young Adult Old

(B)

Young

Figure 4

(A)



Young Neurons Adult Neurons Old Neurons

(B)

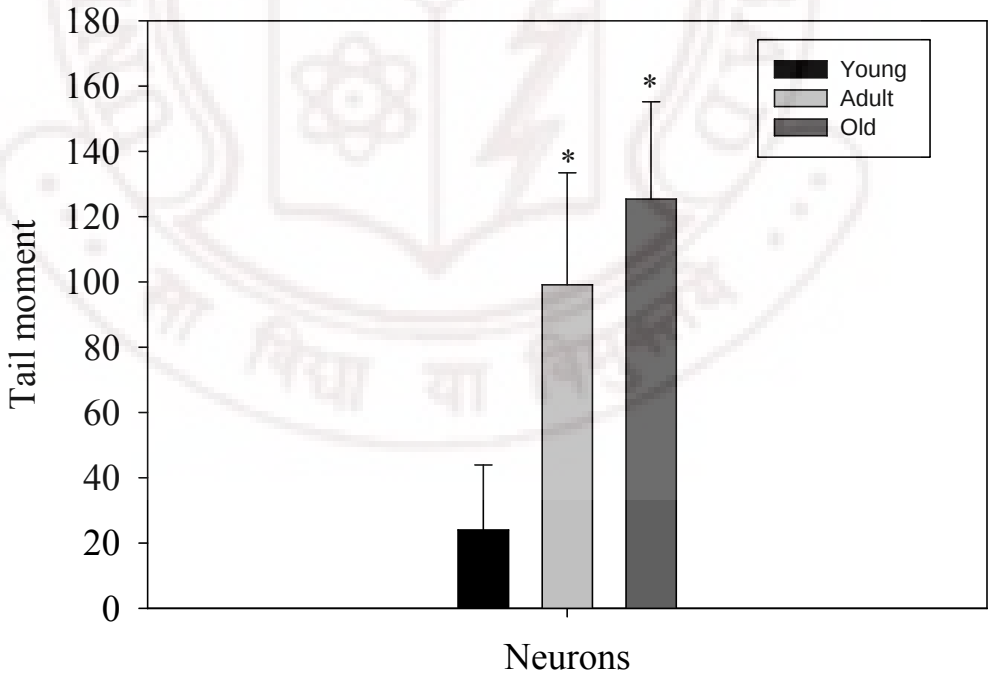
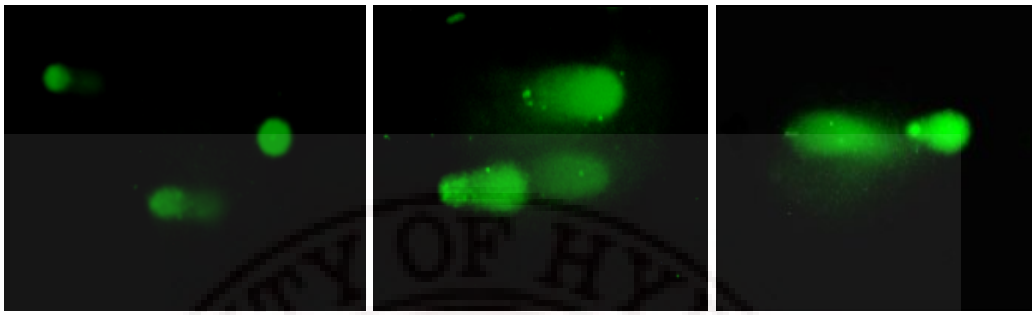


Figure 5

(A)



Young Astrocytes Adult Astrocytes Old Astrocytes

(B)

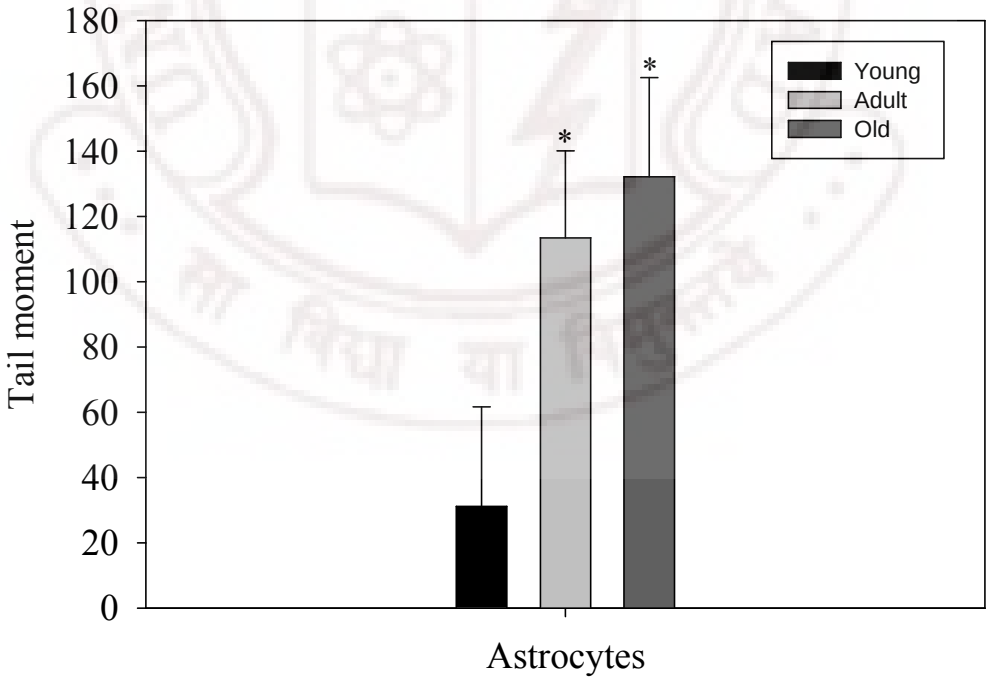
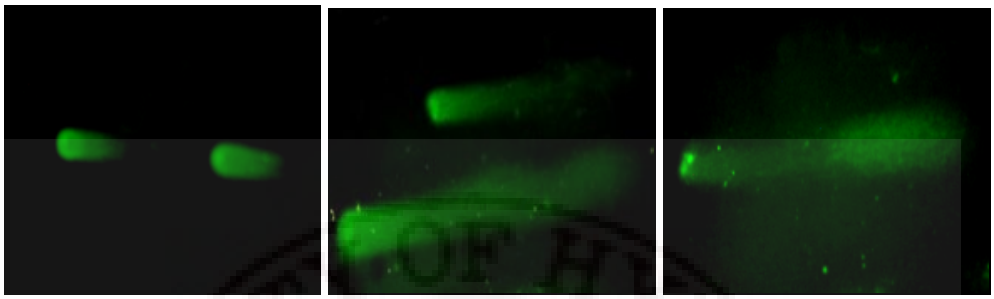


Figure 6

(A)



Young Neurons Adult Neurons Old Neurons

(B)

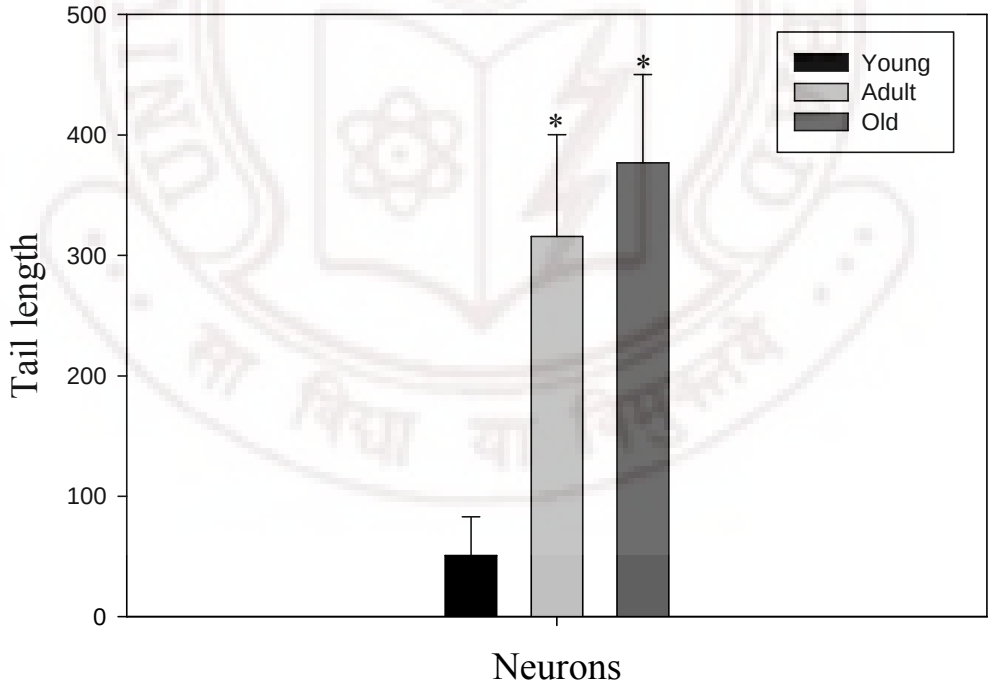
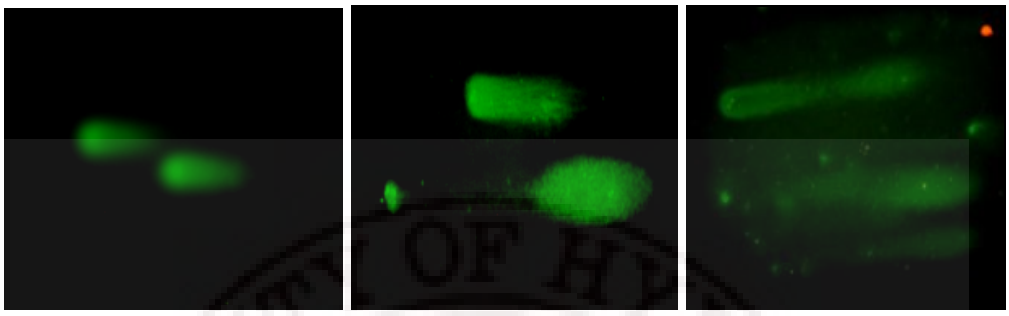


Figure 7

(A)



Young Astrocytes Adult Astrocytes Old Astrocytes

(B)

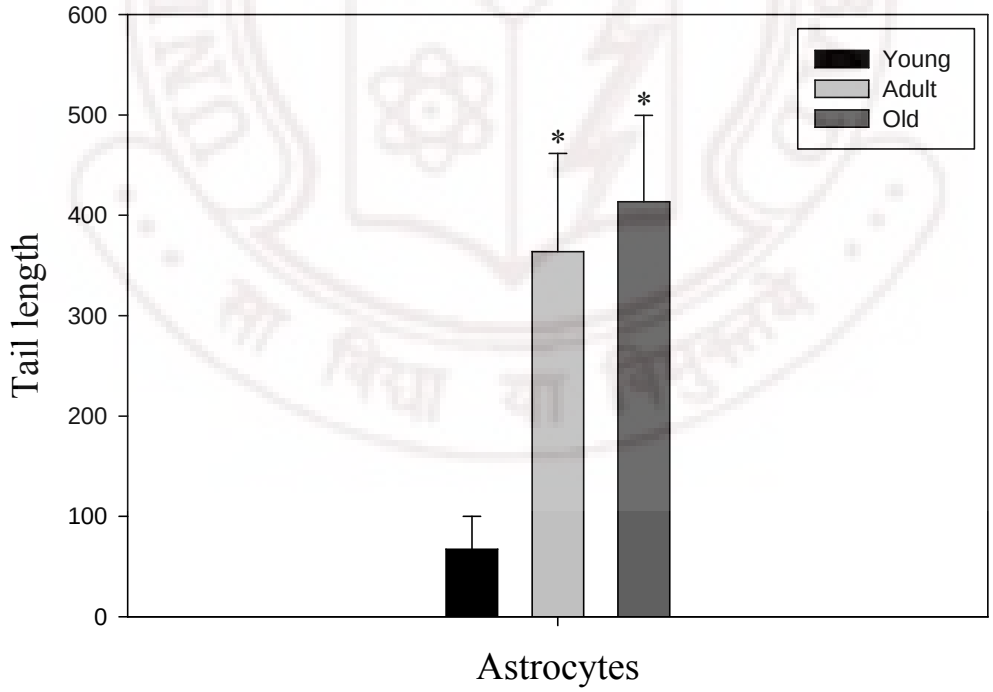


Figure 8

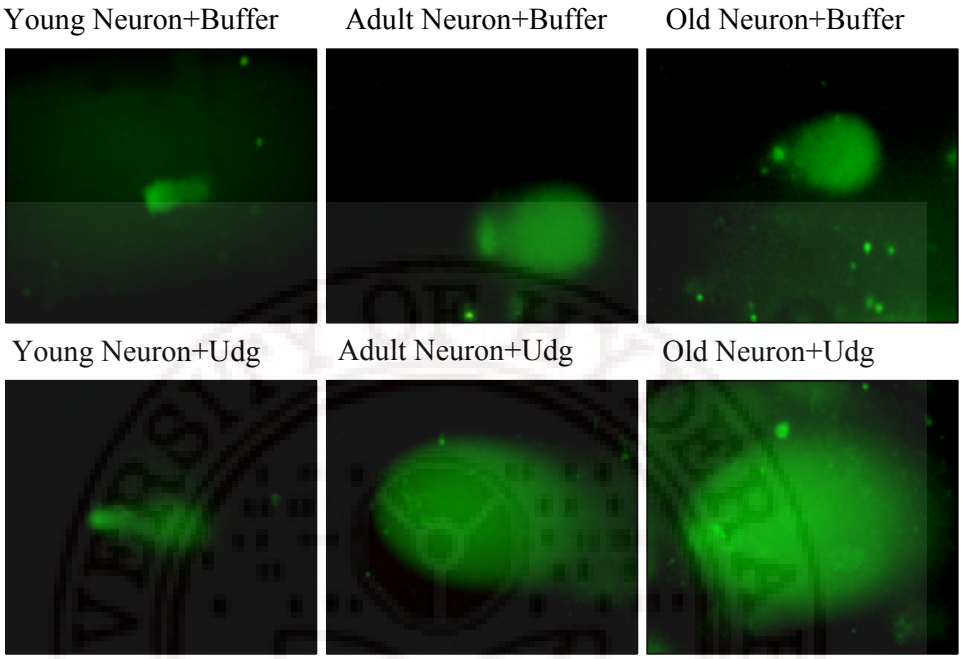

Young Neuron+Buffer
Adult Neuron+Buffer
Old Neuron+Buffer
Young Neuron+Udg
Adult Neuron+Udg
Old Neuron+Udg

Figure 9

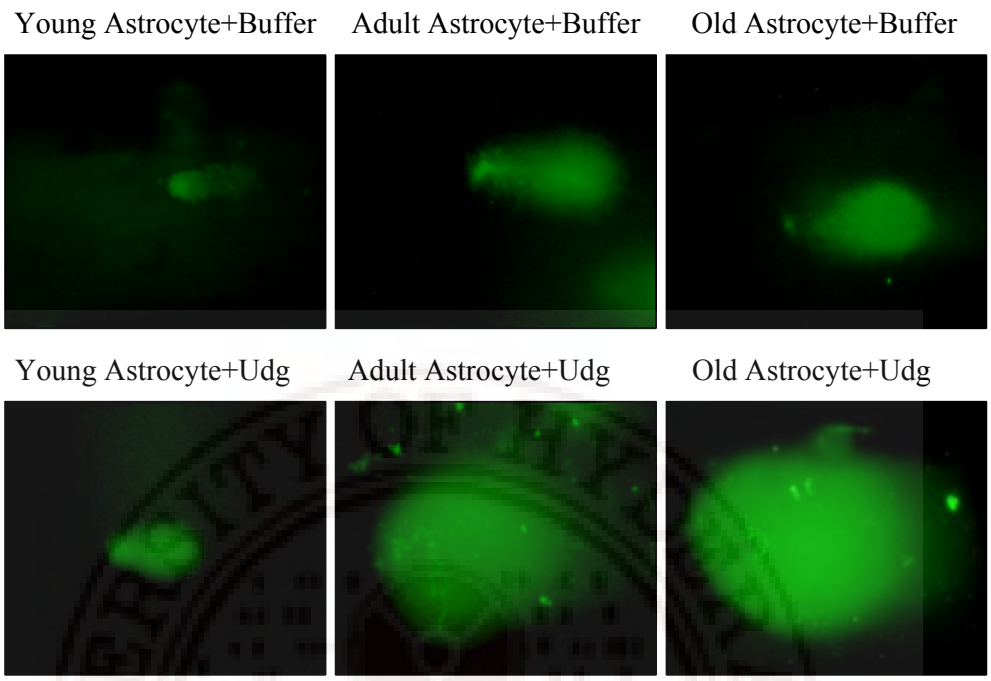
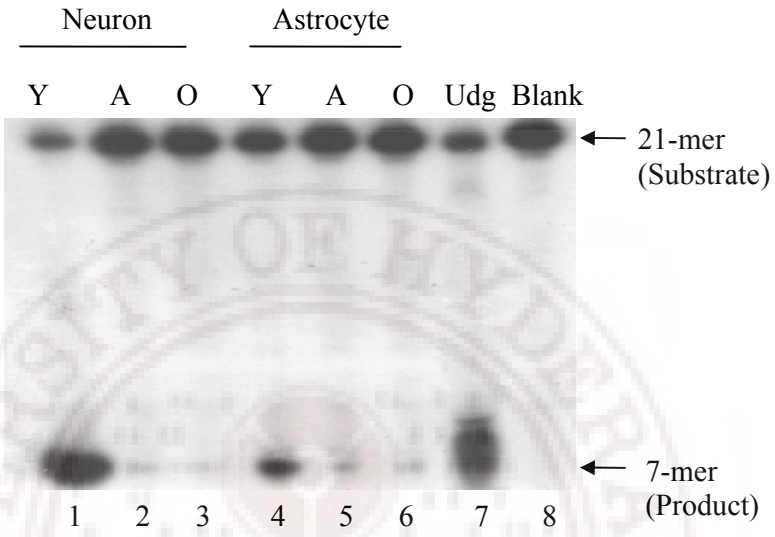

Young Astrocyte+Buffer
Adult Astrocyte+Buffer
Old Astrocyte+Buffer
Young Astrocyte+Udg
Adult Astrocyte+Udg
Old Astrocyte+Udg

Figure 10

(A)

^{32}P 5'-g c c a t t g U g c t a c c g a t c g c g- 3'
3'-c g g t a a c G c g a t g g c t a g c g c- 5'



(B)

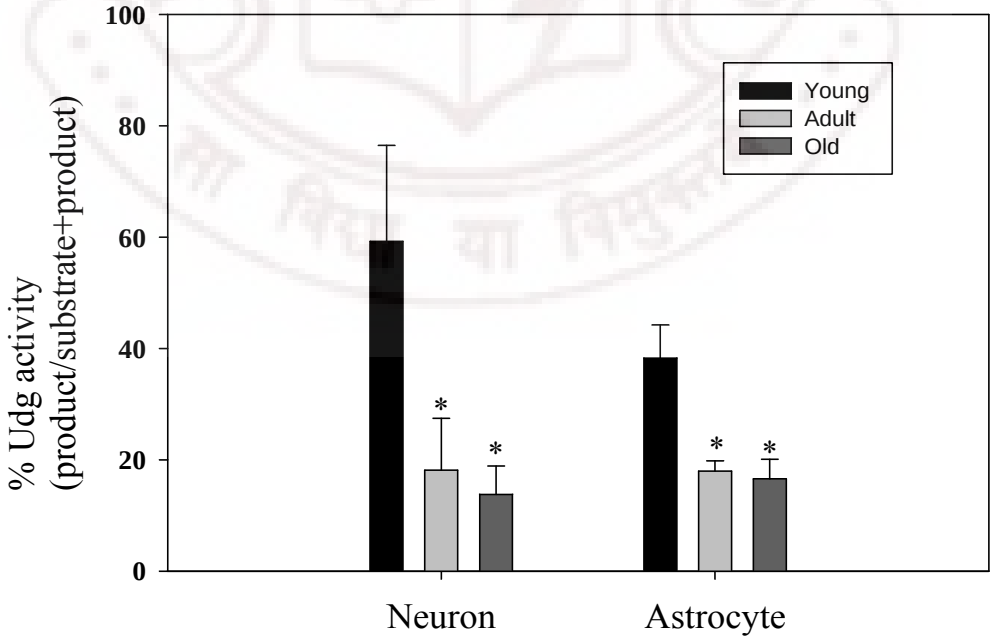
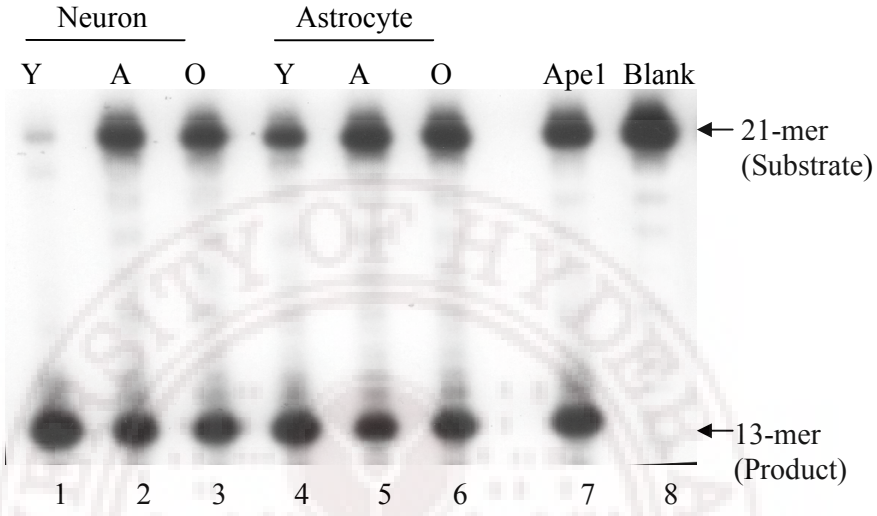


Figure 11

(A)

^{32}P 5'-c g c g a t c g g t a g c F c a a t g g c-3'
3'-g c g c t a g c c a t c g C g t t a c c g-5'



(B)

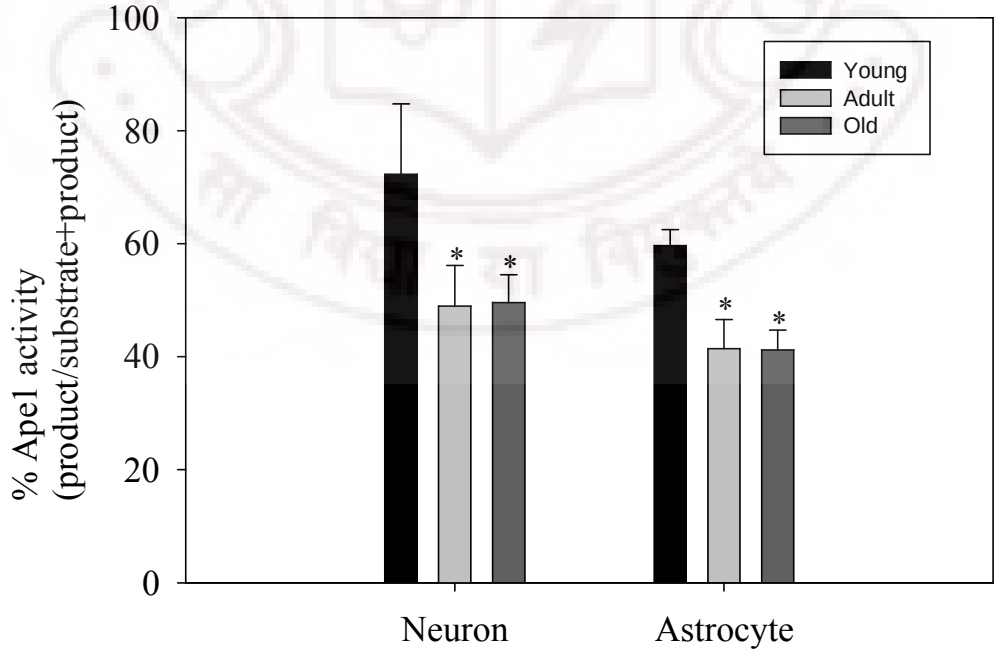


Figure 12

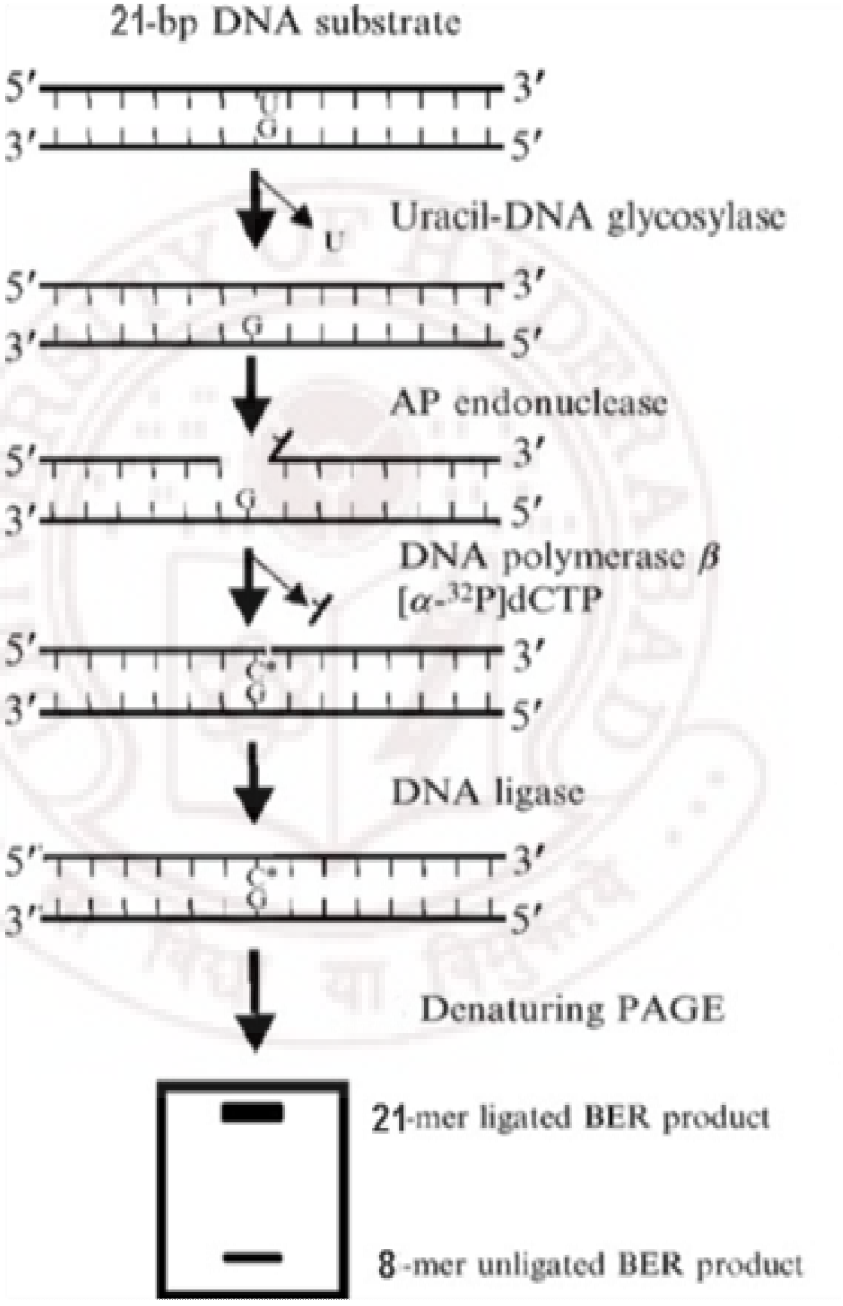

21-bp DNA substrate
5′
3′
U
G
3′
5′
Uracil-DNA glycosylase
U
AP endonuclease
DNA polymerase β
[α-32P]dCTP
DNA ligase
Denaturing PAGE
21-mer ligated BER product
8-mer unligated BER product

Figure 13

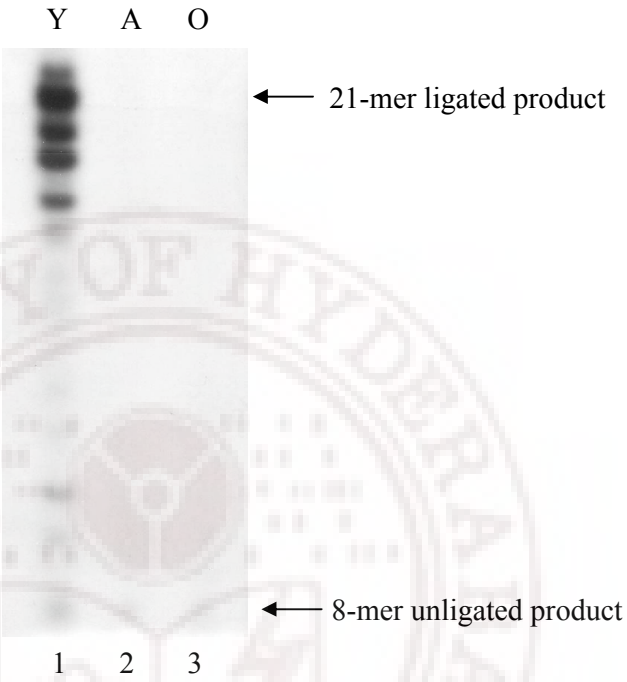


Figure 14

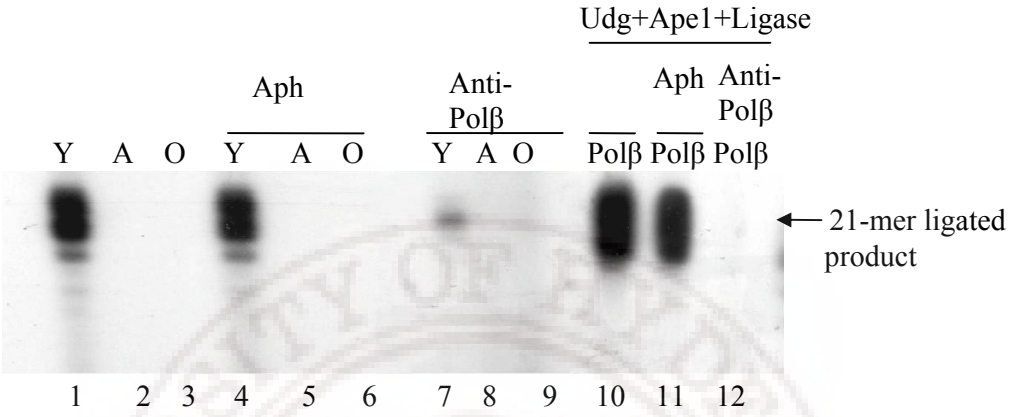

Udg+Ape1+Ligase
Aph
Anti-Polβ
Aph
Anti-Polβ
Y A O Y A O Y A O Polβ Polβ Polβ
21-mer ligated product
1 2 3 4 5 6 7 8 9 10 11 12

Figure 15

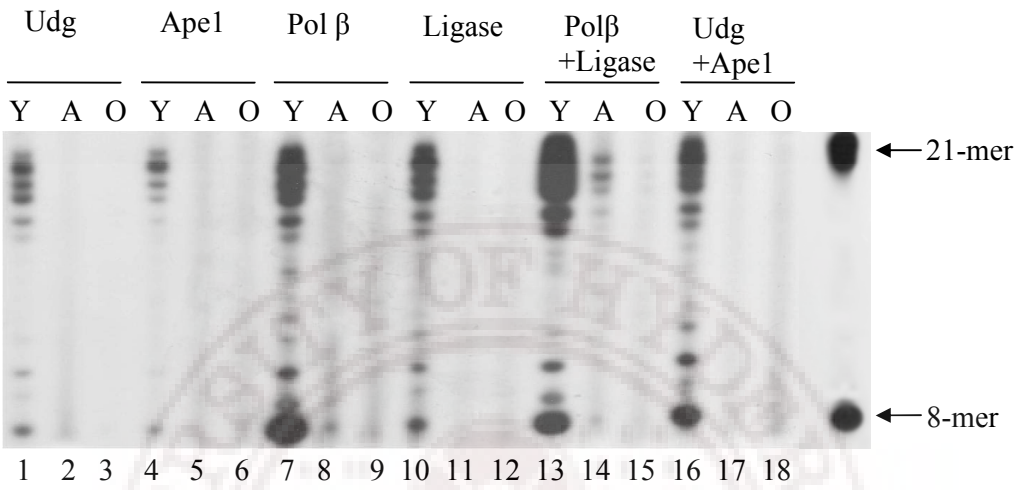

Udg
Ape1
Pol β
Ligase
Polβ +Ligase
Udg +Ape1
Y A O Y A O Y A O Y A O Y A O Y A O
21-mer
8-mer
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 16

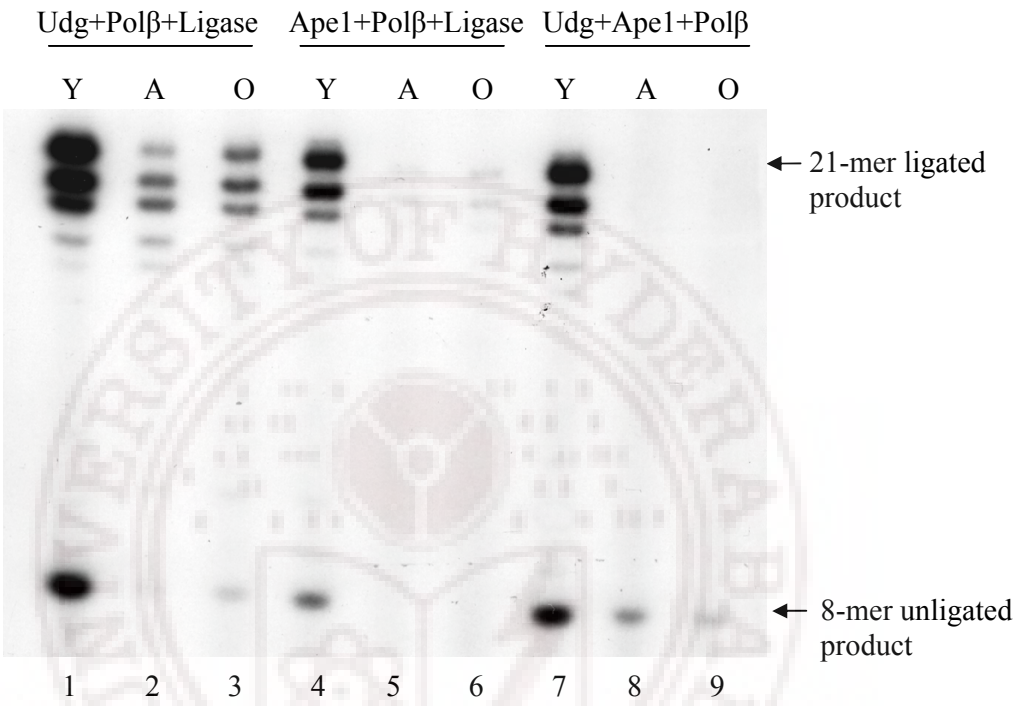

Udg+Polβ+Ligase
Ape1+Polβ+Ligase
Udg+Ape1+Polβ
Y A O Y A O Y A O
21-mer ligated product
8-mer unligated product
1 2 3 4 5 6 7 8 9

Figure 17

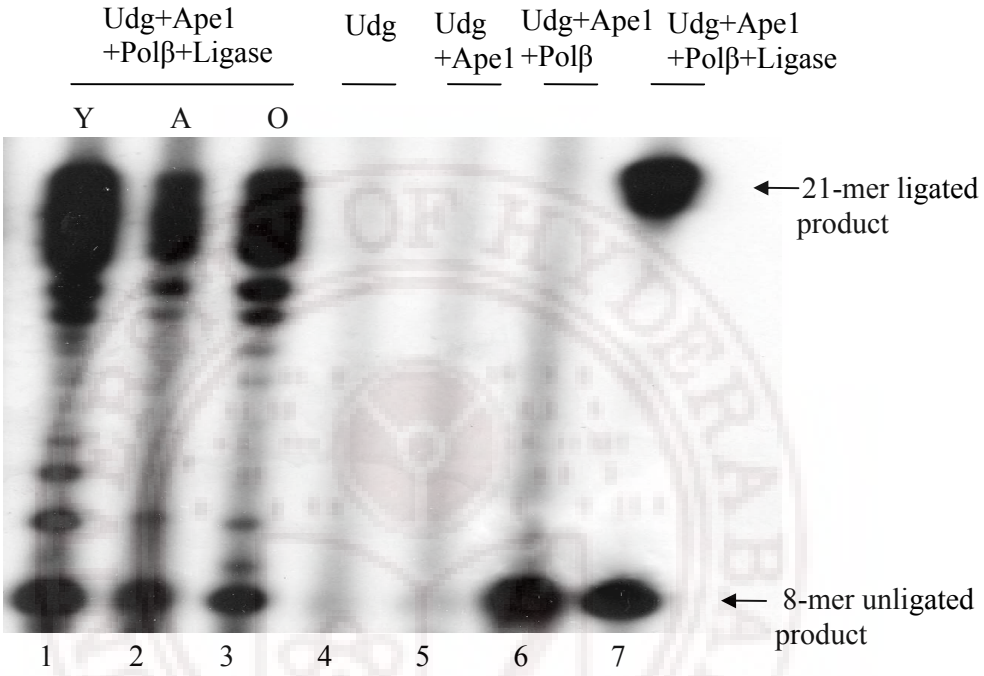
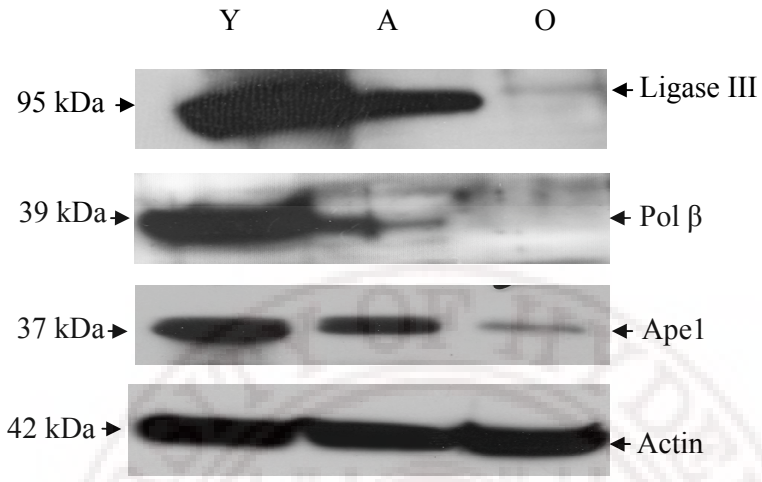

Udg+Ape1
+Polβ+Ligase
Udg
Udg
+Ape1
Udg+Ape1
+Polβ
Udg+Ape1
+Polβ+Ligase
Y
A
O
21-mer ligated
product
8-mer unligated
product
1
2
3
4
5
6
7

Figure 18

(A)


Y
A
O
95 kDa
Ligase III
39 kDa
Pol β
37 kDa
Ape1
42 kDa
Actin

(B)

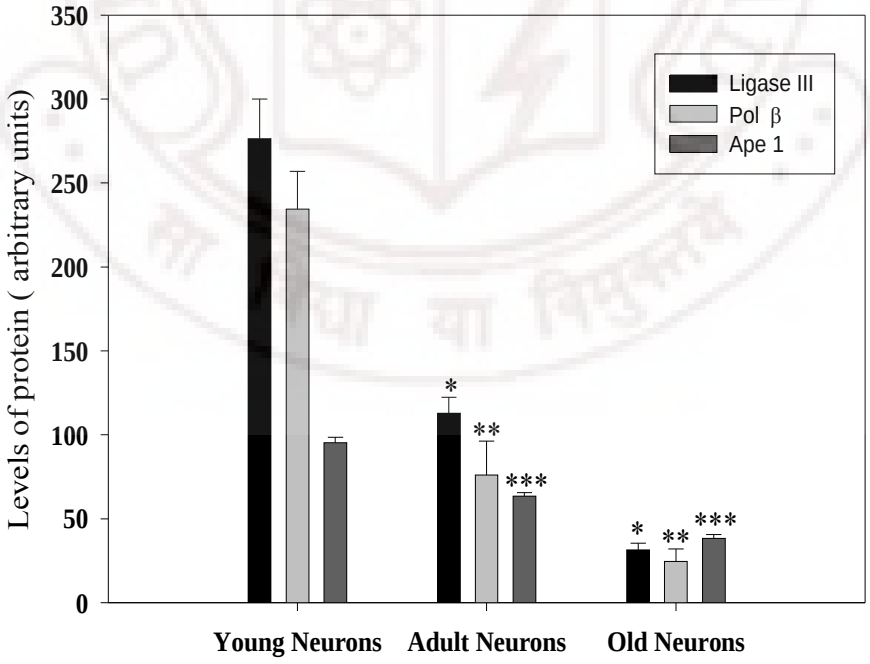

Levels of protein (arbitrary units)
350
300
250
200
150
100
50
0
Ligase III
Pol β
Ape 1
*
**
***
*
**
***
Young Neurons
Adult Neurons
Old Neurons

Figure 19

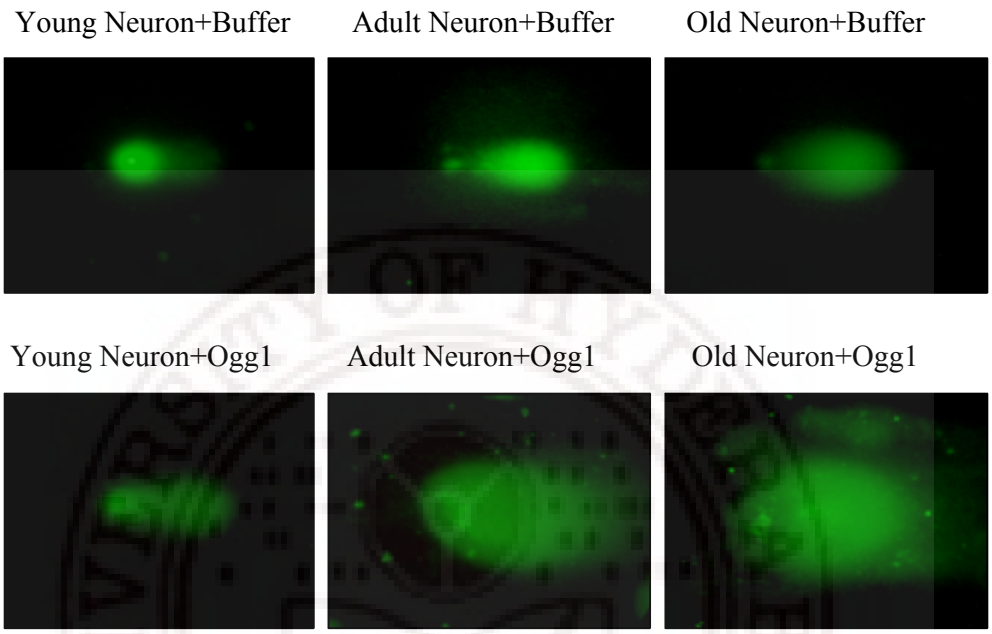

Young Neuron+Buffer
Adult Neuron+Buffer
Old Neuron+Buffer
Young Neuron+Ogg1
Adult Neuron+Ogg1
Old Neuron+Ogg1

Figure 20

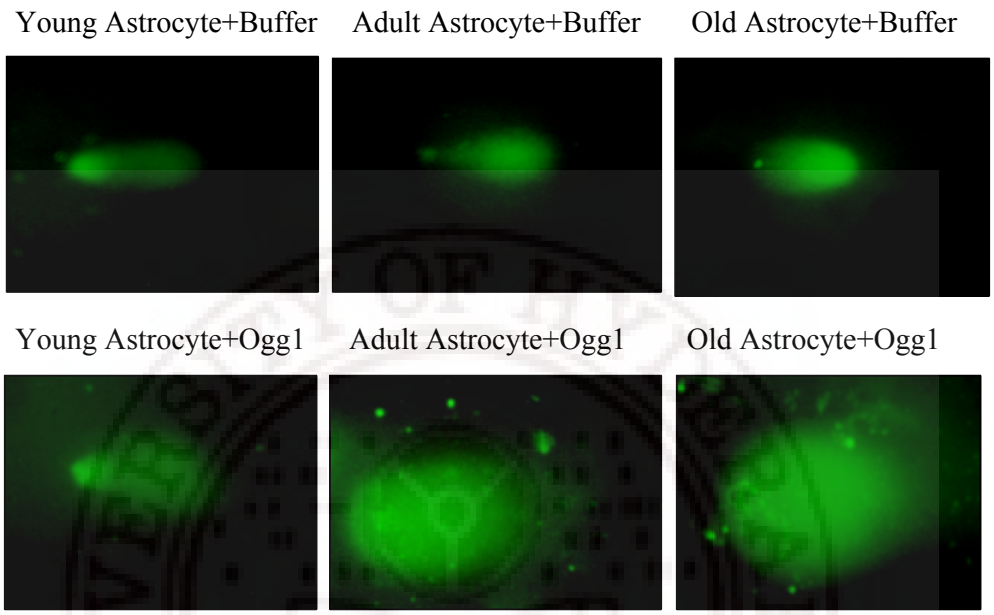
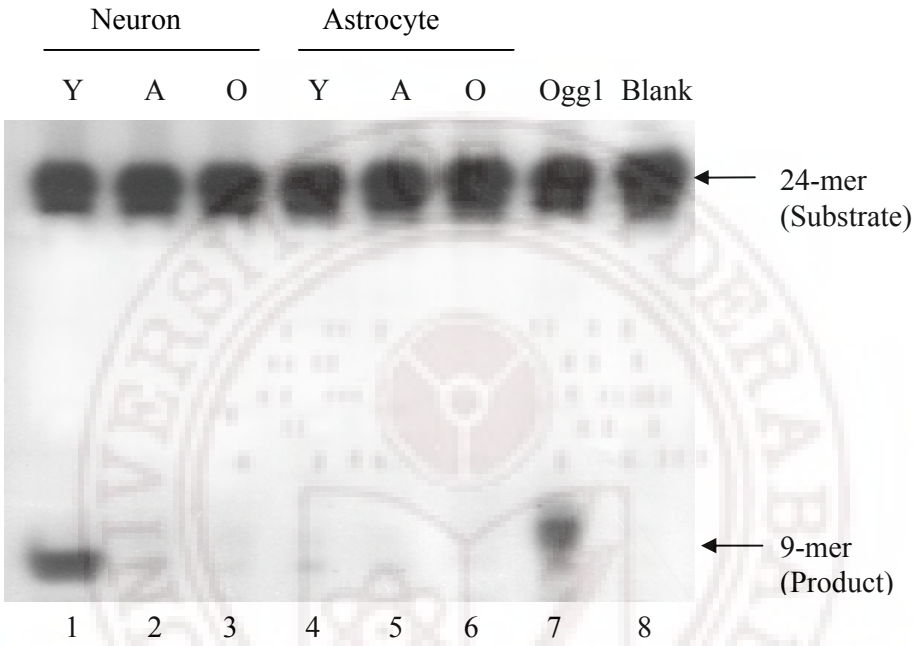

Young Astrocyte+Buffer
Adult Astrocyte+Buffer
Old Astrocyte+Buffer
Young Astrocyte+Ogg1
Adult Astrocyte+Ogg1
Old Astrocyte+Ogg1

Figure 21

(A)

Sequence:
P^{32} 5' GAACTAGTG**O**ATCCCCCGGG CTGC 3'
3' CT TGATCACCTAGGGGGCCCGACG 5'
Where **O** is 8-oxoguanine.



(B)

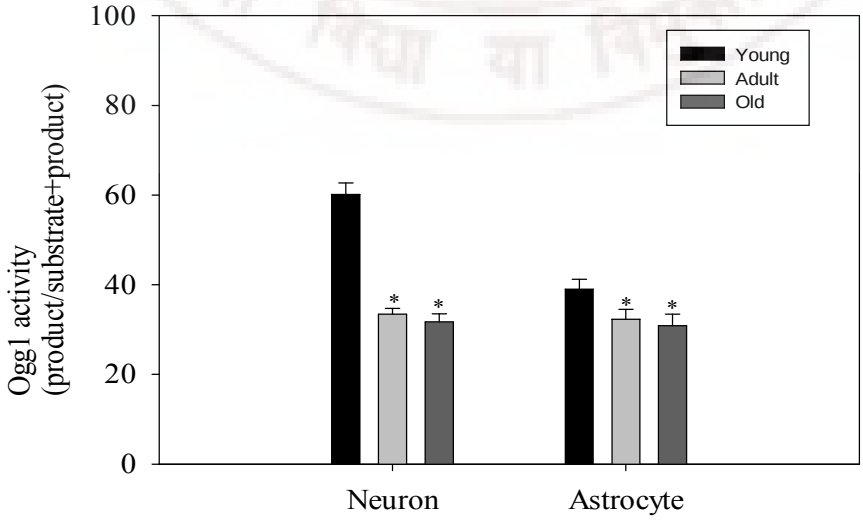


Figure 22

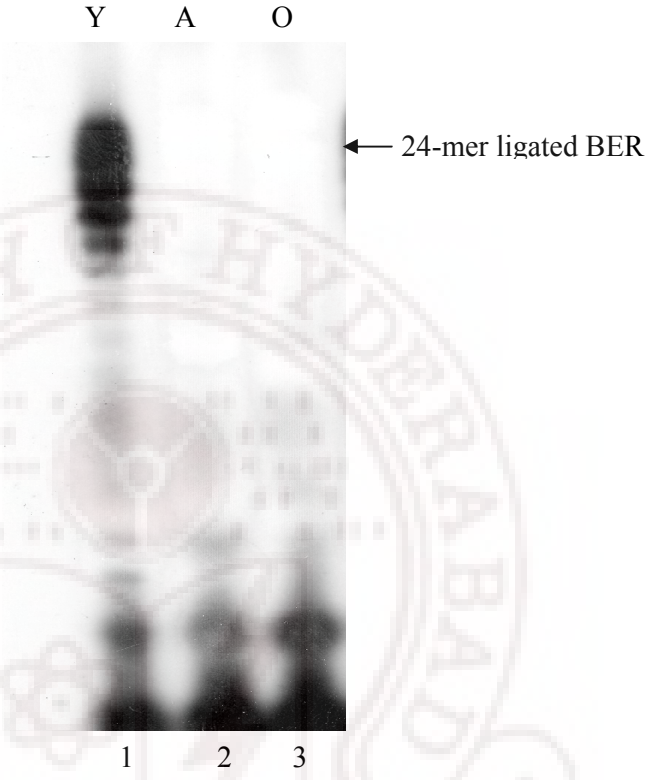


Figure 23

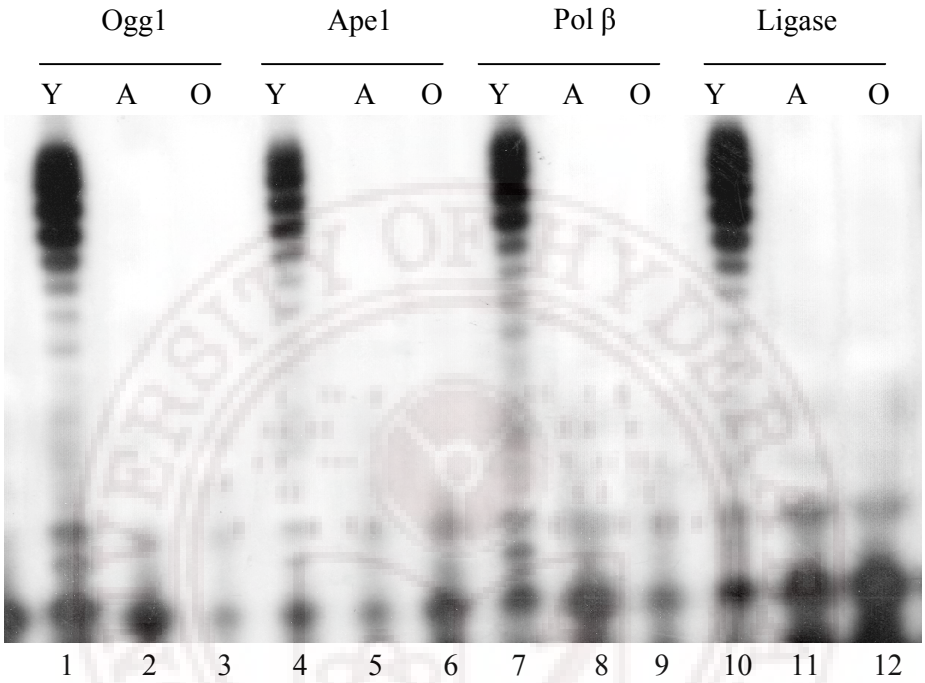


Figure 24

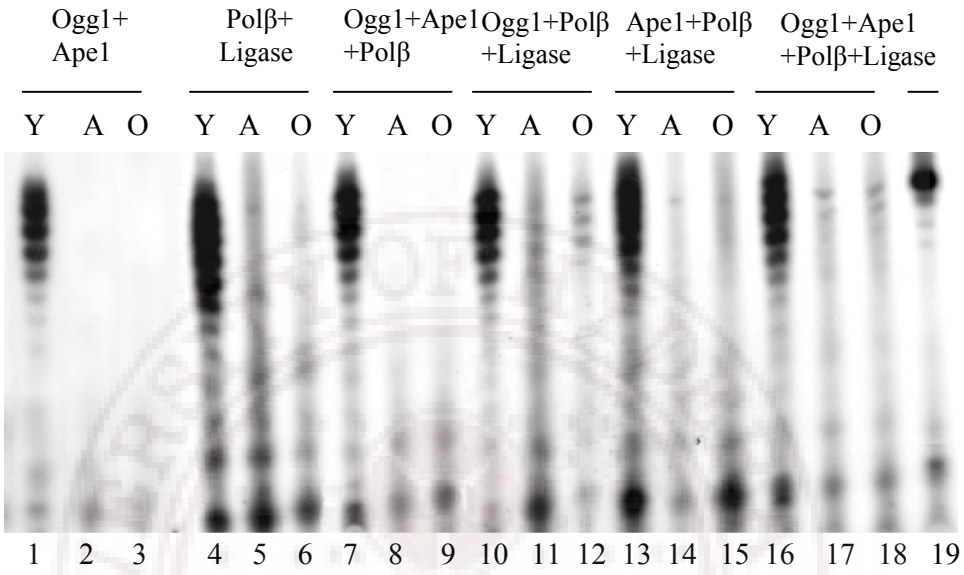
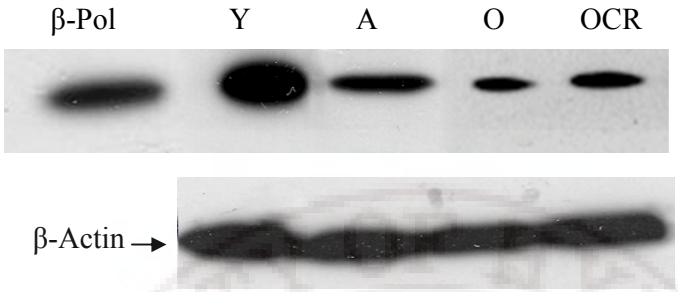

Ogg1+ Ape1
Polβ+ Ligase
Ogg1+Ape1 +Polβ
Ogg1+Polβ +Ligase
Ape1+Polβ +Ligase
Ogg1+Ape1 +Polβ+Ligase
Y A O Y A O Y A O Y A O Y A O Y A O
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 25

(A)


β-Pol
Y
A
O
OCR
β-Actin

(B)

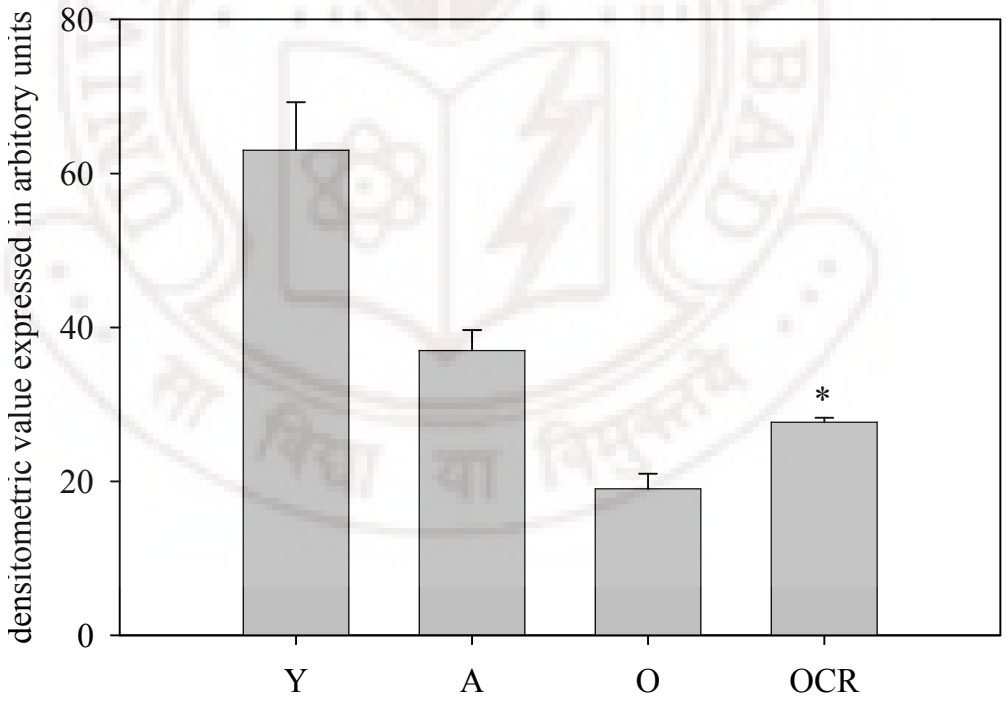
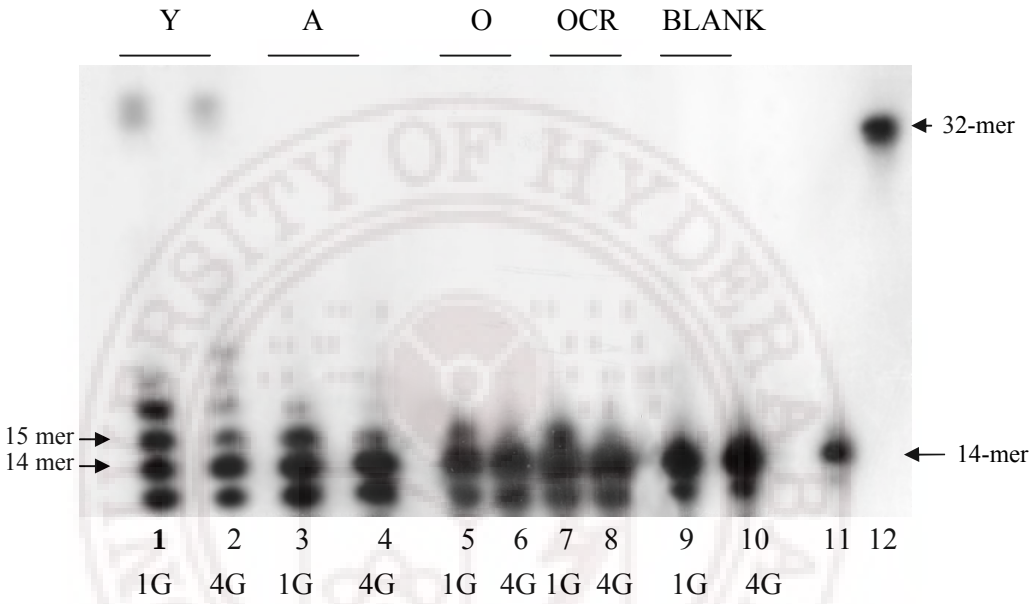

densitometric value expressed in arbitory units
80
60
40
20
0
*
Y
A
O
OCR

Figure 26

(A)



(B)

1 Gap Oligo:
Oligo 2, the 14-mer, upstream primer Oligo 4, the 17-mer, downstream primer

32P-5'-c g a g c c a t g g c c g c - a g a t t t t t g c g g t g c c - 3'
3'-g c t c g g t a c c g g c g g t c t a a a a a c g c c a c g g -5'(Oligo 1, the 32-mer)

4 Gap Oligo:
Oligo 2, the 14-mer, upstream primer Oligo 3, the 14-mer, downstream primer

32P-5'-c g a g c c a t g g c c g c - - - - t t t t t t g c g g t g c c -3'
3'-g c t c g g t a c c g g c g g t c t a a a a a a c g c c a c g g -5'(Oligo 1, the 32-mer)

Figure 27

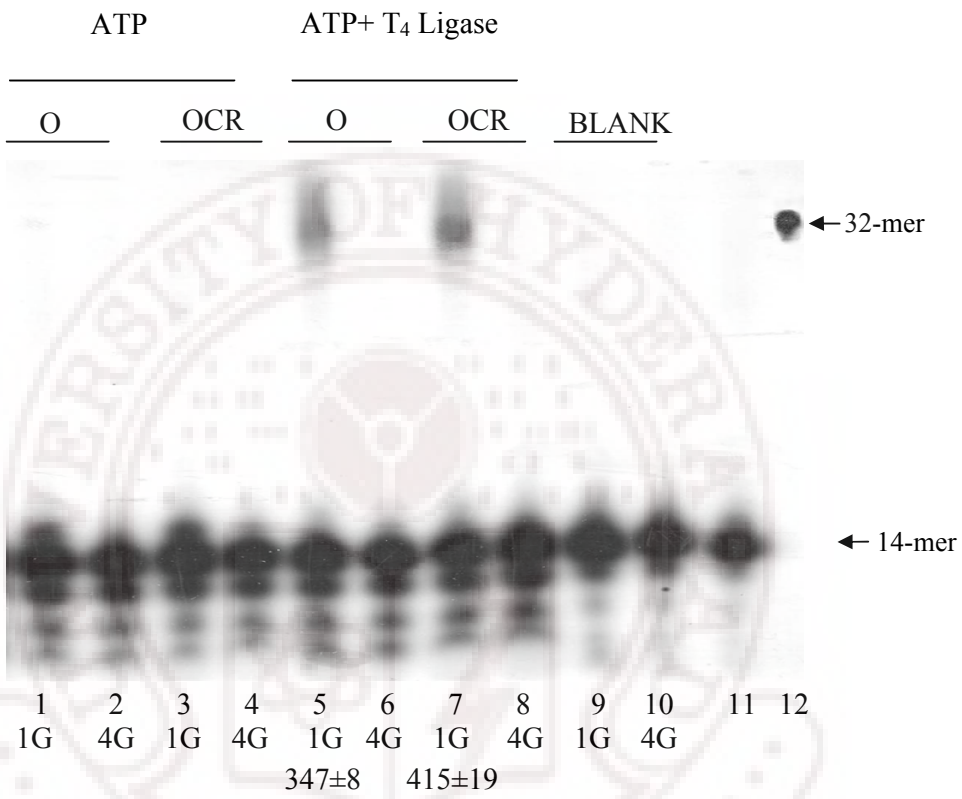
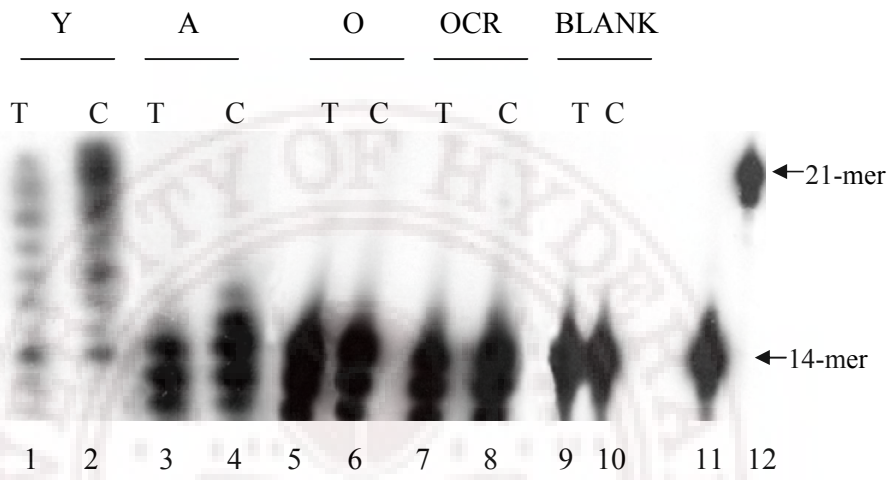

ATP
ATP+ T4 Ligase
O
OCR
O
OCR
BLANK
32-mer
14-mer
1 2 3 4 5 6 7 8 9 10 11 12
1G 4G 1G 4G 1G 4G 1G 4G 1G 4G
347±8
415±19

Figure 28

(A)



(B)

Correct duplex (G-C)
32P 5'-c g c g a t c g g t a g c G -3' (Oligo-A, 14-mer)
3'- g c g c t a g c c a t c g C g t t a c c g - 5' (Oligo-B, 21-mer)

Mismatched duplexes (G-T)
32P 5'–c g c g a t c g g t a g c G - 3'(Oligo-A, 14-mer)
3'- g c g c t a g c c a t c g T g t t a c c g- 5' (Oligo-C, 21-mer)

Figure 1

An outline of pathways of base excision repair (BER): On the left side is "Short patch" pathway and the right side "Long patch" pathway.

Crossing over of the pathways can occur at points 2 and 7. Abbreviations: DB, damaged base; GLY-DNA, glycosylase; Pol β/ δ/ ε, DNA polymerase β/ δ/ ε respectively; LIG I/III, DNA-ligase I/III; PARP1, poly (ADP-ribose) polymerase 1; dNTPs, deoxynucleoside triphosphates.

Figure 2

(A) Morphology of neurons isolated from 'Young' (7 days postnatal), 'Adult' (6 months) and 'Old' (≥24 months) rat brain cerebral cortex by Ficoll gradient (28% ficoll/22% ficoll). Image taken at 40 X magnification from an inverted microscope.

(B) Western blot shows neuronal extracts prepared from young rats probed with anti-neuron specific enolase (NSE) and anti-glial cell fibrilary acidic protein (GFAP).

Figure 3

(A) Morphology of astrocytes isolated from 'Young' (7 days postnatal), 'Adult' (6 months) and 'Old' (≥24 months) rat brain cerebral cortex by Ficoll gradient (28% ficoll/22% ficoll/10% ficoll). Image taken at 40 X magnification from an inverted microscope.

(B) Western blot shows astroglial extract prepared from young rat probed with anti-neuron specific enolase (NSE) and anti-glial cell fibrilary acidic protein (GFAP).

Figure 4

Alkaline single cell gel electrophoresis or comet assay of neurons prepared from 'Young' (7 days postnatal), 'Adult' (6 months) and 'Old' (≥24 months) rat brain cerebral cortex.

(A) Fluorescence photomicrographs showing comets of neurons prepared from young, adult and old rat brain cortex. Neurons were embedded within agarose on a glass slide, then lysed, and DNA is "unwound" under alkaline conditions followed by alkaline electrophoresis (pH>13). Following SYBR Green I staining, nuclei were visualized under epifluorescence microscope, and images were captured for comet moment analysis. Damaged DNA migrates towards the anode, resulting in an appearance of a comet, which can be analyzed quantitatively for DNA SSBs.

(B) Bar graph showing tail moment expressed in arbitory units of neurons prepared from young, adult and old rat brain cortex. 50 randomly chosen comets were analyzed. Values are expressed in mean±standard deviation (SD). Results from three independent experiments are shown. *These values are significantly different ($p<0.05$ for adult and old neurons) from the corresponding values at young neurons.

Figure 5

Alkaline single cell gel electrophoresis or comet assay of astrocytes prepared from 'Young' (7 days postnatal), 'Adult' (6 months) and 'Old' (≥24 months) rat brain cerebral cortex.

(A) Fluorescence photomicrographs showing comets of astrocytes prepared from young, adult and old rat brain cortex. Other notations same as Figure 4.

(B) Bar graph showing tail moment expressed in arbitory units of astrocytes prepared from young, adult and old rat brain cortex. 50 randomly chosen comets were taken for analysis. Values are expressed in mean±standard deviation(SD). Results from three independent experiments are shown. *These values are significantly different ($p<0.05$ for adult and old astrocytes) from the corresponding values at young astrocytes.

Figure 6

Neutral single cell gel electrophoresis or comet assay of neurons prepared from 'Young' (7 days postnatal), 'Adult' (6 months) and 'Old' (≥24 months) rat brain cerebral cortex.

(A) Fluorescence photomicrographs showing comets of neurons prepared from young, adult and old rat brain cortex. Neurons embedded within agarose on a glass slide, then lysed, and DNA is followed by neutral electrophoresis (pH~8.2). Following SYBR Green I staining, nuclei were visualized under epifluorescence microscope, and images were captured for comet analysis. Damaged DNA migrates towards the anode, resulting in an appearance of a comet, which can be analyzed quantitatively for DNA DSBs.

(B) Bar graph showing tail length expressed in arbitory units of neurons prepared from young, adult and old rat brain cortex. 50 randomly chosen comets were taken for analysis. Values are expressed in mean±standard deviation(SD). Results from three independent experiments are shown. *These values are significantly different ($p<0.05$ for adult and old neurons) from the corresponding values at young neurons.

Figure 7

Neutral single cell gel electrophoresis or comet assay of astrocytes prepared from 'Young' (7 days postnatal), 'Adult' (6 months) and 'Old' (≥24 months) rat brain cerebral cortex.

(A) Fluorescence photomicrographs showing comets of astrocytes prepared from young, adult and old rat brain cortex. Other notations same as Figure 6.

(B) Bar graph showing tail length expressed in arbitory units of astrocytes prepared from young, adult and old rat brain cortex. 50 randomly chosen comets were taken for analysis. Values are expressed in mean±standard deviation(SD). Results from three independent experiments are shown. *These values are significantly different ($p<0.05$) for adult and old astrocytes) from the corresponding values at young astrocytes.

Figure 8

Measurement of uracil sites in young brain (Y, 7 days postnatal), adult brain (A, 6 months) and old brain (O, ≥ 2 years) neurons by single cell gel electrophoresis or comet assay.

Three different experiments have been conducted. A typical representative image of comet is shown.

Figure 9

Measurement of uracil sites in young brain (Y, 7 days postnatal), adult brain (A, 6 months) and old brain (O, ≥ 2 years) astrocytes by single cell gel electrophoresis or comet assay.

A typical representative image of comet is shown out of three different experiments.

Figure 10

Uracil DNA glycosylase activity (Udg) in 'young', 'adult' and 'old' rat neuronal and astroglial extracts.

Neuronal and astroglial extracts prepared from different age of rats were incubated with 200 fmol of 5'[γ^{32}P]-kinased 21-mer DNA oligonucleotide duplex containing uracil at position 8 in the assay buffer for 20 minutes at 37 ^{0}C, generating the specific 7-mer cleavage product. The samples were then processed as described in Materials and Methods, Chapter 2. A typical autoradiogram from three different experiments is shown. (A) Lanes 1–3, neuronal extracts from young brain (Y, 7 days postnatal), adult brain (A, 6 months) and old brain (O, ≥ 2 years). Lanes 4–6, astroglial extracts from young brain (Y, 7 days postnatal), adult brain (A, 6 months) and old brain (O, ≥ 2 years). Lane 7 is 0.5 units of pure Uracil DNA glycosylase (Udg). Lane 8 is without any extracts (enzyme blank). One unit of Udg releases 50 pmol of uracil from an oligomeric substrate containing single dUMP residue in 15 minutes at 37 °C in a 50 µl reaction volume.

(B) Quantitative analysis of Udg activity in neuronal and astroglial extracts as the function of age, determined by densitometric measurements on autoradiogram from three independent experiments. The Udg activity was calculated as the relative amount of 7-mer product to 21-mer substrate (product/substrate+product). Values are expressed in mean±SD.

* These values are significantly different ($P<0.05$ for adult and old) from the corresponding value at 'young' (ANOVA, Holm-Sidak method).

Figure 11

Apurinic endonuclease 1 (Ape1) activity in 'young', 'adult' and 'old' rat neuronal and astroglial extracts.

Neuronal and astroglial extracts prepared from different age of rats. Equal amount of protein (200 ng) from each age was incubated with the 5' [γ ^{32}P]-kinased 21-mer DNA duplex (200 fmol) containing tetrahydrofuran analog (F) of an AP site at position 14 in incision buffer, for 10 mintues at 37 ^{0}C, generating the specific 13-mer cleavage product. The samples were then processed as described in Materials and Methods, Chapter 2. A typical autoradiogram from three different experiments is shown. (A) Lanes 1–3, neuronal extracts from young brain (Y, 7 days postnatal), adult brain (A, 6 months) and old brain (O, $\geq$ 2 years). Lanes 4–6, astroglial extracts from young brain (Y, 7 days postnatal), adult brain (A, 6 months) and old brain (O, $\geq$ 2 years). Lane 7 is 1 unit of pure Ape1. Lane 8 is without any extracts (enzyme blank). One unit is the amount of enzyme required to cleave an AP site oligonucleotide within an oligonucleotide duplex at the rate of 1 pmol/hour at 37 ^{0}C.

(B) Quantitative analysis of Ape1 activity in neuronal and astroglial extracts as the function of age, determined by densitometric measurements on autoradiogram from three independent experiments. The incision activity was calculated as the relative amount of 13-mer product to 21-mer substrate (product/substrate+product). Values are expressed in mean±SD. * These values are significantly different ($P<0.05$ for adult, old, neurons and astrocytes) from the corresponding value at 'young' (ANOVA, Holm-Sidak method).

Figure 12

DNA polymerase β dependent base excision repair (BER) activity assay.

Schematic diagram illustrates the principle of BER assay. Uracil DNA glycosylase will remove uracil (U) and AP endonuclease incise the abasic site on its 5' side. DNA polymerase β (Pol β) will label the repaired DNA strand by adding a [α^{32}P]-dCMP residue, thereby generating a repair unligated intermediate (8-mer). When the 5'-dRP residue is removed, the nicked substrate can be ligated to generate a full length ligated product (21-mer).

Figure 13

Uracil initiated base excision repair activity in 'young'(Y), 'adult' (A) and 'old' (O) rat neuronal extracts.

DNA oligoduplex containing uracil at position 8 was incubated for 20 minutes at 37 ^{0}C with 10 μg of neuronal extract in the presence of [α ^{32}P]-dCTP, dNTPs and buffer as described in Materials and Methods, Chapter 2. The reaction products were purified and separated on a denaturating 20% polyacrylamide sequencing gel, exposed to X-ray film and visualized by autoradiography. A typical autoradiogram from three different experiments is shown. Lanes 1–3, neuronal extracts from young brain (Y, 7days), adult brain (A, 6 months) and old brain (O≥, 2 years). Position of 21-mer, ligated product and 8-mer, unligated products are shown.

Figure 14

Uracil initiated base excision repair activity in 'young'(Y), 'adult' (A) and 'old' (O) rat neuronal extracts in presence of aphidicolin and neutralizing polyclonal DNA polymerase β antibody.

Neuronal extracts prepared from rat brain and pure Pol β were incubated with aphidicolin (Aph) or neutralizing polyclonal anti-DNA polymerase β (anti-pol β) or any other factor as indicated, on ice for 45 minutes. Then, BER assay was performed by using DNA oligoduplex containing uracil present at position 8 as substrate by incubating for 20 minutes at 37 ^{0}C in the presence of [α ^{32}P]-dCTP, dNTPs and buffer as described in Materials and Methods. The reaction products were purified and separated on a denaturating 20% polyacrylamide sequencing gel, exposed to X-ray film and visualized by autoradiography. A typical autoradiogram from three different experiments is shown. Lanes 1–3, neuronal extracts from young brain (Y, 7days), adult brain (A, 6 months) and old brain (O≥, 2 years). Lanes 4–6, Y, A and O with 50 μM of Aph. Lanes 7-9, Y, A and O with 1 μl anti-Pol β. Lane 10, pure 0.5 units Udg, 1 unit Ape1, 0.1 units Pol β and 20 units Ligase. Lane 11, pure Udg, Ape1, Pol β and Ligase with Aph. Lane 12, pure Udg, Ape1, Pol β and Ligase with anti- Pol β.

Figure 15

Uracil initiated base excision repair activity in 'young', 'adult' and 'old' rat neuronal extract supplemented with Udg, Ape1, Pol β, Ligase.

A representative autoradiogram is shown. Lanes 1–3, neuronal extracts from young brain (Y, 7 days), adult brain (A, 6 months) and old brain (O≥, 2 years) with 0.5 units of Udg. Lanes 4-6, neuronal extracts (Y, A and O) supplemented with 1 unit of Ape1. Lanes 7-9, neuronal extracts (Y, A and O) supplemented with 0.1 units of Pol β. Lanes 10-12, neuronal extracts (Y, A and O) supplemented with 20 units of T_4 DNA Ligase (Ligase). Lanes 13-15, neuronal extracts from Y, A and O rat brain supplemented with Pol β and Ligase. Lanes 16-18 is supplemented with Udg and Ape1. Position of 8-mer and 21-mer marker as shown. Other notations are same as Figure 13.

Figure 16

Uracil initiated base excision repair activity in 'young', 'adult' and 'old' rat neuronal extract supplemented with Udg, Ape1, Pol β and Ligase in different combinations.

A representative autoradiogram is shown. Lanes 1–3, neuronal extracts from young brain (Y, 7days), adult brain (A, 6 months) and old brain (O≥, 2 years) supplemented with Udg, Pol β and Ligase. Lanes 4-6 are supplemented with Ape1, Pol β and Ligase. Lanes 7-9 are supplemented with Udg, Ape1 and Pol β. Other notations are same as Figure 13.

Figure 17

Uracil initiated base excision repair activity in 'young', 'adult' and 'old' rat neuronal extract supplemented with Udg, Ape1, Polβ and Ligase.

Lanes 1-3, neuronal extracts from young brain (Y, 7days), adult brain (A, 6 months) and old brain (O≥, 2 years) supplemented with Udg, Ape1, Polβ and Ligase. Reconstitution of uracil-initated BER was performed with supplementation of pure enzymes. Lane 4, only Udg. Lane 5, Udg with Ape1. Lane 6, Udg, Ape with Pol β. Lane 7, Udg, Ape1, Pol β with Ligase. Position of 8-mer unligated and 21-mer ligated product as shown.

Figure 18

Levels of BER enzyme proteins in 'young', 'adult' and 'old' rat neuronal extracts.

(A) Representative western blots show the expression level of Ligase III, Pol β and Ape1 in neuronal extract prepared from rats at different ages.

(B) Quantitative analysis of Ligase III, Pol β and Ape1 in neuronal extract as the function of age, determined by densitometric measurement on autoradiogram from three independent experiments. Values expressed as mean±SD.

*, ** and *** are significantly different ($P<0.05$ for adult and old) from the corresponding value at 'young' (ANOVA, Holm-Sidak method).

Figure 19

Measurement of 8-oxoG sites in young brain (Y, 7 days postnatal), adult brain (A, 6 months) and old brain (O, ≥ 2 years) neurons by single cell gel electrophoresis(comet assay).

Three different experiments have been conducted. A typical representative image of comet is shown.

Figure 20

Measurement of 8-oxoG sites in young brain (Y, 7 days postnatal), adult brain (A, 6 months) and old brain (O, ≥ 2 years) astrocytes by single cell gel electrophoresis(comet assay).

A typical representative image of comet is shown out of three different experiments.

Figure 21

8-oxoguanine DNA-glycosylase/AP lyase activity (Ogg1) in 'young', 'adult' and 'old' rat neuronal and astroglial extracts.

Neuronal and astroglial extracts prepared from different age of rats were incubated with 200 fmol of 5'[γ^{32}P]-kinased 24-mer DNA oligonucleotide duplex containing 8-oxoG at position 10 in the assay buffer for 120 minutes at 32 ^{0}C, generating the specific 9-mer cleavage product. The samples were then processed as described in Materials and Methods, Chapter 2. A typical autoradiogram from three different experiments is shown. (A) Lanes 1–3, neuronal extracts from young brain (Y, 7 days postnatal), adult brain (A, 6 months) and old brain (O, ≥ 2 years). Lanes 4–6, astroglial extracts from young brain (Y, 7 days postnatal), adult brain (A, 6 months) and old brain (O, ≥ 2 years). Lane 7 is 0.2 units of pure human 8-oxoguanine DNA glycosylase (Ogg1). Lane 8 is without any extracts (enzyme blank). One Unit of Ogg1 catalyzes the cleavage of 1 pmole of a [^{32}P]-oligonucleotide probe in 1 hour at 37 °C, at an 8-oxoG:C base pair within a duplex oligo. (B) Quantitative analysis of Ogg1 activity in neuronal and astroglial extracts as the function of age, determined by densitometric measurements on autoradiogram from three independent experiments. The incision activity was calculated as the relative amount of 9-mer product to 24-mer substrate (product/substrate+product). Values are expressed in mean±SD. *These values are significantly different ($P<0.05$ for adult and old) from the corresponding value at 'young' (ANOVA, Holm-Sidak method).

Figure 22

8-oxoG initiated base excision repair activity in 'young', 'adult' and 'old' rat neuronal extracts.

A representative autoradiogram showing the product of 8-oxoG initiated base excision repair synthesis incorporation following incubation of double-strand 8-oxoG containing oligonucleotide present at position 10 with neuronal extracts prepared from different age of rats were incubated with 25µg of protein extracts for 120 minutes at 32 ◦C in the presence of [α^{32}P]-dGTP. A typical autoradiogram from three different experiments is shown. Lanes 1–3, neuronal extracts from young (Y), adult (A) and old (O) rat brain.

Figure 23

8-oxodG initiated base excision repair activity in 'young', 'adult' and 'old' rat neuronal extracts supplemented with 0.2 units of Ogg1, 0.5 units of Ape1, 0.2 units of Pol β, 20 units of T_4 DNA Ligase alone.

A typical autoradiogram from three different experiments is shown. Lanes 1–3, neuronal extracts from young brain (Y, 7 days postnatal), adult brain (A, 6 months) and old brain (O, ≥ 2 years) with 0.2 units Ogg1. Lanes 4-6, neuronal extracts from Y, A and O with 0.5 units Ape1. Lanes 7-9, neuronal extracts from Y, A and O with 0.2 units Pol β. Lanes 10-12, neuronal extracts from Y, A and O with 20 units T_4 DNA Ligase (Ligase).

Figure 24

8-oxodG initiated base excision repair activity in 'young', 'adult' and 'old' rat neuronal extracts supplemented with different combinations of 0.2 units of Ogg1, 0.5 units of Ape1, 0.2 units of Pol β, 20 units of T_4 DNA Ligase alone.

A typical autoradiogram from three different experiments is shown. Lanes 1–3, neuronal extracts from young brain (Y, 7 days postnatal), adult brain (A, 6 months) and old brain (O, ≥ 2 years) with Ogg1 and Ape1. Lanes 4-6, neuronal extracts from Y, A and O with Pol β and Ligase. Lanes 7-9, neuronal extracts from Y, A and O with Ogg1, Ape1 and Pol β. Lanes 10-12, neuronal extracts from Y, A and O with Ogg1, Pol β and Ligase. Lanes 13-15, neuronal extracts from Y, A and O with Ape1, Pol β and Ligase. Lanes 16-18, neuronal extracts from Y, A and O with Ogg1, Ape1, Pol β and Ligase. Lane 19, with all pure enzymes Ogg1, Ape1, Pol β and Ligase.

Figure 25

Effect of calorie restriction on the levels of Pol β.

(A) Western blot showing the level of Pol β (39 kDa) in young, adult, old control and calorie restricted old neurons. Pure human DNA polymerase β (Pol β) is kept as a positive control in this blot. The relative levels of Pol β protein in various extracts were quantified using Image J 1.41 (NIH, USA). The densitometric value of 39 kDa band of Pol β, expressed as mean ±standard deviation from three different experiments, is 63±6.2, 37±2.6, 19±2 and 27.66±2 for young, adult old control and calorie restricted old rat neurons respectively. The increase in the levels of Pol β in calorie restricted old neuronal extracts when compared is significantly different from old control neuronal extracts at $P<0.01$(Student's-test). The average percentage increase in level in calorie restricted as compared to old control was 45.6. Y, A, O and OCR represents 7 days young rats, 6 months adult rats, 30 months old control rat and 30 months old calorie restricted rat. Lower panel showing β-actin levels in the same amount of extracts used in upper panel.

Figure 26

DNA gap repair activity in neuronal extracts prepared from young (7 days), adult (6 months), old and calorie restricted 30 months old rats.

(A) Lanes 1-2, neuronal extract from young rats. Lanes 3-4, neuronal extract from adult rats. Lanes 5-6, neuronal extract from old rats. Lanes 7-8, neuronal extract from calorie restricted old rats. Lanes 9-10, enzyme blanks. Lanes 1, 3, 5, 7 and 9 are with 1gap (1G) substrate. Lanes 2, 4, 6, 8 and 10 are with 4 gap (4G) substrate. Lanes 11 and 12 are standard 14-mer and 32-mer.

(B) The oligo duplex substrates with one and four nucleotide gaps used in the study are also shown. An oligo duplex having a 1 nucleotide gap in one strand is prepared by annealing a previously 5'-kinased oligo 2 (14-mer) and a oligo 4 (17-mer) to unlabeled oligo 1, the 32-mer. Such an annealing would produce a 1- gap oligo. It may be noted that the strand with 1 nucleotide gap is also having a ^{32}P label on 5' side and a non radioactive phosphate on the 5' side of down stream 17-mer. Similarly, annealing a 5'-kinased oligo 2 and oligo 3, to oligo 1, the 32-mer would yield a duplex with a 4 nucleotide gap. The strand with 4 nucleotide gap is also having a ^{32}P label on 5' side and a non radioactive phosphate on the 5' side of down stream 14-mer.

Y, A, O and OCR represents 7 days young rats, 6 months adult rats, 30 months old control rat and 30 months old calorie restricted rat.

Figure 27

DNA gap repair activity in neuronal extracts prepared from control and calorie restricted 30 months old rats. Effect of supplementing the neuronal extracts with 1mM ATP together with T_4 DNA Ligase.

Lanes 1-4, neuronal extracts from control and calorie restricted rats supplemented with 1mM ATP. Lanes 5-8, neuronal extracts from control and calorie restricted rats supplemented with 1mM ATP +20 units of T_4 DNA ligase. One unit of ligase activity is defined as (according to the supplier) that amount of enzyme required to achieve 50% ligation of lambda/Hind III digest in 30 minutes at 16 ^{0}C in 20 μl of the reaction mixture and at a 5'-DNA termini concentration of 0.12 μM. The densitometric values of 32-mer band (mean ±standard deviation calculated from three different experiments) in lanes 5 and 7 are shown at the bottom of those lanes. Lanes 9-10 enzyme blanks. Lanes 1, 3, 5, 7 and 9 are with 1gap (1G) substrate. Lanes 2, 4, 6, 8 and 10 are with 4 gap (4G) substrate. Lanes 11 and 12 are standard 14-mer and 32-mer. Other notations are same as in Figure 26.

Figure 28

Template driven primer extension activity in neuronal extracts prepared from young (7 days), adult (6 months), old and calorie restricted 30 months old rats.

(A) Lanes 1-2, neuronal extract from young rats with a oligo duplex substrate having a mismatched base pair (GT) or a correctly matched base pair (GC) at 3' position of the primer respectively; Lanes 3-4, neuronal extract from adult rats with GT and GC substrates respectively; Lanes 5-6, neuronal extract from old control rats with GT and GC substrates respectively; Lanes 7-8, neuronal extracts from calorie restricted old rats with GT and GC substrate respectively; Lanes 9-10, GT and GC substrate enzyme blanks respectively.

(B) The structures of the two oligo duplexes used in these experiments are also shown in the figure. The 5' kinased oligo-A, 14-mer is hybridized with (oligo-B, 21-mer) and (oligo-C, 21-mer) to form correct duplex (G-C) and mismatched duplex (G-T) in equimolar concentration. Y, A, O and OCR represent 7 days young rats,6 months adult rats, 30 months old control rat and 30 months old calorie restricted rat. Lanes 11and 12 show the mobility of standard 14 and 21-mers.

CONTENTS

List of abbreviations

AD	: Alzheimer's disease
AP	: Apurinic/Apyrimidinic
APH	: Aphidicolin
APE 1	: AP endonuclease 1
AT	: Ataxia Telengiectasia
ATP	: Adenosine Triphosphate
BER	: Base Excision Repair
BSA	: Bovine serum Albumin
CNS	: Central nervous system
CR	: Calorie restriction
CS	: Cockayne's syndrome
dATP	: 2'-deoxyadenosine 5'- triphosphate
dCTP	: 2'-deoxycytidine 5'- triphosphate
dGTP	: 2'-deoxyguanosine 5'- triphosphate
dTTP	: 2'-deoxythymidine 5'- triphosphate
DNA	: Deoxyribonucleic acid
dRPase	: 5'- 2-deoxyribose-5-phosphate lyase
DSB	: Double strand break
DTT	: Dithiothreitol
EDTA	: Ethylene diamine tetra acetic acid
FA	: Fanconi Anaemia
FEN 1	: Flap structure specific endonuclease-1
GFAP	: Glial cell fibrilary acidic protein
IR	: Ionizing radiation
kDa	: Kilodaltons

μg : microgram

μl : Microliter

NER : Nucleotide excision repair

NHEJ : Non Homologous End Joining

NSE : Neuron specific enolase

OGG1 : 8-oxoguanosine DNA glycosylase

PAGE : Polyacrylamide gel electrophoresis

PARP-1 : Poly ADP- ribose Polymerase 1

PCNA : proliferating nuclear antigen

POL β : DNA polymerase beta

PD : Parkinson's disease

PMSF : Phenylmethyl Sulfonyl Flouride

POPOP : 2, 2'–p-Phenylene–bis [5-phenyloxazole]

PPO : 2, 5-Diphenyl–1, 3–Oxazole

SSB : Single Strand Break

TBS : Tris buffered saline

TCA : Trichloroacetic acid

TCR : Transcription coupled repair

UDS : Unscheduled DNA synthesis

UDG : Uracil-DNA glycosylase

UV : Ultra Violet

WS : Werner Syndrome

XP : Xeroderma Pigmentosum

XRCC1 : X-ray repair cross complementing gene 1

Table 1

Approximate frequencies of occurrence of endogenous DNA damage in mammalian cells.

Damage	Events per cell per day	Reference
Depurination	10,000	(Lindahl and Nyberg 1972)
Depyrimidination	600	(Lindahl and Karlstrom 1973)
Deamination	100-300	(Lindahl and Nyberg 1974)
Single-strand breaks (Including all types of base damage viz. oxidative damage, adduct formation with reducing sugars, methylation,cross-links and so forth)	10,000	(Saul 1985)
Double-strand break	9	(Bernstein and Bernstein 1991)
Interstand cross link	8	(Bernstein and Bernstein 1991)
DNA-protein cross link	unknown	(Bernstein and Bernstein 1991)

Table 2

Name	Sequence
UG	5’g c c a t t g U g c t a c c g a t c g c g 3’
	3’c g g t a a c G c g a t g g c t a g c g c 5’
F	5’ c g c g a t c g g t a g c F c a a t g g c 3’
	3’ g c g c t a g c c a t c g C g t t a c c g 5’
O	5’ g a a c t a g t g O a t c c c c c g g g c t g c 3’
	3’ c t t g a t c a c C t a g g g g g c c c g a c g 5’

U: deoxy-uracil; F: Tetrahydrofuran abasic site analog; O: 8-oxo-guanine

Table 3

Detection of uracil sites in isolated neurons and astrocytes prepared from Young (7 days), Adult (6 months) and Old (≥2 years) rat cerebral cortex by comet assay following with or without treatment with uracil-DNA glycosylase (Udg).

Sample	Buffer	Udg	Net amount of uracil sensitive sites.
	Tail moment (mean±SD)	Tail moment (mean±SD)	
Young Neurons	14.96 ± 12.06	22.77 ± 15.22	7.81
Adult Neurons	115.71 ± 30.79	141.78 ± 65.29 *	26.07
Old Neurons	134.70 ± 41.83	185.34 ± 74.63 *	50.64
Young Astrocytes	18.80 ± 15.77	24.83 ± 16.84	6.03
Adult Astrocytes	121.92 ± 35.91	141.72 ± 57.39 *	19.8
Old Astrocytes	135.72 ± 39.22	175.44 ± 61.71 *	39.72

Values are expressed in mean ± (SD).

* These values are significantly different ($P<0.05$ for adult and old) from the corresponding value at 'young' (ANOVA, Holm-Sidak method).

Table 4

Detection of 8-oxoG sites in isolated neurons and astrocytes prepared from Young (7 days), Adult (6 months) and Old (≥2 years) rat cerebral cortex by comet assay following with or without treatment with 8-oxoguanine DNA glycosylase (Ogg1).

Sample	Buffer Tail moment (mean±SD)	Ogg1 Tail moment (mean±SD)	Net amount of 8-oxoG sites
Young Neurons	14.46 ± 10.09	22.75 ± 20.99	8.29
Adult Neurons	94.76 ± 33.10	138.18 ± 78.09*	43.42
Old Neurons	114.01 ± 21.24	175.64 ± 54.96*	61.63
Young Astrocytes	20.04 ± 14.89	30.74 ± 24.56	10.7
Adult Astrocytes	100.42 ± 36.95	135.05 ± 43.31*	34.63
Old Astrocytes	116.59 ± 23.10	169.49 ± 54.59*	52.9

Values are expressed in mean ± standard deviation (SD).

*These values are significantly different ($P<0.05$ for adult and old) from the corresponding value at 'young' (ANOVA, Holm-Sidak method).

Table 5

DNA polymerase β activity in young, adult and old control and calorie restricted rat neuronal extracts with 'activated' calf thymus DNA as template primer.

Sample	Specific activity of Pol β	% increase in specific activity in calorie restricted compared to control
Young Neurons	7859 ±1143	
Adult Neurons	780 ± 88	
Old Neurons	229 ± 86	-
Old calorie restricted Neurons	369 ± 61*	61.18

Values are mean ± S.D. and expressed as picomole of the radioactive deoxynucleotide incorporated into the acid insoluble fraction in 1 hour/milligram protein. Three independent experiments were performed in the case of young and adult neurons and five independent experiments were done in case of old control and calorie restricted old neurons respectively.* indicate values are significantly different from old control neuron, $P<0.01$ (Student' t-test).

CHAPTER 1

INTRODUCTION

INTRODUCTION

Aging is the progressive accumulation of changes with time associated with or responsible for the ever-increasing susceptibility to disease and death, which accompanies advancing age. These time-related changes are attributed to the aging process. This process may be common to all living things, for the phenomenon of aging and death is universal. If so, both aging and the rate of the aging process are under genetic control to some extent for the manifestations of aging, and life span differs between species and individual members of a species. Further, like all chemicals and chemical reactions, the manifestations of aging, which reflect the chemical composition and the rate of the aging process should be subject to environmental influences (Harman 1981).

Many theories of aging have been proposed in this century, and very often the proposer attempts to explain the whole complex process of aging in terms of a single theory. These attempts are probably doomed to failure, since it has become obvious that aging is multicausal or multifactorial.

There is evidence supporting at least 5 common characteristics of aging in mammals (Troen 2003).

1. Increased mortality with age after maturation.
2. Changes in biochemical composition in tissues with age.
3. Progressive decrease in physiological capacity with age.
4. Reduced ability to respond adaptively to environmental stimuli with age.
5. Increased susceptibility and vulnerability to disease.

Aging is a universal, intrinsic, progressive and deleterious process. Understanding it is of major interest to scientist, physicians as well as to the general population. Critical to this understanding is to formulate comprehensive theories of aging with high predictive and explanatory power. More than 300 theories have been postulated over the years in an effort to adequately explain the phenotype of aged organisms. Some theories have been proven to be related to aging where as others show high probability or possibility of affecting aging, while others has been disproven. Modern technology and research techniques have allowed the researchers to test many of these theories of aging. One theory, which enjoys considerable logic and rationale, is the DNA damage and repair theory.

DNA DAMAGE AND REPAIR THEORY

Alexander (1967) was the first to suggest that DNA damage per se, apart from its role in inducing mutations, may be a primary cause of aging. This theory postulates that the DNA damage, which is bound to occur in the body of an organism, is repaired efficiently up to a certain age of an organism, but thereafter is compromised in a predetermined manner. Thus, from some point of lifespan, DNA repair capacity decreases, therefore DNA damage accumulates. This accumulation of DNA damage leads to the breakdown of all the vital process in the cell finally leading to death.

Hart and Setlow (1974) observed a direct relationship between maximum achievable lifespan of a species and its capacity for UV induced unscheduled DNA synthesis (UDS) [a measure of DNA repair capacity] in fibroblast from seven

species. Similar observations were made using fibroblast from primates (Hart and Daniel 1980) between two mouse species with a difference in Lifespan of 2.5 fold (Hart *et al.* 1979), in skin cells of humans (Sutherland *et al.* 1980) and in lens epithelial cells from rat, rabbit, dog, cow and horse (Treton and Courtois 1982).

Wei *et al.* (1993) demonstrated in basal cell carcinoma skin cancer patients that the normal decline in DNA repair with increased age may account for the increased risk of skin cancer that begins in middle age, suggesting that the occurrence of skin cancer in the young may represent precocious aging.

Cortopassi and Wang (1996) demonstrated that the rate of mitochondrial mutagenesis in laboratory mouse is exponential and is 40 times faster than humans, which is consistent with the lifespan of mice. Zahn *et al.* (2000) showed in two mouse strains that in the strain with shorter longevity, damage increases and the repair deficiency is drastically different from those with higher longevity. These findings of strong coupling of the DNA status to aging as well as longevity suggest causative relations. De Boer *et al.* (2002) showed in mice with a mutation in XPD, a gene encoding a DNA helicase that is mutated in the human disorder trichothiodystrophy (TTD), that aging in TTD mice is caused by unrepaired DNA damage that compromises transcription, leading to functional inactivation of critical genes and enhanced apoptosis.

There are also a few studies that do not support DNA damage contributing to aging. Studies *in vitro* of senescent fibroblast showed minimal decrease in DNA repair (Hart and Setlow 1976).

Similarly, another study showed that the capacity to repair DNA single and double strand breaks mediated by ionizing radiation is not altered during *in vitro* cellular senescence (Mayer *et al.* 1989). However, there is extensive correlative evidence that DNA damage and mutations increase with age. In addition, there are studies that have demonstrated a corresponding decrease of DNA repair. This decrease in DNA repair may in part account for the increased DNA damage levels and mutation frequencies observed with age.

In mammals, long-lived neurons, differentiated muscle cells, and other differentiated cell types that do not divide or divide only slowly, accumulate DNA damage with age. These cells are likely candidates to govern the rate of mammalian aging. In brain, the level of DNA repair is low, endogenous damages accumulate with age, mRNA synthesis declines, and protein synthesis is reduced. Furthermore, cell loss occurs, tissue function declines, and functional impairments directly related to the central processes of aging occur. Thus, for the brain, there appears to be a direct relationship between the accumulation of DNA damage and the important feature of aging. In contrast to non-dividing or slowly dividing cells, at least some types of rapidly dividing cell populations appear to cope with DNA damage by replacing lethally damaged cells through replication of undamaged ones. Examples include duodenum and colon epithelial cells, and hemopoitic cells of bone marrow (Bernstein and Bernstein 1991).

It is the opinion of this lab that the DNA-damage and repair theory occupies a central role in explaining the mechanisms of the aging phenomenon at a basic and fundamental level. This concept has the necessary depth to compliment many other theories of aging either partly or fully. Moreover, the work presented in this thesis pertains to the DNA-repair capacity of brain cells during aging. In view of this, an attempt is made below to briefly review the existing knowledge about DNA-damage and DNA-repair in aging tissues with a special emphasis on brain.

DNA DAMAGE

Living cells face the tremendous task of maintaining an intact genome during the lifespan. The genetic information of all organisms and many viruses is stored in the form of a stable DNA molecule. Since loss of DNA signifies loss of genetic information, DNA has to be maintained. This is in contrast to other biological macromolecules, which can be degraded and replaced by newly synthesized molecules. DNA repair and replication are flanked by a continuous surveillance of genome integrity. When DNA damage or a replication block is detected, checkpoints are activated that delay cell cycle progression. At the same time, DNA repair genes and other factors are activated to remove the damage or replication block, or in situation where the DNA damage is too extensive, to initiate programmed cell death. In this way, premature progression into the next phase of the cell cycle is prevented, and changes in the genetic material in the form of somatic or heritable mutations are obviated.

The nature of the genetic component involved in aging is complex. Several possible mechanisms have been identified, which may contribute to the aging process. The most obvious change is seen in gene expression of altered forms of proteins or altered levels of particular proteins. Alterations in the integrity of DNA itself could contribute to the aging process. Many thousands of mutations may occur in each cell per day as a result of oxidative damage (Lindahl 1993). Though the DNA remains intact to a large extent during the life of an animal, the efficiency of the DNA repair machinery may decline with age (Bohr and Anson 1995, Walter *et al.* 1997). There are observations supporting that DNA repair may be more efficient in cells from longer lived species (Burkle *et al.* 1992, Grube and Burkle 1992). A plethora of alterations in the native structure of DNA occurs in the cell both due to external and internal factors. In view of the highly protective nature of the brain (including the blood brain barrier), the major enemy for causing DNA damage is from within the brain. The net rate of accumulation of a particular type of DNA damage depends on both the rate of its occurrence and the rate of its removal by repair enzymes (Hart and Setlow 1974).

DNA DAMAGES BY ENDOGENOUS FACTORS

AP- Sites

Apurinic/apyrimidinic damages can occur under physiological conditions by hydrolytic cleavage of the purines/pyrimidines from the deoxyribose phosphate backbone of DNA.

It is estimated that a mammalian cell at 37 ^{0}C loses about 10,000 purines and 500 pyrimidines from its DNA by spontaneous hydrolysis (Lindahl *et al.* 1977), such damage should be promptly removed from DNA, as it is a non-coding lesion that can lead to misincorporation during replication and transcription (Friedberg *et al.* 1995). The amount of DNA depurination caused by non enzymatic (spontaneous) hydrolysis that occurs in a single long lived, non replicating mammalian cell, such as human neuron, was estimated to be about 10^{8} purine bases during the lifespan. This accounts for about 3% of the total number of purines in the cell's DNA (Lindahl and Nyberg 1972). Thus, DNA is significantly unstable at the temperatures at which mammalian cells normally exist.

Mismatches and altered bases

Normal metabolic reactions may affect spontaneous deamination of bases in DNA. The products of deamination are mutagenic and would therefore interfere with correct transcriptional events in the brain. The deamination of cytosine to uracil is one of the ways by which uracil, a base in RNA, can occur in DNA. Bases in the DNA can also be modified through alkylation in a non-enzymatic way by compounds like S-adenosylmethionine that leads to the formation of N^{7}-methylguanine, N^{3}-methyladenine and O^{6}-methylguanine (Barrows and Magee 1982, Rydberg and Lindahl 1982). The methylated bases are eventually converted to a strand break. Oxidative damage to the bases in cellular DNA can be caused by products of oxidative metabolism like superoxide radical (O_2^{-}), hydroxyl radical (OH), hydrogen peroxide (H_2O_2).

DNA DAMAGE BY EXOGENOUS FACTORS

Dimers of Pyrimidines

Dimerized pyrimidines are very stable at extreme pressures and temperatures and pose a real threat to genomic integrity. UV light of wavelength around 260 nm induces the formation of chemical bonds between adjacent pyrimidines in DNA to form pyrimidine dimers. Tice and Setlow (1985) estimated that the rate at which UV irradiation induces pyrimidine dimers in human skin is 50,000 per cell per hour. Exposure to both near and far UV light forms several photoproducts (Rao 1993). Damage from UV light to the brain is quite limited since the brain is very well protected by the skull. Even so, UV induced damage is routinely used as model system with various tissues including brain (Rao 1997).

Single strand breaks (SSB)

SSBs are the most prevalent DNA-damage in mammalian cells. SSBs may be formed from AP sites at alkaline pH or removal of modified base by suitable glycosylase in the initial step of base excision repair. UV and ionizing radiations can cause SSBs by generation of free radicals directly or indirectly (Mullaart *et al.* 1990). SSBs could be a good marker for the DNA damage status in any cell.

Double strand damages: Cross-links and Double strand breaks (DSB)

Ultraviolet light, X-ray and gamma ray irradiation is known to induce cross-links, DSBs and SSBs. An important class of chemical modification in DNA is interstrand cross-links, since they prevent strand separation needed for replication

and also transcription. About eight or nine interstrand cross-links occur in each mammalian cell per day (Bernstein and Bernstein 1991). It can be assumed that in view of the protective situation of brain and due to its high metabolic activity the major damage to the DNA would emanate from endogenous factors and from exogenous factors that can cross the blood brain barrier. The frequency of occurrence of different DNA damages by various factors is summarized in Table 1.

DNA DAMAGE IN BRAIN

There are some studies that have looked into DNA damage in the brain, and these studies measured the accumulation of DNA damage with respect to age. Most of these studies appear to check the validity of a number of aging theories that have the central theme, the accumulation of genetic damage with age (Szilard 1959, Hart and Setlow 1974, Hayflick 1980, Gensler and Bernstein 1981).

Price *et al.* (1971) showed in mice that accumulation of SSBs is more in brain compared to liver with age. Chetsanga *et al.* (1977) reported that alkaline sucrose gradient sedimentation of DNA of mouse brain showed few bands for the old (30 months) and only one for the young (6 months), indicating degradation of DNA in old animals due to the breaks. Mori and Goto (1982) using single strand specific S1 endonuclease assay showed that young mouse brain DNA contained only 2.0% single strand regions when compared with mice aged 30 months. Interestingly, they did not find any such age associated changes in other organs like liver, kidney, heart, and spleen.

Tan *et al.* (1990) showed that steady the state level of 7-methylguanine, a major product formed by methylating agents both *in vitro* and *in vivo,* went up approximately 2 fold between young and old age. Mandavilli and Rao (1996b) showed that the number of SSBs increase with age in both cell types and in all the regions studied viz., cerebral cortex, cerebellum, hippocampus, hypothalamus and brain stem. The highest number of SSBs was seen in neurons and astrocytes of the cerebral cortex at any age. This also meant that cerebral cortex is the most vulnerable region for suffering DNA damage of this kind.

In contrast to the above findings, there are a small number of studies that reported no age dependent increase in DNA damage in brain. (Ono *et al.* 1976, Su *et al.* 1984, Mullaart *et al*. 1990). The reasons for these discrepancies are not clear as of now.

Alterations with age at the genetic level were also observed. Studies by Kanungo and Thakur (1979) and Chaturvedi and Kanungo (1985) showed enhanced condensation or compaction of chromatin with age in rat brain. Their results also showed a 50% reduction in the RNA-polymerase II activity in rat brain in old age which may be a result of structural changes in chromatin that may occur with increasing age. This aspect was studied by Venugopal and Rao (1991). They showed that both chromatin bound and free RNA polymerase II activities were decreased with age. In studies using enzyme monococcal nuclease as a probe for chromatin structure, Berkowitz *et al.* (1983) observed that DNA from neuronal preparations showed a decreased susceptibility to digestion during aging.

They also observed a dramatic increase in the nucleosome spacing of the chromatin. All the above studies indicate that with the advancement of age, there is an accumulation of DNA damage in brain.

DNA REPAIR

As a major defense against the environmental damage to cells, DNA-repair is present in all the organisms studied to date, including bacteria, yeast, fish, amphibians, rodents and humans. The DNA repair process would minimize cell killing, mutations, persistent DNA damage and errors in replication.

All organisms have therefore developed mechanisms to maintain the integrity of their genome by either preventing damage to DNA or correcting the damage once occurred. The variety of DNA lesions is matched by a multiplicity of avoidance and repair pathways (Eisen and Hanawalt 1999, Wood *et al.* 2001). Although the number of gene products that are involved in DNA repair is large in many organisms (more than 100 genes), nature makes use of a rather limited number of protein domains for DNA repair processes (Aravind and Koonin 1999, Wood *et al.* 2005).

The pathways involved in the repair of DNA damage in eukaryotic cells were initially categorized using damage sensitive mutants of yeast *Saccharomyces cerevisiae.* More recent characterization of repair has been carried out in metazoans exploiting human genetic repair diseases, mutations in mice, mutations in mammalian cell lines and *in vitro* repair systems (Friedberg *et al.* 1995, Friedberg 2005).

There are three major DNA repair pathways to counteract the different types of DNA damage. 1) A simple reversal of damage 2) Recombinational repair including the end joining 3) Excision repair including mismatch repair.

1) A simple reversal of damage

Direct reversal of DNA damage is a simple and important way of dealing with certain DNA lesions. Examples for this mechanism are the removal of alkyl groups by the ubiquitous enzyme alkyltransferase, reversal of the UV-induced pyrimidine dimer by the enzyme photolyase, or direct ligation of DNA single strand breaks (Friedberg et al. 1995, Eisen and Hanawalt 1999). Reversal of damage can take place by a single protein, e.g. O^6–methyl guanine methyltransferase, which removes methyl groups from O^6 –methyl guanine thus avoiding a mismatch formation since O^6 –methyl guanine can pair with both C and T (Mitra and Kaina 1993). In these modes of repair there is no cleavage of DNA, but chemical alterations are simply reversed *in situ*.

2) Recombinational type of repair

Both DNA DSBs and interstrand cross-links are unusual lesions, since they alter both strands of the DNA molecule (Thompson and Schild 1999). If left unrepaired, DSBs lead to broken chromosomes and cell death, and if repaired incorrectly, they can lead to chromosome rearrangements and cancer (Chu 1997). Recombination can occur either by the homologous recombination repair (HRR) or non-homologous end joining (NHEJ), the latter mode being less accurate.

3) Excision repair including mismatch repair

Excision repair is the most prominent and perhaps most universal in maintaining the genomic integrity. Essentially, the overall strategy of this pathway consists of 4 steps. **1**). Recognition of the damage site **2**). Excision of the damaged portion. **3**). Resynthesis of the removed sequence by DNA polymerases **4**). Ligation of the newly synthesized strand by ligases.

The overall excision repair consists of 3 major pathways: nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR).

Nucleotide excision repair (NER)

NER is a highly sophisticated and versatile DNA damage removal pathway. NER removes predominantly bulky DNA adducts and damage that distorts the DNA structure considerably. NER is able to handle a multitude of DNA lesions, the most relevant of which may be the damage inflicted on DNA by the UV component of sunlight (de Laat *et al.* 1999). Substrates for NER are damage due to exposure to UV irradiation, adduct by with a variety of compounds like cisplatin, psolaren and carcinogens like acetlyaminoflourine etc. The available evidence suggests that the overall process resembles that in *E. coli*, but there are many differences in detail and the specific proteins involved. NER differs from BER in that the excision patch is quite long in NER when compared to the shorter patch in BER.

In the case of UV induced damage, incision occurs precisely at 6 bases 3' to the damage and 22 bases 5' to the damage, thus releasing a 29 nucleotide fragment (Tanaka and Wood 1994). It is for this reason that NER is considered as 'long patch repair'.

Mismatch repair (MMR)

MMR plays a significant postreplicative role in safeguarding the integrity of the genome virtually in all organisms from bacteria to mammals. This repair pathway corrects base–base and insertion/deletion (I/D) mismatches that have escaped the proofreading function of replicative polymerases. The human and the bacterial DNA MMR systems are very similar not only in structure, but also in function.

MMR confers to the genome a 100–1000 fold protection against mutations arising during DNA replication (Loeb 1994). The MMR machinary scans and repairs newly replicated DNA by excising the mutated strand in either direction to the mismatch. In its absence, cells assume a mutator phenotype in which the rate of spontaneous mutation is greatly elevated. The discovery that defects in MMR segregate with certain cancer predisposition syndromes highlights its essential role in mutation avoidance. Mutations in one of the human DNA MMR genes, hMSH2, account for approximately half of all cases of genetically linked hereditary non-polyposis colorectal cancer (Hemminki *et al.* 1994, Fishel *et al.* 1993), and inactivation of the mouse MSH2 gene results in a lymphoproliferative disorder and a predisposition to malignancy (de Wind *et al.* 1995).

The human system has a number of homologues for each bacterial protein. The human MMR system may be regulated in several different biological situations. Studies with immunohistochemistry showed that the hMSH2 protein resides in proliferative portions of oesophageal and intestinal epithelium (Wilson *et al.* 1995, Marra *et al.* 1996, Leach *et al.* 1996) and increases atleast 12 fold in proliferating cells (Marra et al. 1996).

Base excision repair (BER)

The BER pathway consists essentially of 4 steps and can be divided into two sub pathways; one termed 'short patch or single nucleotide replacement pathway' and the other 'long patch pathway' involving the insertion of upto 13 nucleotides (Figure 1). Step one of short patch pathway (left panel of Figure 1) consists of the recognition and cleavage of an altered base (DB) from the deoxyribose phosphate moiety by an appropriate DNA-glycosylase. This enzyme also allows the AP endonuclease (Ape1) to reach the site (Figure 1.1). Multiple DNA glycosylases with varying substrate specificity are continuously scanning the DNA. For example, eight human nuclear glycosylases have been cloned to date (Scharer and Jiricny 2001). Some DNA-glycosylases recognize and remove 8-oxoguanine opposite C (Rosenquist *et al.* 1997, Radicella *et al.* 1997), oxidative forms of bases like thymine glycol, cytosine glycol, dihydrouracil (Hilbert *et al.* 1997) and alkylated adenine like 3-methyl adenine, ethenoadenine and hypoxanthine (Chakravarti *et al.* 1991, Samson *et al.* 1991).

In the second step (Figure 1.2), the DNA chain at the 5'-side of the abasic site is cleaved by a major endonuclease Ape1 specific for abasic damage. Ape1 is the major AP endonuclease in humans, also known as HAP1, APEX, and REF1 (Demple *et al.* 1991, Seki *et al.* 1992, Robson *et al.* 1992). The enzyme flips out the baseless deoxyribose and cleaves it on the 5' side. Also, like in the case of the 1st step, this enzyme, still bound to DNA, attracts and interacts with DNA Polymerase β (Pol β), which is involved in the next step in the repair pathway. The glycosylase dissociates from DNA at this point.

In the third step, Pol β fills up the one-nucleotide gap and also releases the 5'-2-deoxyribose-5 phosphate (dRP). At the same time, DNA ligase III - XRCC1 (X-ray repair cross-complementing, gene I) complex arrives at the site.

In the fourth step, DNA ligase III seals the nick and Pol β dissociates from the site. Subsequently the XRCC1 and ligase III complex comes off from the site, leaving behind repaired DNA (Figure 1 left panel, B).

Human XRCC1 not only complexes with DNA ligase III but also interacts with other core enzymes involved in BER and is therefore thought to play a crucial role in protein exchanges in the pathway. Eukaryotes, in contrast to prokaryotes, contain more than one DNA ligase, and these enzymes have distinct roles in DNA metabolism. Five DNA ligase activities, I-V, have been purified from mammalian cell extracts. Ligase III is more specifically involved in DNA repair and recombination.

The predominant route for BER is the 'short patch or single nucleotide pathway' shown on the left side of Figure 1.

The overall process of BER is characterized by the sequential binding of proteins to DNA as well as among themselves in pairs facilitating the repair process to occur efficiently and swiftly (Kohler *et al.* 1999, Hoeijmakers 2001).

When the dRpase (5'-2-deoxyribose-5-phosphate lyase) activity of Pol β cannot act on the chemical structure of the terminal sugar phosphate after AP endonuclease incision (Step 2) (for example, reduced or oxidized abasic site) the repair synthesis would nevertheless continue but in a strand displacement manner. According to recent reports, Pol β also initiates regular long-patch BER, which involves synthesis of upto 13 nucleotides beginning at the damage site (Frosina *et al.* 1996, Klungland and Lindahl 1997, Stucki *et al.* 1998, Matsumoto *et al.* 1999, Dantzer *et al.* 2000, Podlutsky *et al.* 2001, Prasad *et al.* 2001). Poly (ADP-ribose) polymerase-1 (PARP-1) is required for a switch to initiate long-patch BER when the repair product cannot be ligated after incorporation of the first nucleotide by Pol β (Dantzer *et al.* 2000, Podlutsky *et al.* 2001, Prasad *et al.* 2001). In case of long patch repair, Pol β is replaced by Pols δ or ε, which conduct strand displacement synthesis (Fortini *et al.* 1998, Stucki *et al.* 1998).

This long patch repair requires the activator proliferating nuclear antigen (PCNA) and a 'flap' structure specific endonuclease-1 (FEN1) activity to cut the flap like structure produced by strand displacement synthesis (Wu *et al.* 1996, Klungland and Lindahl 1997). Nealon *et al.* (1996) suggested that while Pol β is

the major BER polymerase in human cells, other polymerases also contribute to a significant extent.

DNA REPAIR IN BRAIN

Neurons are postmitotic and are the longest living cells in the body. The information regarding the ability of brain cells to carry out specific types of DNA repair reactions is scanty although several DNA repair enzymes have been identified in brain (Waser *et al.* 1979, Kuenzle 1985, Mazzarello *et al.* 1992, Rao 1993, Weng and Sirover 1993).

Alexander (1967) first noticed that the DNA-repair system is lower once cells are differentiated into a postmitotic state. Gensler (1981a, 1981b) found that unscheduled DNA synthesis (measure of DNA repair capacity) in response to UV irradiation is markedly lower in mature hamster brain than in mature hamster lung or kidney cells. Korr and Schultze (1989) demonstrated through autoradiography, that DNA repair was low in various types of cells of adult mouse brain *in vivo*. Even though the level of DNA repair for some types of damages in neurons is low, this repair might be sufficient to cope with DNA lesions if they occur at a low rate in these cells. Studies from this laboratory, Subba Rao and Subba Rao (1984a), showed measurable levels of Pol β, which is considered a repair enzyme in adult and aging rat brain. Further studies also showed there is no change in the fidelity of this enzyme between young, adult and old ages (Subba Rao *et al.* 1985). A putative 'House keeping' DNA repair enzyme, a non-specific alkaline DNase of rat brain

exhibited high activity during adult and old ages (Venugopal and Rao 1993, Rao 1990).

Hanawalt *et al.* (1992) showed not only significant DNA repair in a model neuronal cell system, but also the tenet of genomic heterogeneity of DNA repair is applicable to the postmitotic system such as brain.

Brain has different cell types and relatively few studies have examined the expression of DNA repair proteins in different brain cell types. APE/Ref1 mRNA levels were shown to be particularly high in certain hypothalamic nuclei, as well as in hippocampus and cerebellum (Wilson *et al.* 1996). APE/Ref1 is a multifunctional enzyme that has both an AP endonuclease activity that is essential for glycolase initiated BER, and also acts as a redox-sensing factor for transcription by fos and jun (Flaherty *et al.* 2001). Duguid *et al.* (1995) found that APE/Ref1 was expressed heterogeneously throughout the brain with high levels in hippocampal neurons. Verjat *et al.* (2000) using *in situ* hybridization reported heterogeneous expression of 8-oxoG glycolyase 1(Ogg1) mRNA in different regions of the brain. The highest levels were observed in the hippocampus, cerebral cortex, cerebellum and several hypothalamic and brain stem cell groups.

LeDoux *et al.* (1996) using primary cultures of homogenous populations of each of the three glial cell types in the brain studied DNA repair and they found that repair of O^6–methylguanine by methyl guanine methytranferase was better in astrocytes than in oligodendrocytes or microglial cells.

Studies have been conducted to examine DNA repair by mitochondria (LeDoux and Wilson 2001, Susan *et al.* 1998). The studies indicated that astrocytes have higher DNA repair capacity compared to other glial cell types. Chen *et al.* (2000) detected BER of oxidative damage in mitochondrial extracts from adult rat brain, as well as from cultured cortical neurons and astrocytes. Mandavilli *et al.* (2000) found that endogenous mitochondrial DNA damage in the caudate putamen and cerebellum was higher in 1 year old than 22 day old mice, suggesting an age related decrease in mitochondrial DNA repair.

BRAIN and BER

Brain is very well protected externally (including the blood brain barrier) and it can be assumed that most of the damage to the DNA of brain would be due to endogenous factors and from such exogenous factors that can cross the blood brain barrier. There is ample amount of evidence, that BER would be the main guardian to ensure genomic stability in the highly metabolic organ brain. For example, Pol β is the enzyme that takes part in BER compared to other polymerases found in the nuclei of the mammalian cells, particularly in filling the single nucleotide gap (Wood and Shivji 1997, Wilson and Thompson 1997, Fortini *et al.* 1998). Waser *et al.* (1979) found that Pol β constitutes 90% of the DNA polymerase activity in adult rat brain and it was concluded that Pol β is the major repair enzyme. It thus can be expected that genomic maintenance in brain cells can be largely taken care of a Pol β dependent BER pathway. As outlined above, many proteins known to be participating in BER are found in brain also (Rao 2002).

The status of BER in health and disease assumes great importance with the accumulating knowledge of several neurodegenerative diseases that appear in old age and their molecular link to genomic stability (Martin 1999, Subba Rao 2007).

DNA POLYMERASE β

Replication of DNA is carried by enzymes called DNA polymerases (Brautigam and Steitz 1998). To date, the number of DNA polymerases has increased to atleast 19 since the initial discovery of DNA Pol α (DNA polymeraseα) in eukaryotic cells in 1957. In early 1970, Pols β and γ (DNA polymerase β and DNA polymeraseγ) were discovered, leading to the simple concept that Pol α was the enzyme responsible for nuclear DNA replication, Pol β for DNA repair, Pol γ for mitochondrial DNA replication. Later in 1980's, Pol δ (DNA polymerase δ) and Pol ε (DNA polymerase ε) were discovered and intensive work on them suggested that a particular Pol might have more than one functional task and that a particular DNA synthetic event may require more than one Pol (Stucki *et al.* 2001). In most recent times several other DNA polymerases have been discovered (polymerases η, κ, λ, μ, ρ etc.,) and these polymerases show the interesting ability to copy a DNA strand with a lesion with varied specificity. It is likely that the precise function of these newly discovered polymerases will be known in the next few years (Hubscher *et al.* 2002).

Pol β is the smallest eukaryotic polymerase and it was proposed as a DNA repair enzyme 20 years ago (Hubscher *et al.* 1979). It is found in vertebrates and lacks intrinsic accessory activities such as 3'-5' exonuclease, endonuclease, dNMP turnover, RNaseH, or the reverse of the DNA synthesis reaction, pyrophosphorolysis (Baril *et al.* 1971, Chang and Bollum 1971, Matsukage *et al.* 1974, Kumar *et al.* 1990a). Pol β is expressed independent of the cell cycle stage (Zmudzka *et al.* 1988), but regulation of the enzyme is in a tissue specific fashion. Pol β is composed of a single 39 kda polypeptide containing 335 amino acid residues and the secondary structure predictions suggest ordinary globular structure with α-helix content (Zmudzka *et al.* 1986, SenGupta *et al.* 1986). Both human and rat enzymes were cloned 15 years ago and extensively studied over the years by Dr.Sam Wilson and his group (Zmudzka *et al.* 1986, SenGupta *et al.* 1986). The recombinant human enzyme purified from *E.coli*, does not have exonuclease or endonuclease activity like natural enzymes, and the recombinant enzyme is similar to the natural enzyme (Abbotts *et al.* 1988). The recombinant enzyme has the same template-primer specificity as the natural enzyme and has a reactive epitope for anti-β-polymerase IgG.

Pol β is folded into distinct domains each associated with a specific functional activity. An 8 kDa amino terminus domain is connected to a 31 kDa domain by a protease sensitive hinge region (Prasad *et al.* 1998).

These two isolated protein domains have dedicated biochemical activities (Kumar et al. 1990a, Kumar *et al.* 1990b, Casas-Finet *et al.* 1991, Prasad *et al.* 1993, Prasad

et al. 1994, Piersen *et al.* 1996). The 31 kDa domain catalyzes nucleotidyl transferase reaction where as the 8 kDa domain has a lyase activity (dRpase) that removes the 5'deoxyribose phosphate generated after incision by an AP endonuclease during BER (Matsumoto and Kim 1995) and also single strand binding activity (Prasad *et al.* 1994). During the past several years of research, evidence has accumulated which confirms a role for DNA Pol β in the mammalian AP site BER pathway. Lack of DNA Pol β in DNA Pol β deficient cells or in the presence of neutralizing antibody leads to a reduction in DNA repair activity, which strongly suggests a role for DNA Pol β in the BER pathway *in vivo* (Dianov *et al.* 1999). It was found that Pol β fills a gap of upto 6 nucleotides in one of the strands of a double stranded DNA very efficiently and in a processive manner if the down stream primer has a 5'-phosphate. On the other hand, if the down stream primer has a 5'-OH or there is no downstream primer at all (no gap at all therefore simply extending the primer using the other strand as template) then the addition of nucleotides to the primer is slow and distributive (Prasad *et al*. 1994, Singhal and Wilson 1993). Thus the most preferred substrate for Pol β seems to be a double stranded DNA with a gap of less than 6 nucleotides (the most preferred being single nucleotide gap) with a 5'-phosphate on the down stream primer.

It must however be mentioned that Pol β may be slow and distributive, but not inactive, towards simple template primers without any gap in the primer strand (Wang and Korn 1982). By biochemical and physical experiments the binding site

for the 8kda domain is shown to be six nucleotides (Casas-Finet *et al.* 1992) and the intact enzyme covers about 12 nucleotides.

Pol β associates with other enzymes of the BER pathway such as DNA ligase I, AP endonucelase, and XRCC1-DNA ligase III. It has been demonstrated (Prasad *et al.* 1996, Dimitriadis *et al.* 1998) recently that Pol β and DNA ligase I interact and form a tight complex in solution. Other roles for Pol β have been envisaged (Wilson 1998). *In vitro* DNA repair studies have shown that Pol β has a role in repair of monofunctional DNA adducts by Hela nuclear extracts (Dianov *et al.* 1992) and of UV damaged DNA (Jenkins *et al.* 1992) and abasic lesions in DNA (Matsumoto and Bogenhagen 1989) by *Xenopus laevis* oocyte extract.

It has also been implicated in meiotic events associated with synapsis and recombination. It dynamically localizes to the synaptonemal complexes formed by chromosomal pairs in meiosis (Plug *et al.* 1997). The 67 kDa *S.cerevisiae* homolog of mammalian Pol β encoded by nonessential Pol 4 gene has been implicated in DSB repair. It probably utilizes a NHEJ mechanism. Sugo *et al.* (2000) have shown in mice by targeted disruption of the Pol β gene retarded growth, and the mice died of respiratory failure immediately after birth. The increased apoptotic cell death observed in the developing central and peripheral nervous system suggest that Pol β plays an essential role in neurogenesis.

DNA REPAIR AND HUMAN NEUROLOGICAL DISORDERS OF AGING.

DNA repair disorders refer to a group of conditions that are characterized by a failure of distinct cellular DNA repair mechanisms to function properly. The consequences of these failures are far reaching and extend to abnormalities related to normal growth and development, aging (normal and premature), programmed cell death, and cancer inherited conditions. Some of these inherited disorders closely associated with defective DNA-repair are mentioned briefly below.

XERODERMA PIGMENTOSUM (XP)

XP is a DNA repair disorder related to the NER pathway. It is an autosomal recessive disorder characterized by cutaneous photosensitivity, pigmentary changes, and a propensity for the early development of malignancies in sun exposed mucocutaneous areas, including the eye (Kraemer *et al.* 1987). Photosensitivity and the high cancer incidence observed in XP patients are due to mutations in genes that are unique to global genomic repair and have no role in transcription-coupled repair; for example XPC and DDB1 (XPE), and replication polymerase η (Epstein *et al.* 1970, Day 1975, Kraemer *et al.* 1994a, Kraemer *et al.* 1994b, Eveno *et al.* 1995, Kraemer 1997, Subba Rao 2007). Most cases are symptomatic in childhood, except for an adult variant form.

The predominant symptoms include sun sensitivity, photophobia, and, in about 20% of the patients, neurological abnormal (Kraemer *et al.* 1987, Vermeulen *et al.* 1994). Mutations in seven different genes have been reported in patients with XP. These include genes involved in complementation groups (XPA, ERCC3 [XPB],

XPC, ERCC2 [XPD], DDB1, ERCC4 and ERCC5) that have a role in NER (Subba Rao 2007).

COCKAYNE SYNDROME (CS)

CS was first reported in 1936 by Edward Alfred Cockayne, a British physician. CS is an early-onset, progressive neurological disorder characterized by dwarfism, microencaphaly, mental retardation, sensitivity to sunlight, retinal degeneration, partial deafness, and facio-skeletal and/orgait abnormalities, but no increased cancer incidence (Dov Soffer 1979). In terms of its neuropathology, the CS brain shows increased fibrosis, neuronal dystrophy, and an accumulation of senile plaques and/or neurofibrillary tangles along with progressive demyelination or dysmyelination (Bohr *et al.* 2005). This syndrome is not associated with cancer or loss of personality. The two proteins found to be mutated in this syndrome, ERCC8 and ERCC6, have been shown to be required for transcription-coupled repair (Subba Rao 2007).

ATAXIA TELANGIECTASIA (AT) AND NIJMEGEN BREAKAGE SYNDROME (NBS)

AT is characterized primarily by cerebellar degeneration, immunodeficiency, genome instability, clinical radiosensitivity, and cancer predisposition. NBS shares all these features except cerebellar deterioration. The cellular phenotypes of AT and NBS are almost indistinguishable, however, and include chromosomal instability, radiosensitivity, and defects in cell cycle checkpoints normally induced by ionizing radiation (Shiloh 1997).

The protein product of the gene responsible for AT, designated ATM, is a member of a family of kinases characterized by a carboxy-terminal phosphatidylinositol 3-kinase-like domain. The NBS1 protein is specifically mutated in patients with NBS and forms a complex with the DNA repair proteins Rad50 and Mre11 (Zhao *et al.* 2000). Each of these proteins functions in differing aspects of DSB resolution and/or DNA-damage-checkpoint responses. It is hypothesized that the neurological dysfunction of the associated disorders arises from (i) a defect in the processing of DSBs presumably by the NHEJ pathway and/or (ii) an inappropriate DNA-damage response, quite possibly during neural development (Kulkarni and Wilson 2008).

TRICHOTHIODYSTROPHY (TTD)

TTD is a DNA repair disorder related to the mutation of genes in the NER pathway. It is an autosomal recessive disorder characterized by mental abnormalities, with sulfur deficient brittle hair and skin photosensitivity, growth retardation and neurological abnormalities (Subba Rao 2007, Kulkarni and Wilson 2008).

ALZHEIMER'S DISEASE (AD)

AD is a degenerative disorder of the central nervous system in humans. The disorder is thought to be caused by misfolding of β-amyloid and Tau proteins, which aggregate and deposit as plaques and neurofibrillary tangles (NFTs) in AD brains (Smith *et al.* 2000). AD is characterized by progressive neuronal degeneration, which is regarded as a feature of accelerated aging. In AD, neurons

in cerebral cortex, basal forebrain, and locus ceruleus are progressively lost. There are evidences to suggest that 8-hydroxyguanine, 8-hydroxyadenine, and 5,6-diamino-5-formamidopyrimidine accumulate abnormally in both nuclear and mtDNA isolated from vulnerable brain regions in amnestic mild cognitive impairment, the earliest clinical manifestation of AD, and that there are decreases in 8-oxoguanine glycosylase activity in the nuclear fraction of AD hippocampal and parahippocampal gyri (HPG), superior and middle temporal gyri (SMTG), and inferior parietal lobule (IPL) (Lovell and Markesbery 2007, Markesbery and Lovell 2006, Lovell *et al.* 2000).

It is noteworthy that the two DNA repair pathways that are most likely to be adversely affected in AD are BER and end NHEJ, due to limited DNA base damage processing by DNA glycosylases, reduced DNA synthesis capacity by DNA Pol β and reduced NHEJ activity as well as protein levels of DNA PK catalytic subunits (Fishel *et al.* 2007, Shackelford 2006, Weissman *et al.* 2007).

PARKINSON'S DISEASE (PD)

PD, which is characterized by muscle rigidity, tremors, and in extreme cases, a loss of physical movement (akinesia), is caused by degeneration of dopaminergic cells in the brain's susbtantia nigra, the region that controls voluntary movement, produces the neurotransmitter dopamine, and regulates mood (Kulkarni and Wilson 2008). Increased levels of oxidative stress as well as expression of the mitochondrial BER enzyme 8-oxoguanosine DNA glycosylase (Ogg1) have been

reported to occur in the substantia nigra region of the brain in patients with PD (Fukae *et al.* 2005). Robbins *et al.* (1983) found that cell lines from six patients with PD were significantly more sensitive to X-rays than were normal cell lines. Sensitivity to UV irradiation was normal in these patients. These results suggest that such a DNA repair defect could cause rapid, abnormal accumulation of spontaneously occurring DNA damage in PD and AD neurons *in vivo*, which results in premature death.

HUNTINGTON'S DISEASES (HD)

HD is an autosomal-dominant disorder characterized by involuntary choreiform movements, loss of cognitive function, and a massive loss of neurons in the striatum (Albin *et al.* 1990, Butterfield *et al.* 2001). It is caused by CAG trinucleotide expansion in the coding region of the HD gene on chromosome 4; this expansion leads to a toxic glutamine-rich protein. Oxidative stress seems to be a feature of the disease; patients harbor elevated levels of oxidative biomarkers, such as malondialdehyde, 3-nitrotyrosine, and heme oxygenase-1 and exhibit oxidative fragmentation of DNA in their cortical and striatal neurons (Butterfield *et al.* 2001, Kovtun *et al.* 2007). Recent studies have demonstrated a more direct link between oxidative stress and HD by correlating CAG expansion to the activity of the BER enzyme Ogg1. In particular, the DNA glycosylase Ogg1 is proposed to promote a ''toxic oxidation cycle'' in which strand displacement and slippage during BER of oxidized bases can result in expansion of CAG triplets during gap-filling synthesis (Kovtun et al. 2007).

FRIEDREICH'S ATAXIA, AND MYOTONIC DYSTROPHY TYPES 1 AND 2

Friedreich's ataxia is an autosomal-recessive disorder characterized by progressive ataxia and loss of limb deep-tendon reflexes (contraction of muscles in response to stimuli such as tapping of muscle tendons). Most of the clinical features are the result of degeneration and atrophy of sensory neurons, spinal-cerebellar tracts, and sensory fibers in the peripheral nerves (Harding 1981, Koutnikova *et al.* 1997). In Friedreich's ataxia, expansion of the trinucleotide GAA occurs in the first intron of the gene on chromosome 9 that codes for the frataxin (FRDA) protein, whereas in myotonic dystrophy either the CAG (type 1) or the CCTG (type 2) expansions are seen in the a zinc finger protein 9 (*CNBP*) gene (Dere and Wells 2006, Subba Rao 2007).

SPINOCEREBELLAR ATAXIA WITH AXONAL NEUROPATHY-1 AND TRIPLE-A SYNDROME.

In spinocerebellar ataxia with axonal neuropathy-1 (SCAN1), there is a progressive degeneration of postmitotic neurons. El-Khamisy *et al.* (2005) have recently demonstrated that this neurodegenerative disease results from a mutation in the gene encoding tyrosyl DNA phosphodiesterase 1 (TDP1). TDP1 in human cells is required for the repair of chromosomal SSBs arising from abortive topoisomerase I activity or oxidative stress. TDP1 is part of the multi protein SSB repair complex and directly interacts with DNA ligase IIIα, and this complex is inactive in SCAN1 cells. These findings suggest that the TDP1-dependent SSB

repair pathway is defective in differentiated neurons of SCAN1 patients. Normally, SSBs or gaps are repaired in neurons through BER, in which both DNA ligase IIIα and XRCC1 participate, along with polynucleotide kinase (Rao 2003b). It therefore seems that the TDP1-dependent SSB repair is a slightly different mode of repair, and could be of considerable importance in brain cells where it deals with SSBs resulting from a variety of causes.

Triple-A (achalasia–addisonian–alacrima) syndrome, which is due to a repair defect of DNA SSBs was found to be caused by a mutation in a gene called AAAS (located on chromosome 12q13), which codes for a protein named ALADIN.

HUTCHINSON-GUILFORD PROGERIA SYNDROME (HGP)

HGP is an extremely rare genetic disease that accelerates the aging process to about seven times the normal rate. Because of this accelerated aging, a child of ten years will have similar respiratory, cardiovascular, and arthritic conditions that a 70-year-old would have. Currently, there is no cure for this disease, and because of its rare nature, no definitive cause can be pinpointed. Some physical features of progeria children include dwarfism, wrinkled/aged-looking skin, baldness, and a pinched nose. Mental growth is equivalent to other children of the same age. Most children with progeria live no longer than their early teenage years. Cultured HGP fibroblasts have been reported to have decreased ability to repair single strand breaks following gamma irradiation (Epstein *et al.* 1974, Little *et al.* 1975).

WERNER SYNDROME (WS)

Mutations in the RECQL2 gene, encoding for a DNA helicase, are responsible for WS (Gray *et al.* 1997, Nehlin *et al.* 2000, Mohaghegh and Hickson 2001, Shen and Loeb 2001). WS is characterized by caricatural premature aging associated with graying of the hair often before the age of 20 years. Malignancy occurs in 10% of the cases. The features of WS are scleroderma-like skin changes, especially in the extremities, cataract, subcutaneous calcification, premature arteriosclerosis, diabetes mellitus, and a wizened and prematurely aged faces. Fujiwara *et al.* (1977) showed that the elongation rate of DNA chains during replication was significantly slower in WS skin fibroblast cells than in normal cells. These cells exhibited normal repair of X-ray induced and SSBs and UV induced repair synthesis. The finite replicative life span of human cells *in vitro*, the Hayflick phenomenon (Hayflick 1965), is due to the stochastic loss of replicative ability in a continuously increasing fraction of newborn cells at every generation.

Normal human fibroblasts achieve approximately 60 population doublings in culture, while WS cells usually achieve only about 20 population doublings (Faragher *et al.* 1993).

BLOOM'S SYNDROME (BS)

BS is due to mutations in the RECQL gene, a DNA helicase involved in DNA replication and repair (Ellis *et al.* 1995, Karow *et al.* 1997, Kitao *et al.* 1999). BS is characterized by growth deficiency, sun-sensitive facial reddening, sub- or infertility, variable degrees of immunodeficiency, and predisposition to cancers of

many sites and types (German 1995). Cells from patients with Bloom's syndrome are genomically unstable and show elevated levels of both homologus recombination and sister chromatid exchange.

FANCONI'S ANEMIA (FA)

FA is an inherited autosomal recessive disorder. It is classically diagnosed between 2 and 15 years of age. The disease is caused by a genetic defect that prevents cells from fixing damaged DNA or removing toxic, oxygen-free radicals that damage cells. It is characterized by refractory anemia progressing to pancytopenia, congenital and developmental abnormalities, and an increased incidence of malignancy. Fanconi cells are deficient in repair of dihydrooxydihydro thymine residues and hypersensitive to cross-linking agents such as mitomycin C and cis-platinum (Fujiwara *et al.* 1987).

More than 150 human genetic disease syndromes have been characterized as having some potential relationship to the normal biology of aging. Approximately, 40% of infant mortality results from genetically determined conditions. The great abundance of human genetic variation raises the possibility that certain mutations will affect genes concerned with longevity. Whether or not any of this life shortening mutation reflects alterations in some of the genes that might relate to longevity is unclear.

In some syndromes, evidence of both elevated DNA damage and premature aging is observed. These include AT, CS, WS. Neurodegeneration is seen in AT, CS, XP, HD, PD, and AD.

SCOPE OF THE PRESENT STUDY

The present study constitutes the continued effort of this laboratory to assess the validity of the hypothesis that accumulation of DNA-damage and decreased DNA-repair capacity is at least one of the major naturally chosen genetic switches for initiating the phenomenon of aging and its associated disabilities. The emphasis of this work is on brain cells.

A major outcome of these extended efforts was the finding that DNA Pol β is the predominant DNA polymerase in brain and this activity markedly decreases by adult hood itself and the trend continues through old age. Through an indirect biochemical approach, both SSBs and DSBs in DNA were found to increase in both neurons and astroglia. Similarly, Pol β dependent primer extension activity was drastically decreased in adult and old ages, and it could be restored back to higher levels by supplementation of the neuronal extracts with purified recombinant Pol β. Subsequent preliminary work had also shown that gap repair through the BER pathway is also adversely affected in aging neurons and supplementation of the neuronal extracts with Pol β and DNA ligase could restore this activity (Rao *et al.* 2001, Rao 2002, Rao 2003b, Rao 2007).

The work presented in this thesis constitutes a logical extension of earlier work with the following objectives:

1. The assessment of DNA strand breaks is an important parameter in many biological studies, and it is important for us to assess DNA damage in brain cells

with respect to the age of the animal. It was attempted to examine visually actual DNA damage in brain cells at different ages instead of assessing the damage indirectly through a biochemical method as was done earlier. For this, the technique of Single Cell Gel Electrophoresis (the so called "Comet Assay") was used under different conditions to view and assess the damage through appropriate software. The results showed that most of the damage is seen in adult hood itself with a further increase through old age.

The results are presented in Chapter 3.

2. The uracil in the genomic DNA mainly arises from deamination of cytosine residues and is repaired by BER. It is now well established that Pol β is an important component of the BER pathway.

In the present study, the effect of age on uracil initiated BER activity in rat cortical neurons was investigated. The results showed that accumulation of uracil gradually increases in neurons and astrocytes from the rat cerebral cortex with age. Overall BER activity is adversely affected due to a deficiency in Pol β and Ligase in aging neurons.

The results are presented in Chapter 4.

3. Oxidative damage produced by endogenously and exogenously generated reactive oxygen species (ROS) has been implicated in mutagenesis and carcinogenesis and may play an important role in the pathogenesis of aging. Oxidized guanine, 8-hydroxyguanine (8-oxoG), is abundantly produced in DNA

exposed to ROS and such DNA damage is primarily repaired by BER. Hence 8 oxoG initiated BER has been studied in brain cells at different ages.

The results showed increasing accumulation of 8-oxoG in neurons and astrocytes from aging rats. The 8-oxoG driven BER is markedly decreased in aging neurons and partial restoration of the activity could be achieved through supplementation of the extracts with Pol β and DNA ligase.

The results are presented in Chapter 5.

4. Reduced DNA repair potential and accumulation of DNA damage would have a profound influence on the aging process of the individual. It is well established now that dietary calorie restriction (CR) confers the benefit of a reduced rate of ageing and therefore extended life span. CR appears to increase genetic stability by enhancing BER capacity and reducing age-related accumulation of certain DNA damage. However, so far information related to the beneficial affects of CR on DNA repair remain at the enzyme activity level and the repair process at the molecular level needs to be examined. Also, it is not known whether CR continued well into old age would sustain the beneficial affects. In the present study an attempt is made to examine the Pol β activity and also the actual process of DNA gap repair and primer extension in isolated cortical neurons subjected to extended CR.

In the present study the effect of 40% CR imposed during an extended period during the life span (from 6 months to 30 months) of rats on the activity of Pol β

and DNA short gap repair and template driven primer extension was evaluated. Cortical neuronal extracts prepared from CR rats showed significantly higher Pol β activity and protein levels when compared to control 30 month old rats. Further, single nucleotide gap repair with a slightly improved efficiency in CR neurons, could be visualized after supplementation of the extracts with T_4 DNA ligase. No impressive primer extension activity is seen in either CR or old control neurons.

These results are taken to convey that extended CR leads to improved Pol β activity and therefore Pol β dependent DNA gap repair activity.

The results are presented in Chapter 6.

In Chapter 7 all the results presented in earlier chapters have been discussed in light of the existing information.

CHAPTER 2

MATERIALS AND METHODS

ANIMALS

Cohorts of Wistar strain rats in-bred over generations and maintained in our animal house were used. The three age groups studied were 7 days postnatal, 6 months and ≥2 years. We designated these three age groups as 'Young', 'Adult', and 'Old' respectively. Rats were maintained in a pathogen free environment with a 12h light-dark cycle. Food and water were provided ad libitum.

CHEMICALS

Highly polymerized calf thymus DNA, 'Activated' calf thymus DNA, Bovine serum albumin (BSA), Adenosine triphosphate (ATP), Leupeptin, Pepstatin A, Phenylmethyl sulfonyl Flouride (PMSF), Dithiothreitol (DTT), Sephadex G-50, Trypsin Type V from bovine pancreas, Trypsin inhibitor type II from soyabean, Sigmacote, Triton X-100, Tween-20, Acrlylamide, Bis-acrylamide, Ammonium persulphate,N,N,N',N'-Tetramethyl-1-2-diaminomethane(TEMED) were purchased from Sigma chemical Co., St.Louis, MO, USA. Unlabeled nucleotides, 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxycytidine 5' triphosphate (dCTP), and 2'-deoxyguanosine 5'- triphosphate (dGTP) and 2'-deoxythymidine 5'-triphosphate (dTTP) were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Nicotinamide adenine dinucleotide (NAD), Dimethyl sulphoxide (DMSO) was from Sisco Research Laboratory, Bombay, India. Radiolabeled [α^{32}P]-dCTP, [γ^{32}P]-ATP were purchased from BARC, Bombay, India. Ficoll 400 was purchased from Amersham Pharmacia Biotech, Uppsala, Sweden. 2,5-Diphenyl-1,3-Oxazole (PPO) and 2,2'-p-Phenylene-bis[5-henyloxazole] (POPOP) were purchased from

Beckman instruments Inc., Fullerton, CA, USA. GF/C filters were purchased from Schleicher and Schuell, Dassel, Germany. Nitex nylon screens of definite pore sizes were purchased from Small parts Inc., Miami, Florida, USA. PAGE purified synthetic deoxyoligonucleotides, T_4 DNA ligase, T_4 polynucleotide kinase (T_4 PNK), Uracil DNA Glycosylase (Udg), Horse-radish peroxidase conjugated anti-mouse IgG antibody, Horse-radish peroxidase conjugated anti-rabbit IgG antibody were supplied by Bangalore Genei, Bangalore, India. PAGE purified synthetic deoxyoligonucleotides containing deoxy-uracil or tetrahydrofuran (F) were supplied by Integrated DNA Technologies, Coralville, IA, USA. PAGE purified synthetic deoxyoligonucleotides containing 8-oxoguanine (8-oxoG), Human Apurinic Endonuclease-1(Ape1), Human 8-oxoguanine DNA Glycosylase (hOgg1), Comet Assay™ kit were supplied by Trevigen Inc, Gaithersburg, MD. Human DNA polymerase β and Rabbit polyclonal DNA polymerase β antibody were a gift from Drs. Rajendra Prasad and Samuel Wilson, NIEHS, Triangle Park, North Carolina, USA. Monoclonal anti mouse β-actin (AC-15), Monoclonal anti mouse Ape1 (13B8E5C2), Monoclonal anti mouse DNA ligase III (6G9), Monoclonal DNA polymerase β (18S) from Novus Biologicals, Littleton, CO, USA. Super-signal® West Pico chemiluminescent substrate kit was purchased from Pierce, Rockford, IL, USA. All other chemicals used were of analytical grade.

Isolation of Neuronal and Astroglial enriched fractions from Young, Adult and Old rat brains.

Reagents

1) Isolation medium: 8% glucose (w/v), 5% fructose (w/v) and 2% ficoll in 10 mM KH_2PO_4 - NaOH buffer, pH 6.0.
2) 0.1% (w/v) Trypsin in isolation medium.
3) 0.1% (w/v) Trypsin inhibitor in isolation medium.
4) 7% (w/v) ficoll in isolation medium.
5) 10% (w/v) ficoll in isolation medium.
6) 22% (w/v) ficoll in isolation medium.
7) 28% (w/v) ficoll in isolation medium.

Neuronal and astroglial cell enriched fractions from the rat cerebral cortex of different ages were prepared essentially as standardized in this laboratory (Rani *et al.* 1983). The rats were decapitated, brain removed and taken in isolation medium in ice. The entire cerebral hemispheres were removed. Grey and white matter was separated from cerebral cortex and grey matter was sliced into very small pieces and incubated at 37 ^{o}C for one hour in the 0.1% trypsin. Grey matter from young was incubated in isolation medium at 37 ^{o}C for 30 minutes. After the incubation trypsin containing medium was carefully removed and an equal amount of 0.1% soyabean trypsin inhibitor in isolation medium was added and chilled on ice for 5 minutes. The remaining procedure was carried out at 0-4 ^{o}C.

The medium containing trypsin inhibitor was discarded and the tissue was washed with ice cold isolation medium and passed through nylon membranes of pore sizes 105μm, 80μm, and 48μm. The tissue was placed on 105μm nylon mesh stretched over a porcelain Hirsch funnel, and gently stirred by using a glass rod to aid the screening process. During this process, the tissue was kept moist by addition of ice cold isolation medium. The cell suspension obtained after passage through the 105μm mesh was then passed through 80μm nylon mesh and finally through 48μm nylon mesh three times each. The resulting crude cell suspension was centrifuged at 760xg for 15 minutes. The supernatant thus obtained was discarded and the crude cell rich pellet which consisted of both neurons and astrocytes was suspended in 20 ml (10 ml per gram of the tissue) 7% ficoll in isolation medium and centrifuged at 270xg for 10 minutes and the pellet obtained is mostly composed of neurons. The supernatant composed mostly of astrocytes. The crude neuronal pellet was suspended in 5 ml of isolation medium and was loaded onto discontinuous ficoll gradients for further purification. The supernatant was diluted in a ratio of 1:1.125 with isolation medium and centrifuged at 1100xg for 10 minutes. Supernatant was discarded and the astrocyte rich pellet obtained was suspended in 5 ml of isolation medium and was loaded onto discontinuous ficoll gradients for further purification. Ficoll gradients were prepared in 50 ml polycarbonate tubes from the bottom up, of 5 ml each of 28%, 22%, 10% ficoll (w/v) in the medium. The neuronal and astroglial cell suspension was loaded onto the 10% ficoll and centrifuged at 7800xg for 20 minutes in swinging bucket rotor.

The layers at each interface were removed carefully with a Pasteur pipette. Neurons were obtained as a pellet in 28% ficoll gradient. Astrocytes were obtained as a layer in 22% ficoll gradient. The interface between 22% and 10% consisted of broken processes and debris and was discarded. Cells, both neurons and astrocytes were collected from the gradient, then washed with 5ml of medium without ficoll three times at 1500xg for 10 minutes, and then in 5ml of phosphate buffered saline (1 X PBS pH 7.4) thrice at 1500xg for 10 minutes. Counting of the cells was done and viability of the cells was determined by trypan blue exclusion and was found to be > 85%. The cells were routinely examined for their characteristic morphology.

Comet (alkaline condition) assay:

DNA strand breaks in neurons and astrocytes were evaluated using Trevigen Comet Assay kit (Trevigen, Gaithersburg, MD). Cells were resuspended in ice cold PBS (Ca^{2+} and Mg^{2+} free) to a concentration of $1x10^5$ cells/ml. Briefly, an aliquot of 50 µl of cells ($1x10^5$ cells/ml) was added to 500 µl of molten LMAgarose (1% low-melting agarose) kept at 42 ^{0}C. Fifty microliters were immediately pipetted and evenly spread onto an area of the comet slides. The slide was incubated at 4^0C in the dark for 10 minutes to accelerate gelling of the agarose disc and then transferred to prechilled lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% sodium lauryl sarcosinate, 1% Triton X-100, pH 10] for 60 minutes at 4 ^{0}C.

A denaturation step was performed in alkali solution (0.3 M NaOH, 1 mM EDTA, pH>13) at room temperature for 20 minutes, in the dark. The slide was then transferred to prechilled alkaline electrophoresis solution pH >13 (300 mM NaOH, 1 mM EDTA) were subjected to electrophoresis at 1 V/cm, 300 mA for 40 minutes in the dark at 4 ^{0}C. At the end of the electrophoresis, the slides were washed with neutralization buffer (0.4 M Tris-HCl, pH 7.4); the slide was immersed in ice cold 100% ethanol at room temperature for 5 minutes and air dried. DNA was stained with 50 µl of SYBR Green 1 dye (Trevigen) (1:10 000 in Tris-EDTA buffer, pH 7.5) for 20 minutes in the refrigerator and immediately analyzed using an Olympus digital camera attached to an Olympus BX51 epifluorescence microscope.

Comet (neutral condition) assay:

The comet assay (Trevigen Inc., Gaithersburg, MD) was performed according to manufacturer's protocol by using neutral conditions to mainly detect double-strand breaks. Cells were resuspended in ice cold PBS (Ca^{2+} and Mg^{2+} free) to a concentration of $1x10^{5}$ cells/ml. Briefly, an aliquot of 50 µl of cells ($1x10^{5}$ cells/ml) was added to 500 µl of molten LMAgarose (1% low-melting agarose) kept at 42 ^{0}C. Fifty microliters were immediately pipetted and evenly spread onto an area of the comet slides.

The slide was incubated at 4 ^{0}C in the dark for 10 minutes to accelerate gelling of the agarose disc and then transferred to prechilled lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% sodium lauryl sarcosinate, 1% Triton X-100, pH 10] for 60 minutes at 4 ^{0}C. The excess lysis buffer from the slides was tapped off and the slides were washed twice with 1X Tris-buffered EDTA solution (TBE) for 10 minutes each. The slides were placed in a horizontal electrophoresis chamber and covered with TBE buffer. Electrophoresis was carried out at the rate of 1.0 V/cm for 20 minutes. The slides were removed from the electrophoresis chamber, washed in deionized water for 5 minutes and immersed in ice cold 100% ethanol for 5 minutes. Subsequently, the slides were air dried, DNA was stained with 50 µl of SYBR Green 1 dye (Trevigen) (1:10 000 in Tris-EDTA buffer, pH 7.5) for 20 minutes in the refrigerator and immediately analyzed using an Olympus digital camera attached to an Olympus BX51 epifluorescence microscope. Fifty comets per slide were randomly analyzed and fluorescent images were scored for comet parameters. For each slide, 50 randomly chosen comets were analyzed using an Olympus BX51 epifluorescence microscope with an excitation filter of BP 450-480 nm and a barrier filter of 515 nm. Images were captured by an Olympus digital camera and microscopic evaluation of the comet images was quantified using the image analysis system of Comet Score™ (TriTek Corp.). Tail length and Tail moment were measured using the Tritek CometScore^{TM} Freeware v1.5 image analysis software.

Procedure for calorie restriction:

Experiments were carried out in accordance with procedures established by the Animal Ethics Committee by the University of Hyderabad. 20 Cohorts of Wistar strain rats slightly less than 6 months, weighing around 300 gms were obtained from the University of Hyderabad animal house facility. They were fed commercially available 'Rat and Mice feed' purchased from Hindustan Lever, (New Delhi, India), which is complete in all nutritional aspects.

Food was provided ad libitum until 6 months of age. At that time rats were randomly divided into two groups, one receiving 100% of the average ad libitum feed (control group) and other 60% (calorie restricted group). Each group consisted of 10 rats. They were maintained in a clean environment at 23 °C on a 12/12 h light/dark cycle. Calorie restriction was imposed from 6 to 30 months of age at which time the animals were sacrificed. Two animals were combined for analysis. Thus five batches of samples for control and calorie restricted groups were obtained. It must be mentioned here that the control animals with free access to food showed all the signs of morbidity from 24-month onwards and looked very sick and nearing death. One rat actually died even before attaining 30 months of age, whereas all the rats in CR group survived. CR animals group were smaller in size but appeared active and healthy.

Neuronal cell enriched fractions from cerebral cortex of young (7 days), adult (6 months), old control and calorie restricted old rats (30 months) were prepared essentially according to Rani *et al.* (1983). This procedure has been well standardized in this laboratory over the past several years and the viability (Trypan blue exclusion test) of neurons and the purity of the fraction thus obtained range from 80 to 95%.

Preparation of neuronal and astroglial extracts:

The final preparation of the neuronal and astroglial cells were suspended in extraction medium consisting of 20 mM Tris–Hcl, pH 7.5, 0.1 mM DTT, 1 mM EGTA, 10% glycerol, 0.5% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate), 0.1 mM PMSF, 5 mM β-mercaptoethanol, 1 mM $MgCl_2$, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 0.5 M KCl. Leupeptin, pepstatin A, and PMSF were added just before use. The suspension was sonicated for 5 s, three times with the setting at 5 in a Branson sonifier and incubated on ice for 1 hour. The suspension was centrifuged at 100 000 g for 1h in a Sorvall ultracentrifuge, model-80 and the clear supernatant was used as the source for DNA polymerase β and other enzymes/factors needed for BER. Protein concentration was estimated by the method of Bradford (1976).

DNA polymerase β Assay:

Briefly, the reaction mixture (50µl) contained 40 mM Tris-HCl pH 8.0, 8 mM $MgCl_2$, 1 mM DTT, 4 mM ATP, 25 µM cold dCTP (unlabeled), 100 µM of each dGTP, dTTP, dATP, 1 µ curie of [α^{32}P]-dCTP (hot dCTP), 5 µg of activated calf thymus DNA and 5 or 10 µg of enzyme extract. The reaction was carried out in 37 °C for 20 minutes and stopped on ice by adding 1 ml of chilled 10% TCA containing 10mM tetrasodium pyrophosphate. 200 µg of BSA and calf thymus DNA were added as carriers. The samples were incubated on ice for 5 minutes and then centrifuged for 15 seconds at 12,000 rpm at room temperature. The supernatant was discarded and pellet dissolved in 400 µl of 0.2 N NaOH. After the whole pellet had dissolved in NaOH then 10% TCA solution (1 ml) was added to the dissolved pellet and centrifuged for 5 minutes at 6,000 rpm at room temperature. The supernatant was discarded and the pellet was resuspended in 500 µl of chilled 5% TCA. The whole solution along with the precipitate was transferred onto 2.5 cm glass fiber filters (Schleicher & Schuell) and washed six times each with chilled 5% TCA and 95% ethanol. The washed filters were allowed to dry by keeping in the oven at 40 ^{0} C for 20 minutes or keeping in the hood overnight. The dried filters were taken in toluene based scintillation fluid containing 5gm PPO and 0.5 gm POPOP per liter having 0.1% triton-X-100 and the radioactivity was counted in a Wallac 1409 counter. The specific activity is expressed as pmol of dCMP incorporated into acid insoluble fraction / milligram protein/hour.

The sequences of the oligonucleotides used in this study are presented in Table 2. Oligonucleotides containing deoxy-uracil (U), tetrahydrofuran (F) (Integrated DNA Technologies, Coralville, IA, USA) and 8-oxoG (Trevigen, Gaithersburg, MD) were 5'[^{32}P]-kinased by incubating with [γ^{32}P]-ATP (BRIT, Mumbai, India) in the presence of T_4 polynucleotide kinase (Bangalore Genei,India). Unincorporated free [γ^{32}P]-ATP was separated from the reaction mixtures using sephadex G-50 columns. The ^{32}P-kinased oligonucleotides were then annealed to the complementary strands in the presence of 50 mM NaCl and 5 mM $MgCl_2$ by heating the samples at 70 ^{0}C for 10 minutes and allowing them to be slowly cool to room temperature. For base excision repair synthesis incorporation, unlabeled substrates (UG and O) were annealed as described above.

Uracil DNA-glycosylase (Udg) activity assay:

Udg activity in neuronal and astroglial extracts were determined by excising uracil from a 21-mer [^{32}P]-kinased oligonucleotide duplex containing uracil at position 8 (Table 2). Briefly, the 20 µl reaction contained 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM DTT, 25 µg/ml bovine serum albumin, 200 fmol of 5'-kinased uracil containing oligonucleotide duplex and 10 µg of neuronal and astroglial extract. Reactions were incubated at 37 °C for 20 minutes and reaction was terminated with 10 µl of 3 X alkaline loading buffer (300 mM NaOH, 97 % formamide and 0.2 % bromophenol blue), the samples are heated 95 ^{0}C for 10 minutes then fast cool to 4 ^{o}C.

The denatured samples were loaded along with markers and subjected to 20% polyacrylamide sequencing gel electrophoresis with 7 M urea in 90 mM Tris–borate EDTA buffer, pH 8.3 at 2300 V for 3 h. Analysis of the substrate and product was done by autoradiography and quantified by using Image J (NIH, USA) of the required spots by calculating the relative amount of the 7-mer oligonucleotide product with the unreacted 21-mer substrate (product/product + substrate). Negative controls consisted of the reaction mixture and oligonucleotide in the absence of nuclear extract.

Apurinic endonuclease 1 (Ape1) Assay:

The Ape1 activity was analyzed using a quantitative in vitro assay that measures the incision of a 21-mer oligonucleotide duplex containing a synthetic tetrahydrofuran (F) AP site at position 14 (Table 2). Briefly, 200 fmol of [^{32}P]-kinased and purified oligonucleotide duplex was incubated with 200 ng of neuronal and astroglial extracts in a 20 µl reaction mixture containing 10 mM HEPES-KOH, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$. The reaction mixtures were incubated for 10 minutes at 37 °C, and stopped by the addition of 10 µl of loading dye (95% formamide, 50 mM EDTA, 0.1% of xylene cyanol, 0.1% of bromphenol blue), and heated at 95 °C for 5 minutes. The denatured samples were loaded along with markers and subjected to 20% polyacrylamide sequencing gel electrophoresis with 7 M urea in 90 mM Tris–borate EDTA buffer, pH 8.3 at 2300 V for 3 h.

Analysis of the substrate and product was done by autoradiography and quantified by using Image J (NIH, USA) of the required spots by calculating the relative amount of the 13-mer oligonucleotide product with the unreacted 21-mer substrate (product/product + substrate).

Base excision repair (Udg-BER) assay:

Briefly, the standard reaction mixture (50 µl) contained 100 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 2 mM ATP, 0.5 mM NAD, 5 mM diTris phosphocreatine, 10 units of creatine phosphokinase, 3 pmol 21-bp oligodeoxynucleotide duplex containing a uracil residue at position 8, dATP, dGTP, and dTTP at 20 µM each, 20 nM of unlabeled dCTP, 10 µCi of [α^{32}P]-dCTP (3000 Ci/mmol)). The BER reaction was initiated by addition of 10 µg neuronal extract. The reaction mixture was incubated at 37 °C for 20 minutes; Reactions were stopped by the addition of stop solution (50 mM EDTA, 0.3 M NaCl). The DNA was extracted with phenol: chloroform: isoamylalcohol (25:24:1, v/v/v) and precipitated with 3 volumes of chilled ethanol and 50 µg of glycogen. The precipitates were collected by centrifugation and washed with 70% ethanol, dried under a vacuum, and resuspended in 5 µl of 80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue. After incubation at 95 °C for 5 minutes, the DNA was electrophoresed 20% polyacrylamide sequencing gel electrophoresis with 7 M urea in 90 mM Tris–borate EDTA buffer, pH 8.3 at 2300 V for 3 h. The gel was dried, kept for autoradiography with intensifying screen in -80 ^{0}C.

Enrichment assays were performed by adding 0.5 units of Udg, 1 unit of Ape1, 0.1 units of Pol β, 20 units of T_4 DNA ligase (Ligase) independently or in different combination to neuronal extracts. Afterward, Udg-BER assays were performed as described above.

8-oxoguanine DNA glycosylase (Ogg1) activity assay:

Ogg1 activity in neuronal and astroglial extracts was determined by excising 8-oxoG from a 24-mer [^{32}P]-kinased oligonucleotide duplex containing 8-oxoG at position 10 (Table 2). Briefly, Ogg1 activity was assayed in a reaction volume 20 µl containing 10 mM Hepes-KOH pH 7.4, 10 mM EDTA, 100 mM KCl, 0.1mg/ml BSA, 200 fmol oligonucleotide duplex and 25 µg protein extract. Reactions were incubated for 120 minutes at 32 °C, Reaction was terminated with 10 µl of 3 X alkaline loading buffer (300 mM NaOH, 97% formamide and 0.2% bromophenol blue), the samples are heated 95 ^{0}C for 10 minutes then fast cool to 4 ^{0}C. The denatured samples were loaded along with markers and subjected to 20% polyacrylamide sequencing gel electrophoresis with 7 M urea in 90 mM Tris–borate EDTA buffer, pH 8.3 at 2300 V for 3 h. Analysis of the substrate and product was done by autoradiography and quantified by using Image J (NIH, USA) of the required spots by calculating the relative amount of the 9-mer oligonucleotide product with the unreacted 24-mer substrate (product/product + substrate).

Base excision repair (8-oxoG-BER) assay:

Briefly, the standard reaction mixture (50 µl) contained 10 mM Hepes-KOH pH 7.4, 10 mM EDTA, 100 mM KCl, 0.1 mg/ml BSA**,** 10 mM $MgCl_2$, 4 mM ATP, 0.5 mM NAD, 5 mM diTris phosphocreatine, 100 µg/ml of creatine phosphokinase, 400 fmol 24-bp oligodeoxynucleotide duplex containing a 8-oxoG residue at position 10, dATP, dCTP, and dTTP at 20 µM each, 20 nM of unlabeled dGTP, 10 µCi of [α^{32}P]-dGTP (3000 Ci/mmol). The BER reaction was initiated by addition of 25 µg neuronal extract. The reaction mixture was incubated at 32 °C for 120 minutes; Reactions were stopped by the addition of stop solution (50 mM EDTA, 0.3 M NaCl). The DNA was extracted with phenol: chloroform: isoamylalcohol (25:24:1, v/v/v) and precipitated with 3 volumes of chilled ethanol and 50 µg of glycogen. The precipitates were collected by centrifugation and washed with 70% ethanol, dried under a vacuum, and resuspended in 5µl of 80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue. After incubation at 95 °C for 5 minutes, the DNA was electrophoresed 20% polyacrylamide sequencing gel electrophoresis with 7 M urea in 90 mM Tris–borate EDTA buffer, pH 8.3 at 2300 V for 3 h. The gel was dried, kept for autoradiography with intensifying screen in -80 ^{0}C.

Enrichment assays were performed by adding 0.2 units of Ogg1, 0.5 units of Ape1, 0.2 units of Pol β, 20 units of Ligase, independently or in different combination to neuronal extracts. Afterward, 8-oxoG-BER assays were performed as described above.

Construction of DNA oligo duplex to generate gap and primer extension substrates

The sequences of the synthetic deoxyoligonucleotides for constructing gapped substrate are given below.

1) 5'-g g c a c c g c a a a a a a t c t g g c g g c c a t g g c t c g -3' (Oligo1, 32-mer)

2) 5'- c g a g c c a t g g c c g c -3' (Oligo 2, 14-mer)

3) 5'- t t t t t t g c g g t g c c-3' (Oligo 3, 14-mer)

4) 5' a g a t t t t t g c g g t g c c-3'(Oligo 4, 17-mer)

1 Gap Oligo duplex:

Oligo 2, 14-mer, upstream primer Oligo 4, 17-mer, downstream primer

32P-5'-c g a g c c a t g g c c g c - a g a t t t t t g c g g t g c c - 3'

3'-g c t c g g t a c c g g c g g t c t a a a a a a c g c c a c g g -5'(Oligo 1, 32-mer)

4 Gap Oligo duplex:

Oligo 2, 14-mer, upstream primer Oligo 3, 14-mer, downstream primer

32P-5’-c g a g c c a t g g c c g c - - - - t t t t t t g c g g t g c c -3’

3’-g c t c g g t a c c g g c g g t c t a a a a a a c g c c a c g g -5’(Oligo 1, 32-mer)

An oligo duplex having a 1 nucleotide gap in one strand is prepared by annealing a previously 5’-kinased oligo 2 (14-mer) and a non-kinased oligo 4 (17-mer) to unlabeled oligo 1, the 32-mer. Such an annealing would produce a 1- gap oligo duplex as shown above. It may be noted that the strand with 1 nucleotide gap is also having a ^{32}P label on 5’ side. Similarly, annealing a 5’-kinased oligo 2 and oligo 3, to oligo 1, the 32 -mer would yield a duplex with a 4 nucleotide gap.

Primer extension duplexes

The sequences of the synthetic deoxyoligonucleotides for constructing primer extension substrates are given below.

1). ^{32}P 5’-c g c g a t c g g t a g c G -3’ (Oligo-A, 14-mer)

2) ^{32}P 5’-g c c a t t g C g c t a c c g a t c g c g-3’ (Oligo-B, 21-mer)

3) ^{32}P 5’-g c c a t t g T g c t a c c g a t c g c g-3’ (Oligo-C, 21-mer)

The 5’ kinased Oligo-A, 14-mer is hybridized with (Oligo-B, 21-mer) and (Oligo-C, 21-mer) to form correct duplex (G-C) and mismatched duplex (G-T) in equimolar concentration in the presence of 50 mM NaCl and 5 mM $MgCl_2$ as shown below.

Correct duplex (G-C)

^{32}P 5’-c g c g a t c g g t a g c G -3’ (Oligo-A, 14-mer)

3’- g c g c t a g c c a t c g C g t t a c c g - 5’ (Oligo-B, 21-mer)

Mismatched duplexes (G-T)

^{32}P 5’–c g c g a t c g g t a g c G - 3’ (Oligo-A, 14-mer)

3’- g c g c t a g c c a t c g T g t t a c c g- 5’ (Oligo-C, 21-mer)

5’-End labeling of the oligos

The 5’-end of the upstream primer (oligo 2, the 14-mer) was phosphorylated using equimolar [γ^{32}P]-ATP (specific activity, 5000Ci/mmol) and T_4 polynucleotide kinase (2.5 units/pmol of substrate) and the reaction was carried out at 37 ^{0}C for 40 minutes in the buffer (70 mm Tris-Hcl, pH 7.6, 10 mM $MgCl_2$ and 5 mM DTT). The downstream primers of gap repair substrate were also phosphorylated at 5’-end in a similar fashion using unlabeled ATP. In primer extension oligo A was 5’kinased in a similar way with [γ32P]-ATP.

Annealing

Equimolar concentrations of the oligos (oligo A to oligo B or C for primer extension and oligo2, 3, 4 and 1 for gap repair) as mentioned above were hybridized in a reaction mixture containing 50 mM NaCl and 5 mM $MgCl_2$ at 70 ^{0}C for 10 minutes followed by gradually cooling to room temperature.

Gap Repair Assay:

DNA gap repair and primer extension assay were done using 400 fmol of the substrates with 10 µg of extract protein in a reaction mixture of 30 µl containing 20 mM HEPES pH 7.5, 1 mM $MgCl_2$, 0.1 mM DTT, 0.1 mg/ml bovine serum albumin, 2% glycerol, and 20 µM of all the four dNTPs. Reaction was carried out at 37 °C for 20 minutes and stopped by heating at 70 °C for 10 minutes. Where ever mentioned, the reaction mixture is supplemented with required amounts T_4 DNA ligase, and ATP. After the reaction, gap or primer extension reaction end products were purified by passing through Sephadex G-50 spin columns and the eluted fractions were freeze dried, resuspended in the minimal amount of double distilled water and denatured at 85 °C for 5 minutes after adding loading dye. The denatured samples were loaded along with markers and subjected to 20% polyacrylamide sequencing gel electrophoresis with 7 M urea in 90 mM Tris–borate EDTA buffer, pH 8.3 at 3000 V for 3 h. Analysis of the reaction repaired products was done by autoradiography and densitometry of the required spots.

CHAPTER 3

Detection of DNA damage in aging neurons and astrocytes by single cell gel electrophoresis (comet assay)

Introduction

DNA damage is defined as any modification of DNA that changes its coding properties or normal function in transcription or replication (Lindahl 1993, Rao 1993). Damage to the native structure of DNA can occur through two main mechanisms: spontaneous damage caused by sources within a cell and damage caused by external sources such as chemicals and radiation. Protracted oxidative, hydrolytic, deamination or alkylation reactions in a cell can modify DNA bases, or even sometimes cause a complete loss of bases within DNA, resulting in strand breakage. Similarly, cellular DNA can be damaged by external sources such as ultraviolet or ionizing radiation (X-rays, γ-rays, α particles and cosmic rays), and an array of chemical substances can induce interstrand and intrastrand cross-links, DNA–protein cross-links, bulky DNA adducts, single-strand breaks (SSBs) and double-strand breaks (DSBs) (Rao 1990, Rao and Loeb 1992, Rao 2002, Rao 2003b, Reddy and Vasquez 2005, Martin 2008). Brain is the master organ of the body. It controls all other functions either directly or indirectly. The brain has two major types of cells, neurons and glial cells. It is known that neurons, once differentiated are nondividing, and even in glial cells only a small fraction of them are dividing in adult and old ages (Korr 1980). Thus, it can be considered that most of the cells in an adult brain are post mitotic.

Further, in the majority of species the final number of differentiated neurons is reached very early in life and therefore, a neuron's life span is almost equal to that of the whole animal. Considering the high metabolic activity in a neuronal cell, it must be of great necessity and importance to maintain the genomic integrity over a long period of time in order to keep up the fidelity of the cellular processes. Thus, the processes of genomic damage and its repair assume special significance in nervous tissue.

The relationship between DNA repair and the phenomenon of brain aging has been the subject of study in this laboratory for the past several years. The consequence of adversely affected DNA repair would be accumulation of DNA damage. Earlier Mandavilli and Rao (1994) from this laboratory had assessed SSBs and DSBs by using nick translation of DNA with *E. coli* Pol I and addition of nucleotides at the terminal 3'-OH by calf thymus terminal deoxynucleotidyl transferase in DNA isolated from young, adult and old rat brain neurons and astrocytes. Results showed that there is an increase in the number of SSBs and DSBs with age in both neuronal and astroglial DNA. Latter Mandavilli and Rao (1996a) from this laboratory measured SSBs and DSBs in permeabilised neurons and astrocytes from young, adult and the aged rat brain by using nick translation with *E. coli* Pol I or calf thymus terminal deoxynucleotidyl transferase and measuring the addition of nucleotides at the terminal 3'-OH end.

They have shown that the number of SSBs increases in cerebral cortex neurons with age. There are 7400 breaks in the genomic DNA of an old neuron as compared with 3000 in a young neuron. When the cells are exposed to either MNNG (a methylating agent) or glutamate (excitotoxic at higher levels) before assessment of the breaks, the damage is clearly aggravated at all the ages indicating an increased susceptibility of genomic DNA with age. Even with respect to the DSBs, a steady increase in the number is seen with age in neurons. There is a fourfold increase in the number between young and old ages. Prior treatment of cells with either MNNG or glutamate resulted in the formation of more DSBs at all ages, and an age dependent susceptibility of neurons could be clearly seen.

Measurement of DNA damage through the addition of nucleotides at free 3'-OH groups present in DNA is one method that can be used to assess strand breaks in DNA. There are other methods to measure cellular DNA damage, for example, the so-called comet assay also known as single cell gel electrophoresis.

The present study was undertaken to assess damage to DNA at the single cell level from isolated neurons and astrocytes of different ages through single cell gel electrophoresis or comet assay (Ostling and Johanson 1984). Since assessment of DNA strand breaks is an important parameter in many biological studies, it is important for us to assess DNA damage in brain cells with respect to the age of the animal. Therefore, the present study to assess DNA damage through "Comet assay" in aging rat neurons and astrocytes is performed.

Methods

Preparation of Neuronal and astroglial cell fractions isolated from 'Young' (7 days postnatal), 'Adult' (6 months) and 'Old' (≥24 months) rat brain cerebral cortex was essentially as described in Material and Methods, Chapter 2.

Comet assay

The concept of microgel electrophoresis of dispersed cells was first introduced in 1984 by Ostling and Johanson (1984) as a method to measure DNA single-strand breaks that caused relaxation of DNA supercoils. A modified version was published by Singh and colleagues in 1988, which used alkaline conditions (Singh *et al.* 1988). The idea was to combine DNA gel electrophoresis with fluorescence microscopy to visualize the migration of DNA strands from individual agarose-embedded cells. If the negatively charged DNA contained breaks, DNA supercoils were relaxed and broken ends were able to migrate toward the anode during a brief electrophoresis. If the DNA was undamaged, the lack of free ends and large size of the fragments prevented migration. The alkaline denaturation of DNA and alkaline electrophoresis pH >13 version is capable of detecting DNA single-strand breaks (SSBs), alkali-labile sites (ALS), DNA-DNA/DNA-protein cross-linking, and SSB associated with incomplete excision repair sites (Singh *et al.* 1988, Tice *et al.* 2000)**.** The comet assay also uses neutral electrophoresis buffers that allow only the detection of double-stranded breaks (Singh *et al.* 1988, Lemay and Wood 1999). Determination of the relative amount of DNA that migrated provided a simple way to measure the number of DNA breaks in an individual cell.

Tail moment, a measure of both amount of DNA in the tail and distribution of DNA in the tail, became a common descriptor along with tail length and percentage of DNA in the tail (Olive and Banath 2006). The details of this procedure are described in Material and Methods, Chapter 2.

Neuronal and astroglial cell enriched fractions from cerebral cortex of young (7 days), adult (6 months) and old rats (≥24 months) were prepared essentially according to Rani *et al.* (1983). The morphology of the cells observed under the phase-contrast microscope revealed (Figures 2A and 3A) that the neurons are large cells having a large nucleus and abundant cytoplasm. Thus, they are denser than other cells. The neuron obtained is a neuronal perikaryon retaining stumps of axon and dendrites. The astrocytes have much smaller perikarya with a number of branching processes. This procedure has been well standardized in our laboratory over the past several years and the viability (Trypan blue exclusion test) of neurons and astrocytes and the purity of the fraction thus obtained range from 85 to 95% in all isolates from different ages. Figure 2B, shows western blot of neuronal extract prepared from young rat cortex probed with anti-neuron specific enolase (anti-NSE). The neuronal cell preparation was also characterized by western blot using antibodies for neuron specific enolase (NSE) which is specific marker for neuron and glial cell fibrilary acidic protein (GFAP) which is specific marker for astrocyte.

The result indicated 85-95 % purity of neuronal cells preparation as there is a strong band detected on the blot when neuronal extract probed with NSE and a faint band detected on the blot when neuronal extract probed with GFAP. It may be due to a little astrocytes contamination that invariably occurs while separating neurons and astrocytes from ficoll gradients. Similarly Figure 3B, showing western blot of astrocyte extract prepared from young rat cortex probed with anti GFAP and NSE indicating a good purity of astrocyte preparation as there is no signal detected on the blot when astrocyte extract was probed with NSE.

The comet assay or single cell gel electrophoresis used in this investigation offers a simple, quick, sensitive, reliable, and fairly inexpensive way of measuring DNA damage. I have used the alkaline (pH > 13) version of the assay developed by Singh et al. (1988)(Singh et al. 1988). The pH > 13 version is capable of detecting DNA SSBs, alkali-labile sites (ALS), DNA-DNA/DNA-protein cross-linking, and SSB associated with incomplete excision repair sites (Tice *et al.* 2000). The alkaline version of the comet assay was used to determine the level of DNA strand breaks in isolated neurons and astrocytes prepared from different age groups as shown in Figures 4 and 5. After SYBR Green I staining, young neuronal nuclei appeared very bright and round (Figure 4A). Also, very little migration of DNA is evident, suggesting that very little DNA damage (in the form of DNA strand breaks) is seen in young neurons. However, with advancement of age, there is significant relaxation of DNA from the nucleus, forming a “comet” tail in adult and old neurons (Figure 4A).

As can be seen, the extent of DNA SSBs measured as tail moment (product of percentage of DNA in tail and tail length) also increased in neurons with age. The tail moment value of old neurons increased 5.1 fold as compared to young neurons, while in the case of adult neurons, the increase was 4.1 fold as compared to young neurons (Figure 4B). A similar pattern of DNA migration was found in astrocytes prepared from rat brains of different ages (Figure 5A). The tail moment value of adult and old astrocytes increased 3.6 fold and 4.2 fold respectively as compared to young astrocytes (Figure 5B). Closer evaluation of the data reveals that while in the case of neurons and astrocytes a considerable number of strand breaks accumulated by adulthood themselves, with a further increase in the strand break in old neurons and astrocytes.

I have also used neutral version of the comet assay without treatment with alkaline buffer for electrophoresis to detect DSBs (Singh et al. 1988). These results are shown in Figure 6. The extent of DNA damage measured as tail length under these conditions also increased in neurons with age (Figure 6A). The tail length value of old neurons increased 7.4 fold as compared to young neurons while in the case of adult neurons, there was 6.2 fold increase as compared to young neurons (Figure 6B). A similar pattern of DNA migration in the form a tail was found in astrocytes preparations (Figure 7A). As can be seen, the tail length value in adult and old astrocytes increased 5.3 fold and 6.1 fold respectively as compared to young astrocytes (Figure 7B).

The present results thus provide important information that indeed damage to cellular DNA increases with age in both types of rat brain cells. This substantiates the fundamental premise of the "DNA damage and repair" theory of aging phenomenon that was proposed earlier (Bernstein and Bernstein 1991) and supported by the work from this lab over the years. What is to be noted is the fact that most of the DNA damage seems to accumulate by the time the animal attains adulthood (6 months in the present case). There was of course a further increase in the DNA damage between the ages of 6 and 24 months. However, the magnitude of this increase was less as compared to that between young and adult ages. This may suggest a possible time point of initiation of the aging process.

CHAPTER 4

The effect of age on uracil-initiated base excision repair activity in rat cortical neurons

Introduction:

Aging in the brain is associated with increased DNA damage and reduced DNA repair capacity, which leads to disruption of brain function either as a component of senescence, or as a consequence of age related neurodegenerative disease (Subba Rao and Loeb 1992, Rao 1997, Subba Rao 2007). The native structure of genomic DNA can be damaged in many ways, by external agents and from agents within the cell produced as a part of normal metabolism, giving rise to spontaneous modification, oxidation, deamination and loss of bases (Lindahl 1993). To maintain genomic integrity, base excision repair (BER) is the primary mode of repair in post mitotic tissue like brain, where simple base modifications are more likely to occur than a major damage to DNA (Rao 2007).

Uracil in DNA results from deamination of cytosine, creating a premutagenic U: G mispair. Misincorporation of dUMP instead of dTMP during replication can also result in a U: A pair in DNA (Krokan *et al.* 2002). In general, BER of uracil in DNA is initiated by a uracil–DNA glycosylase (Udg) that cleaves the *N*-glycosidic bond between the base and deoxyribose, leaving an abasic site (AP site) (Lindahl and Wood 1999). Repair of AP sites is initiated by AP endonuclease (Ape1) which binds the AP site and hydrolyzes the phosphodiester bond 5' to the abasic site, generating a 5'terminal sugar phosphate (Lindahl 1990, Lindahl and Wood 1999). The 5'terminal sugar phosphate (dRp) is removed by DNA polymerase β (Pol β) which has an associated AP lyase activity as well (Matsumoto and Kim 1995, Sobol *et al.* 2000). Pol β removes the dRp and adds

one nucleotide to the 3' end of the nick. The most preferred substrate for Pol β seems to be double stranded DNA with a single nucleotide gap in one of the strands, which is the *in vivo* situation after removal of a damaged base by an appropriate glycosylase or by spontaneous depurination (Wang and Korn 1980, Mosbaugh and Linn 1983, Randahl *et al.* 1988, Singhal and Wilson 1993).

BER in mammalian cells is mediated through at least two sub pathways that are differentiated by repair patches and the enzymes involved. One sub pathway is short patch BER, and the other, long patch repair, involves replacement of up to 13 nucleotides (Matsumoto *et al.* 1994, Frosina *et al*. 1996, Wilson 1998). The predominant repair pathway is short patch BER involving excision of a single damaged nucleotide and replacement catalyzed primarily by Pol β (Dianov *et al.* 1992, Klungland and Lindahl 1997). In cases where the terminal sugar phosphate after AP endonuclease incision develops a complex structure that cannot be acted upon by the dRpase activity of the Pol β (For example reduced or oxidized abasic site), repair synthesis would continue in a strand displacement manner. This long patch synthesis is catalyzed by either Pol β or Pol δ/ε with associated proof reading activity. This pathway is stimulated by Proliferating Cell Nuclear Antigen (PCNA) and requires a "flap" structure specific enonuclease-1 (FEN1) (Harrington and Lieber 1994) to cut the flap like structure produced by strand displacement synthesis (Wu *et al*. 1996, Klungland and Lindahl 1997). The primary role of PCNA seems to be stimulation of FEN1 activity and the repair size is about seven nucleotides (Frosina *et al*. 1996).

Besides Pol β, slightly different long patch BER pathway involves Pol δ or Pol ε. Pol β null embryonic fibroblast cells were proficient in repairing oxidative damage, although they were defective in uracil initiated repair (Sobol *et al.* 1996), and the neutralizing antibody to Pol β, which inhibited repair synthesis catalyzed by pure Pol β by approximately 90%, only suppressed repair in crude human cell extracts by a maximum of approximately 70%.

After filling the single nucleotide gap by DNA Pol β, the nick is sealed by DNA ligase. In the short patch repair pathway DNA ligase III along with its partner XRCC1 seals the nick (Cappelli *et al.* 1997), whereas DNA ligase I/III joins the nick in long patch repair (Kim *et al.* 1998). Eukaryotes, in contrast to prokaryotes, contain more than one DNA ligase, and these enzymes have distinct roles in DNA metabolism.

In view of the reported precise role of Pol β in BER, the present study has been extended to examine age-dependent regulation of Pol β-directed BER using a model oligoduplex substrate containing uracil opposite a G in neuronal extracts prepared from aging rat brain cortex.

Methods

Preparation of Neuronal and astroglial cell fractions from 'Young' (7 days postnatal), 'Adult' (6 months) and 'Old' (≥24 months) rat brain cerebral cortex is described in Material and Methods, Chapter 2.

Comet assay:

DNA strand breaks in neurons and astrocytes were evaluated using Trevigen Comet Assay kit (Trevigen, Gaithersburg, MD). Cells were resuspended in ice cold PBS (Ca^{2+} and Mg^{2+} free) to a concentration of $1x10^5$ cells/ml. Briefly, an aliquot of 50 µl of cells was added to 500µl of 1% molten low-melting agarose kept at 42 ^{0}C. Fifty microliters were immediately pipetted and evenly spread onto an area of the comet slides. The slide was incubated at 4 ^{0}C in the dark for 10 minutes to accelerate gelling of the agarose disc and then transferred to prechilled lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% sodium lauryl sarcosinate, 1% Triton X-100, pH 10] for 60 minutes at 4 ^{0}C. Following lysis, the slides were washed three times for 10 minutes with 1X FLARE buffer (25 mM HEPES-KOH at pH 7.4 250 mM KCl, 25 mM EDTA), gently blotted dry with tissue paper and covered with 50 µl of buffer (0.04% 25X FLARE Buffer, 0.01% 100X BSA) or uracil DNA glycosylase in buffer (0.5 units per gel). The slides were incubated in a humidity chamber at 37 ^{O}C for 60 minutes. A denaturation step was performed in alkali solution (0.3 M NaOH, 1 mM EDTA, pH>13) at room temperature for 20 minutes, in the dark. The slide was then transferred to prechilled alkaline electrophoresis Solution pH >13 (300 mM NaOH, 1 mM EDTA) and subjected to electrophoresis at 1 V/cm, 300 mA for 40 minutes in the dark at 4 ^{0}C.

At the end of the electrophoresis, the slides were washed with neutralization buffer (0.4 M Tris-HCl, pH 7.4); and fixed in ice-cold 100% ethanol at room temperature for 5 minutes and air dried. DNA was stained with 50 µl of SYBR Green I dye (Trevigen) (1:10000 in Tris-EDTA buffer, pH 7.5) for 20 minutes in the refrigerator and immediately analyzed using an Olympus digital camera attached to an Olympus BX51 epifluorescence microscope. For each slide, 50 randomly chosen comets were analyzed using an excitation filter of BP 450/480 nm and a barrier filter of 515 nm. Images were captured by the digital camera and microscopic evaluation of the comet images was quantified using the image analysis system of Comet Score™ (TriTek Corp.). Tail moment was measured using the Tritek CometScore^{TM} Freeware v1.5 image analysis software.

Uracil DNA-glycosylase (Udg) Activity Assay:

The details are as described in Materials and Methods, Chapter 2.

Apurinic Endonuclease (Ape1) Assay:

The details are as described in Materials and Methods, Chapter 2.

Base Excision Repair Assay:

In vitro BER was performed essentially as described by (Dianov *et al*. 1992). Unless otherwise specified neuronal extracts prepared from rats of different ages and pure Pol β were incubated with 1 µl of neutralizing polyclonal antibody of Pol β (anti-pol β) or 50 µM of aphidicolin on ice for 45 minutes.

BER was initiated by adding Udg, Ape1, DNA-ligase, uracil containing DNA oligoduplex and 10 μCi [α^{32}P]-dCTP and incubated at 37 ^{0}C for 20 minutes. The details of this procedure are described in Material and Methods, Chapter 2.

Western blot analysis:

Equivalent of 75 μg of protein from neuronal cells of young, adult, and old rats was electrophoresed on a 10% SDS polyacrylamide gel and transferred on to PVDF (Millipore) membrane for western blot analysis. Blot was incubated with 5% non-fat dry milk powder solution in Tris-buffered saline. The membrane was incubated with polyclonal anti-rabbit pol β antibody, monoclonal anti-mouse Ape1 antibody and monoclonal anti-mouse DNA Ligase III antibody. Bound primary antibodies were visualized with the appropriate horseradish peroxidase-conjugated secondary antibodies (Bangalore Genei, India.) and chemiluminescent substrate kit (Pierce, Rockford, IL). Intensity of the band on the film was quantified by using by Image J1.41 software. (NIH, USA).

Result and discussion

Figures 8 and 9 show representative images of DNA migration following electrophoresis of isolated neurons and astrocytes prepared from young, adult and old rat cerebral cortex. The uracil sites in neurons and astrocytes at these three ages were assessed by modified alkaline comet assay. When neurons, prepared from rats of different ages, were incubated with only buffer, increase of DNA migration in the tail was observed with age of the animal (Figure 8). This increase in tail movement is taken to be the increased damage in DNA occurring due to aging.

When the cells were incubated with buffer containing the DNA glycosylase Udg, there was further increase in tail movement, which must be due to the presence of uracil residues accumulating in DNA with age, as this uracil is removed by the added Udg creating single strand breaks, which are reflected by the higher tail movement in the comet assy. Essentially the enhanced tail movement due to the incubation of the brain cells with Udg denotes the increase in the number of uracil sites with age. The pattern of results is the same either with neurons or astrocytes (Figures 8 and 9). Table 3 shows the actual fold increase of uracil sites with age in brain cells. For example, in neuronal DNA the content of uracil between the young and adult ages has increased 3.3 and between young and old ages by 6.5 fold. Similarly adult and old astrocytes accumulate 3.3 and 6.6 fold respectively when compared to young astrocytes. Closer observation of the data suggests that accumulation of uracil in neurons and astrocytes is a gradual process with age (Table 3).

To examine the activity of Udg activity in brain cells, an *in vitro* excision assay was performed. Figure 10A shows the sequences of the oligodeoxynucleotides used in the excision assay. The 21-mer oligonucleotide containing uracil at position 8 was 5'-kinased with [γ^{32}P]-ATP. Complementary oligos was annealed to form DNA oligoduplex. Details of the method are described in Chapter 2 of this thesis. A representative autoradiogram is shown in Figure 10A.

The results indicate that incubation of neuronal and astroglial extracts with the DNA oligoduplex containing uracil for 20 minutes resulted in incision of the radio labeled oligomer, generating a specific 7-mer product (Figure 10A, lanes 1–6). This cleavage product is also seen by the reaction of pure Udg protein (Figure 10A, lane 7), an enzyme known specifically to remove uracil. The Udg activity in neuronal extracts of adult and old rats has decreased significantly when compare to young rats (Figure 10B, $p< 0.05$). However, the decrease seen in adult and old astroglial extracts is of lesser order, although statistically significant (Figure 10B).

Next, the activity of Ape1 in neuronal and astroglial extracts prepared from cerebral cortex of rats of different ages is examined through a similar incision assay as in the case of Udg. Figure 11A shows the sequences of the oligodeoxynucleotides used in this assay. The 21-mer oligonucleotide containing the tetrahydrofuran analog of an AP site (F) at position 14 was 5'-kinased with [γ^{32}P]-ATP. Complementary oligo was annealed to form DNA oligoduplex. Details of the method are described in Chapter 2. A representative autoradiogram is shown in Figure 11A. As can be seen, incubation of brain cell extracts with the F containing DNA oligoduplex for 10 minutes resulted in incision of the radio labeled oligomer, generating a specific 13-mer product (Figure 11A, lanes 1–6). Such cleavage was confirmed through the action of pure Ape1 enzyme (Figure 11A, lane 7). The results indicate an age related decline in APE1 activity in both types of brain cells (Figure 11B, $p<0.05$).

The schematic diagram in Figure 12 illustrates the principle of the BER assay. Uracil DNA glycosylase will remove uracil (U) and AP endonuclease incises the abasic site on its 5' side. Pol β will label the repaired DNA strand by adding a [α^{32}P] dCMP residue, thereby generating a radioactive unligated intermediate (8-mer). When the 5'-dRP residue is removed, the nicked substrate can be ligated to generate a full-length ligated 21-mer with a labeled on the 5' side. In the BER scheme, note that the two distinct activities of Pol β (DNA synthesis and dRP removal) are illustrated as a single step. DNA synthesis is much more rapid than dRP removal and therefore, precedes the generation of a ligatable substrate for DNA ligase. Accordingly, the 8-mer intermediate is observed routinely. BER unligated intermediate and ligated DNA product can be separated by denaturing 20% polyacrylamide sequencing gel electrophoresis.

The effect of age on the overall BER activity with an unlabeled oligo DNA duplex containing uracil at position 8 was measured. During repair, incorporation of a new nucleotide in place of the excised lesion generates an unligated intermediate, 8-mer, which is converted into the full length 21-mer upon ligation with the down stream oligo. A representative autoradiogram is shown in Figure 13. It can be seen that young neurons incorporate [α^{32}P]-dCTP, generating an 8-mer unligated intermediate, which is converted into full length 21-mer upon ligation (Figure 13, lane 1).

Careful observation also reveals that in the case of adult and old neuronal extracts, only a faintly labeled 8-mer not converted into full length 21-mer is seen (Lanes 2 and 3). This finding once again suggests that BER activity is very low in adult and old neurons.

To determine the possible specificity of DNA polymerases involved in uracil initiated BER, the reaction mixture is pre-incubated with some polymerase inhibitor before the initiation of the BER assay. Figure 14 shows the results. Firstly, overall BER activity could be clearly seen in young extracts only, an observation consistent in all our studies. Addition of aphidicolin (50μM), an inhibitor of replicative DNA polymerases, did not have any effect on the activity (lane 4). However, 95% of the 21-mer ligation product has disappeared when young neuronal extracts were pre-incubated with 1 μl of neutralizing polyclonal pol β antibody (lane 7). Also, even in the presence of some other factors required for BER, viz., Udg, Ape 1 and DNA ligase, addition of aphidicolin had no effect on the BER activity (Figure 14, lane 11), but the addition of polyclonal antibody against pol β completely inhibited the BER activity (lane 12). Therefore, it is concluded that brain BER requires Pol β involvement quite specifically.

The basal DNA repair potential of brain appears to be at a low level and BER constitutes the main mode of DNA repair in mammalian brain (Rao 2003b). Pol β, the predominant repair enzyme in neurons and a key factor in the BER pathway, declines with age (Waser *et al*. 1979, Prapurna and Rao 1997, Rao *et al.* 2000, Raji *et al.* 2002).

Recently, Harikrishna *et al.* from our group has shown that DNA gap repair activity declined in aging neurons, primarily because of the declining activities of Pol β and DNA ligase in aging rat neurons (Krishna *et al.* 2005). In view of this information, the BER assay has been performed after supplementing the brain cell extracts with pure Udg, Ape1, Pol β and Ligase independently to assess whether such supplementation could bring back the lost BER activity in adult and old neurons. A representative autoradiogram is shown in Figure 15. In particular, young neuronal extracts supplemented with of pure 0.5 units Udg or 1 unit of Ape1 alone did not change the level of 21-mer ligation product (Lanes 1 and 4), but when supplemented with 0.1 units Pol β or 20 units Ligase alone, formation of the 21-mer product was enhanced (Lanes 7 and 10). In the case of adult and old neurons, no 21-mer product could be visualized, even if Udg, Ape1,Pol β or Ligase were supplemented independently (Lanes 2-3,5-6,8-9 and 11-12). Similar results have been observed when adult and old neuronal extracts were supplemented with Udg together with Ape1 (Lanes 17 and 18). This observation suggested that more than one BER enzyme is deficient in adult and old neurons. When neuronal extracts were supplemented with Pol β together with Ligase, a faint 21-mer product appeared in adult and old neuronal extracts (Lanes 14 and 15) and a prominent 21-mer product was found in young neuronal extracts (Lane 13). These results are taken to indicate that the neuronal extracts from adult and old animals are deficient not only of Pol β but also of Ligase activity.

In the next set of experiments, neuronal extracts were supplemented with Udg, Pol β together with Ligase, and a more improved 21-mer ligation product could be seen in adult and old neuronal extracts (Figure 16, lanes 2 and 3). When neuronal extracts were supplemented with Ape1, Pol β together with Ligase, faint 21-mer ligation products could be seen in adult and old neuronal extracts (Lanes 5 and 6, campare lanes with lanes 2 and 3). When neuronal extracts were supplemented with Udg, Ape1, together with Pol β, no 21-mer spot could be visualized in adult and old neuronal extracts, indicating again that adult and old neuronal extracts are deficient in Ligase activity (Lanes 8 and 9).

As Udg and Ape 1 activity was found to decline with age as described in Figures 10 and 11, and overall BER activity is reduced in adult and old neurons due to deficiency of Pol β and Ligase, the effect of supplementing neuronal extracts with Udg, Ape1, Pol β and Ligase was tested. The results are shown in Figure 17. It is seen that the 21-mer product along with the 8-mer unligated product were found in adult and old neuronal extracts. Results of reconstituting the BER activity with all pure enzymes (Figure 17) illustrating that in the presence of Udg and Ape1, Pol β incorporated [α^{32}P]-dCTP to generate an unligated 8-mer intermediate (Lane 6); when reaction mixture was supplemented with Udg, Ape1, Pol β and Ligase, Pol β incorporated [α^{32}P]-dCTP to generate an unligated 8-mer intermediate, which upon ligation produced a 21-mer quite efficiently (Lane 7).

The protein levels of essential BER enzymes Ape1, Pol β and Ligase III in neuronal extracts were examined through western blotting. A representative western blot is shown in Figure 18A. The expression of both Pol β and Ligase III enzymes was markedly reduced by adult age itself, with further reduction reaching the lowest level by old age, whereas the level of APE1 decreased gradually with age in neurons (Figure 18B). These results are taken to suggest that the age dependent decrease overall BER activity is the result of a deficiency in Pol β and Ligase in aging neurons.

CHAPTER 5

The effect of age on 8-oxoG initiated base excision repair activity in rat cortical neurons

Introduction:

Aging is an inevitable biological process, which is characterized by a general decline in physiological function that leads to morbidity and mortality. There is much evidence that reactive oxygen species (ROS) generated during normal cellular oxygen metabolism are an important source of endogenous DNA damage in all cell types and that the resultant oxidative DNA modification contributes to spontaneous mutation rates. Therefore, oxidative DNA damage has been implicated in carcinogenesis, the aging process and several age-related degenerative diseases (Ames 1989, Ames and Shigenaga 1992, Beckman and Ames 1997, Beckman and Ames 1998, Marnett 2000). The detrimental effects of aging are best observed in post-mitotic tissues like neurons where accumulated DNA damage or a decline in DNA repair capacity is seen (Rao and Loeb 1992, Rao 2007). Neurons are continuously challenged by ROS, which may damage nucleic acids and induce cell death. Thus, effective neuronal repair of oxidative damage is critical to genome maintenance and brain function (Bohr *et al.* 2007).

7, 8-Dihydro-8-oxoguanine or 8-oxogauanine (8-oxoG) is one of the most prevalent products of oxidative attack of DNA and is mainly repaired by the base excision repair (BER) pathway. 8-oxoG is removed from DNA 8oxoG/C pairs by the 8-oxogaunine DNA glycosylase (Ogg1) (Boiteux and Radicella 2000). Ogg1 is a bifunctional enzyme, possessing DNA glycosylase activity (hydrolysis of the N-glycosidic bond of the damaged nucleotide) and AP lyase activity (elimination of the 3'-phosphate, often referred to as β-elimination) (Bjoras *et al.* 1997, Zharkov *et*

al. 2000). Unlike the situation in several other bifunctional DNA glycosylases, the AP lyase activity of Ogg1 is much weaker than its glycosylase activity. Consequently, two products of Ogg1-catalyzed reaction can be detected: the glycosylase activity quickly produces abasic (AP) sites, and a slower AP lyase activity leaves nicked DNA with the nick flanked by a 5'-phosphate and a 3'-terminal α, β-unsaturated aldehyde. If Ogg1 is presented with an AP site-containing substrate, the rate of its cleavage is similar to the rate of β-elimination of a damaged base-containing substrate (Zharkov *et al.* 2000). The 3'-terminal α,β-unsaturated aldehyde resulting from the AP lyase function of Ogg1 is carried out by apurinic endonuclease 1(Ape1) leaving a one nucleotide gap with 3'-OH terminus. Repair is completed by insertion of a single nucleotide by DNA polymerase β, and subsequent ligation of resulting nick is mediated by the XRCC1/Ligase III complex (Vidal *et al.* 2001).

Various reports have shown an accumulation of different oxidative DNA lesions and possibly a decrease in DNA repair capacity of brain in aging. In the present study, this aspect has been extended to examine age-dependent regulation of BER, with a model oligoduplex containing 8-oxoG against a C, in neuronal extracts prepared from aging rat brain cortex.

Methods

Preparation of Neuronal and astroglial cell fractions from 'Young' (7 days postnatal), 'Adult'(6 months) and 'Old' (≥24 months) rat brain cerebral cortex is described in Material and Methods, Chapter 2.

Comet assay:

DNA strand breaks in neurons and astrocytes were evaluated using Trevigen Comet Assay kit (Trevigen, Gaithersburg, MD). Cells were resuspended in ice cold PBS (Ca^{2+} and Mg^{2+} free) to a concentration of $1x10^5$ cells/ml. Briefly, an aliquot of 50 µl of cells was added to 500µl of 1% molten low-melting agarose kept at 42 ^{0}C. Fifty microliters were immediately pipetted and evenly spread onto an area of the comet slides .The slide was incubated at 4 ^{0}C in the dark for 10 minutes to accelerate gelling of the agarose disc and then transferred to prechilled lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% sodium lauryl sarcosinate, 1% Triton X-100, pH 10] for 60 minutes at 4 ^{0}C. Following lysis, the slides were washed three times for 10 minutes with 1X FLARE buffer (25mM HEPES-KOH at pH 7.4 250 mM KCl, 25 mM EDTA), gently blotted dry with tissue paper and covered with 50 µl of buffer (0.04% 25X FLARE Buffer, 0.01% 100X BSA) or 8-oxoguanine DNA glycosylase (Ogg1) in buffer (0.5 units per gel). The slides were incubated in a humidity chamber at 37 ^{0}C for 60 minutes. A denaturation step was performed in alkali solution (0.3 M NaOH, 1 mM EDTA, pH>13) at room temperature for 20 minutes, in the dark.

The slide was then transferred to prechilled alkaline electrophoresis solution pH >13 (300 mM NaOH, 1 mM EDTA) and subjected to electrophoresis at 1 V/cm, 300 mA for 40 minutes in the dark at 4 ^{0}C. At the end of the electrophoresis, the slides were washed with neutralization buffer (0.4 M Tris-HCl, pH 7.4); and fixed in ice-cold 100% ethanol at room temperature for 5 minutes and air dried. DNA was stained with 50 µl of SYBR Green I dye (Trevigen) (1:10000 in Tris-EDTA buffer, pH 7.5) for 20 minutes in the refrigerator and immediately analyzed using an Olympus digital camera attached to an Olympus BX51 epifluorescence microscope. For each slide, 50 randomly chosen comets were analyzed using an excitation filter of BP 450/480 nm and a barrier filter of 515 nm. Images were captured by the digital camera and microscopic evaluation of the comet images was quantified using the image analysis system of Comet Score™ (TriTek Corp.) Tail moment (product of percentage of DNA in tail and tail length) was measured using the Tritek CometScore^{TM} Freeware v1.5 image analysis software**.**

8-oxoguanine DNA-glycosylase (Ogg1) Activity Assay:

The details are as described in Materials and Methods, Chapter 2.

Base Excision Repair Assay:

The details are as described in Materials and Methods, Chapter 2.

Result and discussion:

In general, the alkaline comet assay can measure different types of DNA damage (single strand breaks, alkali labile sites, crosslinking, DNA adducts, SSB associated with incomplete excision repair sites). DNA breaks may relax DNA super coiling and allow relaxed DNA loops to migrate under electrophoresis (Tice *et al.* 2000). Introducing Ogg1 in the assay on naked DNA after lysis was performed, making it possible to detect oxidative DNA damage to Guanine. Ogg1 initiates the repair of 8-oxoG bases by excising them and cutting the sugar-phosphate backbone of the DNA molecule. Thus additional strand breaks are induced at the location of oxidized base substrate, causing DNA relaxation and migration (Collins *et al.* 2001, Smith *et al.* 2006). When using Ogg1 to measure oxidative DNA damage, the usual practice is to incubate a slide (two gels) with buffer alone in parallel along with the +enzyme slide, and to subtract the mean comet score of the control (buffer) slide from the mean score of the +enzyme slide (Collins and Dusinska 2002, Collins 2009).

Figures 19 and 20 show representative images of DNA migration following electrophoresis of isolated neurons and astrocytes prepared from young, adult and old rat cerebral cortex. The 8-oxoG sites in neurons and astrocytes at these three ages were assessed by modified alkaline comet assay. When neurons, prepared from rats of different ages, were incubated with only buffer, an increase of DNA migration in the tail was observed with increasing age of the animal (Figure 19). This increase in tail movement is taken to be increasing DNA damage that occurs

due to aging. When the cells were incubated with buffer containing Ogg1, there was a further increase in the tail movement, which must be due to the increased number of 8-oxoG residues accumulating in DNA with age and this 8-oxoG being removed and DNA being incised by the added Ogg1. Essentially the enhanced tail movement due to the incubation of the brain cells with Ogg1 represents the increase in the number of 8-oxoG sites with age. The pattern of results is the same with either neurons or astrocytes (Figures 19 and 20). Table 4 shows the actual fold increase of 8-oxoG sites with age in brain cells. For example, in neuronal DNA the content of 8-oxoG between the young and adult ages has increased 5.2 and between young and old ages by 7.4 fold. Similarly, adult and old astrocytes accumulate 3.2 and 4.9 fold respectively when compared to young astrocytes. Closer observation of the data revealed that while in the case of neurons and astrocytes considerable amount of 8-oxoG accumulate by adulthood itself, there is a further increase in 8-oxoG in old neurons and astrocytes (Table 4).

As the first step in the DNA BER pathway, excision of DNA base lesions requires the activity of at least two repair enzymes, a specific glycosylase that can recognize and cleave the damaged bases, and an apurinic/ apyrimidinic (AP) endonuclease that incises the sugar phosphate backbone at the remaining abasic residue. To examine the activity of Ogg1 activity in brain cells, an *in vitro* excision/incision assay was performed. Figure 21A shows the sequences of the oligodeoxynucleotides used in the assay.

The 24-mer oligonucleotide containing 8-oxoG (O) at position 10 was 5'-kinased with [γ^{32}P]-ATP. Complementary oligo was annealed to form a DNA oligoduplex. Details of the method are described in Chapter 2. A representative autoradiogram is shown in Figure 21A. The results indicate that incubation of neuronal and astroglial extracts with the DNA oligoduplex containing 8-oxoG for 120 minutes resulted in the incision of the radio labeled oligomer, generating a specific 9-mer product (Figure 21A, lanes 1–6). This cleavage product is also seen in the reaction containing pure Ogg1 (0.2 units) protein (Lane 7), an enzyme known specifically to remove 8-oxoG. The Ogg1 activity in neuronal extracts of adult and old rats has decreased significantly when compare to young rats (Figure 21B, $p< 0.05$). However, the decrease seen in adult and old astroglial extracts is of a lesser order, although statistically significant (Figure 21B).

In order to investigate the effect of age on 8-oxoG initiated BER steps in neurons, we measured the *in vitro* repair of an unlabeled 8-oxoG containing oligoduplex by neuronal extracts in the presence of radioactive dGTP. Repair synthesis incorporates a new nucleotide in place of the excised lesion, and is subsequently converted to the full-length 24-mer product upon ligation. It can be seen that young neurons are able to incorporate radioactive dGMP, which is converted into full length 24-mer repair product upon ligation (Figure 22, lane 1), but adult and old neurons are unable to repair 8-oxo G as seen by the absence of any full length 24-mer radioactive spot on the autoradiogram (Lanes 2 and 3).

In light of this information, the 8 oxoG initiated BER assay was performed after supplementing the brain cell extracts with pure 0.2 units Ogg1, 0.5 units Ape1, 0.2 units Pol β and 20 units T_4 DNA Ligase (Ligase) independently to assess whether supplementation could restore the BER activity in adult and old neurons.

It is seen that only young neurons are able to remove 8-oxo G and incorporate radioactive dGMP into the full length 24-mer ligation product (Figure 23, lanes 1, 4, 7 and 10), where as in case of adult and old neuronal extracts, no 24-mer ligation product could be visualized, even if Ogg1, Ape1, Pol β and Ligase were supplemented independently (Figure 23, lanes 2-3, 5-6, 8-9 and 11-12). Similar results were observed when adult and old neuronal extracts were supplemented with Ogg1 together with Ape1 (Figure 24, lanes 2 and 3). When neuronal extracts were supplemented with Pol β together with Ligase, faint 24-mer product appeared in the case of adult and old neuronal extracts (Lanes 5 and 6) and a prominent 24-mer product was found in young neuronal extracts (Lane 4). These results are taken to indicate that the neuronal extracts from adult and old animals are deficient not only of Pol β but also of Ligase activity.

When neuronal extracts were supplemented with Ogg1, Ape1, together with Pol β, no 24-mer spot could be visualized in adult and old neuronal extracts, indicating again that adult and old neuronal extracts are deficient in Ligase activity (Lanes 8 and 9).

Next neuronal extracts were supplemented with Ogg1, Pol β together with Ligase, which distinctly improved 24-mer ligation in adult and old neuronal extracts as shown by the autoradiography (Lanes 11 and 12). When neuronal extracts were supplemented with Ape1, Pol β together with Ligase, faint 21-mer ligation spots could be seen in adult and old neuronal extracts (Lane 14 and 15, compare lanes with lanes 11 and 12).

As Ogg1 and Ape 1 activity was found to decrease with age (as described in Figure 21), and overall BER activity was reduced in adult and old neurons due to a deficiency in Pol β and Ligase, the effect of supplementing neuronal extracts with Ogg1, Ape1, Pol β and Ligase was tested. It is seen that a significant level of 24-mer product was found in adult and old neuronal extracts (Lanes 17 and 18) although less than that with young extracts (Lane 16). The above results indicate that accumulation of 8-oxoG and a decrease in Ogg1 activity and BER activity due to a deficiency in Pol β and Ligase, are the main causes of aging in neurons.

CHAPTER 6

Dietary calorie restriction from adulthood through old age in rats: improved DNA polymerase β and DNA-gap repair activity in cortical neurons

Introduction:

It is well established now that dietary calorie restriction (CR) confers the benefit of a reduced rate of aging and therefore, extended life span. Since the original seminal observation of McCay *et al.* (1935) that CR, without any compromise in the quality of the diet, extends life span of rats, numerous studies dealing with different species have confirmed this observation (Rao 2003a, Heydari *et al.* 2007). CR increases DNA repair capacity as measured by increased unscheduled DNA synthesis (UDS) in lymphocyte (Licastro *et al.* 1988, Rao *et al.* 1996), hepatocytes and kidney cells (Weraarchakul *et al.* 1989) and enhances the dimethylnitrosamine demethylase activity (Asakura *et al.* 1994). The activity of Pol β, UV and AP DNases, taken as markers for DNA repair potential in brain, were significantly higher in subjects with lower basal metabolic rate (LBMI), who can be considered equivalent to healthy calorie restricted subjects, indicating that CR for prolonged periods shows beneficial effects in terms of DNA repair potential (Rao *et al.* 1996, Raji *et al.* 1998). CR reduces the amount of DNA damage by altering both free radicals and drug metabolism, there by permitting the organism to rapidly detoxify and eliminate xenobiotic agents (Leakey *et al.* 1989).

Basal DNA repair potential of brain appears to be at a low level and BER constitutes the main mode of DNA repair in the mammalian brain (Rao 2003b).

Polβ, the predominant repair enzyme in neurons and a key factor in the BER pathway, declines with age (Waser *et al.* 1979, Prapurna and Rao 1997, Rao *et al.* 2000, Raji *et al.* 2002). DNA gap repair activity was found to decline in aging neurons primarily because of the declining activities of DNA Pol β and DNA ligase in aging rat neurons (Krishna *et al.* 2005). We noticed previously that CR initiated during adult life (6 months) and continued till the age of 24 months, stimulated response on the total DNA polymerase activity. The extent of modification of polymerase activity due to CR is dependent on both the age at which CR was initiated as well as the tissue under study (Prapurna and Rao 1996, Rao *et al.* 1996).

It is, however, clear that so far information related to the beneficial effects of CR on DNA repair remains at the enzyme activity level and the actual repair process at the molecular level needs to be examined. Also, it is not known whether CR continued well into old age would sustain beneficial effects. In the present study, an attempt is made to study the actual process of DNA gap repair and primer extension activities in isolated cortical neurons subjected to extend CR.

Methods

Procedure for calorie restriction:

The details are as described in Materials and Methods, Chapter 2

DNA polymerase β assay:

The details are as described in Materials and Methods, Chapter 2

Procedure for Western blot:

Equivalent of 25 μg of protein from neuronal cell of young, adult, old control and calorie restricted old rat extracts was electrophoresed on a 10% SDS polyacrylamide gel and transferred on to PVDF (Millipore) membrane for western blot analysis. Blot was incubated with 5% non-fat dry milk powder solution in Tris-buffered saline. The membrane was incubated with monoclonal anti-mouse Pol β antibody (Novus Biologicals). Bound primary antibodies were visualized with the appropriate horseradish peroxidase-conjugated secondary antibodies (Bangalore Genei, India.) and chemiluminescent substrate kit (Pierce, Rockford, IL). Intensity of the band on the film was quantified by using by Image J1.41 software (NIH, USA).

Gap Repair and primer extension assay.

The details are as described in Materials and Methods, Chapter 2.

Results

Table 5 shows the DNA Pol β specific activity in young, adult, old control and calorie restricted old rat cortical neuronal extracts with 'activated calf thymus DNA' as the substrate.

As can be seen there is considerable reduction of Pol β activity with age and the specific activity of Pol β is higher in calorie restricted neurons (61.18%) when compared to the old control ($P< 0.01$). Similarly, Figure 25 shows a western blot of Pol β in young, adult, old control and calorie restricted neuronal extracts. There is a statistically significant enhancement in the level of Pol β in calorie restricted neuronal extract (45.6%) as compared to the old control ($P< 0.01$).

The gap filling activity of cortical neuronal extracts prepared from young, adult, old control and calorie restricted rats were tested using an oligo duplex substrate with a gap of 1 or 4 nucleotides. The results are shown in Figure 26. Gap repair involves two major steps: the filling of the gap by the addition of the required number of nucleotides, followed by ligation with the downstream primer. This process, if completed properly should give a radioactive spot corresponding to a 32-mer in the autoradiogram with a corresponding decrease in the intensity of the labeled 14-mer. As can be seen, young neuronal extracts (Figure 26, lanes 1 and 2) show detectable gap repair activity, while in adult this activity is barely seen (Lanes 3 and 4), yet in the old control and calorie restricted old neurons a single nucleotide addition product could be visualized as a 15-mer spot (Lanes 5 and 7). However, ligation to the down stream primer did not take place as indicated by the absence of any spot corresponding to a 32-mer. No gap filling activity is observed when the substrate has a 4 nucleotide gap (Figure 26, lane 6 and 8) in both old control and calorie restricted old neuronal extracts.

Supplementation of the extracts with 1 mM ATP did not change the situation (Figure 27, lanes 1-4). On the other hand, when the extracts were supplemented with both 1 mM ATP and 20 units of T_4 DNA ligase, the 1 nucleotide gap is filled and also ligated to give a product of 32-mer both in the case of old control (Lane 5) and calorie restricted (Lane 7) extracts. It is to be noted that the intensity of the 32-spot in the calorie restricted neuronal extract is greater, implying a slightly improved gap repair activity. This result was consistent in three independent experiments with different extracts as can be seen from the densitometric values shown in Figure 27, lanes 5 and 7. Also, as can be seen, no gap filling activity was observed when the substrate had a 4 nucleotide gap (Lanes 6 and 8), even when the extracts were supplemented with ATP and T_4 DNA ligase.

Up regulation of Pol β activity and overall BER in young and old tissue subjected to CR was reported by (Cabelof *et al.* 2003). Since Krishna *et al.* (2005) have shown earlier that Pol β and DNA ligase are the key factors in gap repair whose activity declines in aging neurons, the higher gap repair activity found in calorie restricted neuronal extracts from the old rats in the present study could perhaps be attributed to the presence of slightly higher amounts of Pol β, but with very insufficient or no improvement in the already low DNA-ligase activity due to the advanced age of the animals. This result is once again taken to point out the improved levels of Pol β in CR brain cells.

Earlier work from this laboratory has shown that simple template driven primer extension activity in a recessed oligo duplex by aging neuronal extracts is drastically reduced when compared to young neuronal extracts (Rao *et al.* 2000). However the activity could be restored to a significant level when the aging neuronal extracts were supplemented with 2.5 units of Pol β. Therefore, in the present study, the effect of CR on neurons to extend a primer, with or without a correctly matched base pair at its 3'-end is tested. The results are shown in Figure 28. Significant extension of the primer could be seen only with young neuronal extracts (Lanes 1 and 2), and there was decreased activity in adult neuronal extracts (compare the activity in lanes 1–2 to 3–4). There was no addition of nucleotides to the primer in both old control and calorie restricted aged neuronal extracts (Lanes 5-8). This result is rather intriguing in that the CR neuronal extracts have shown higher levels of Pol β (Figure 25 and Table 5) and yet the primer extension activity is not seen. In the present study, both the old control and CR old animals were sacrificed at 30 months of age, whereas in our earlier studies (Rao *et al.* 2000), the "old" animals were of 24 months age. It thus appears that in the very old animals, the primer extension activity, which essentially represents a synthetic activity, is almost shut off.

The emerging picture from the overall results of this study is as follows. Dietary calorie restriction of 40% imposed on animals from 6 months to 30 months of age leads to improved Pol β levels in cortical neurons. Improvement in 1 nucleotide gap repair is seen in the calorie restricted neuronal extracts only when

the extracts were supplemented with ATP and T_4 ligase. It is possible that the ligase activity might have shown improvement during the earlier periods of calorie restriction phase and could not be sustained until the very old age of 30 months. It is, nevertheless, significant that addition of DNA ligase to extracts from 30 months old animals restored the gap repair activity suggesting the possibility of intervention to maintain good DNA short gap repair even in old animals.

Another interesting clue that has surfaced out of these studies is that calorie restriction does not appear to sustain the activity of template driven addition of nucleotides to a primer probably because post mitotic cells like neurons do not see this activity as a "repair" activity. Thus a distinction seems to be made between gap filling and addition of nucleotides to a primer which has no downstream sequence. Thus, the present observations lend further support to the hypothesis that one of the lifespan extending mechanisms of CR may be to channel the limited energy resource available towards an essential maintenance process like DNA repair rather than towards reproductive and anabolic activities (Rao 2003a).

CHAPTER 7

General discussion
Summary

General discussion

Aging is regarded as an elusive and inevitable phenomenon occurring in all higher organisms. Many theories have been proposed to explain this process. One such theory which enjoys considerable logic and rationale is the DNA damage and repair theory. DNA repair potential of an organism could play a vital role in the maintenance of genomic integrity, the failure of which could result in disease and among many other things, aging as well (Hart and Setlow 1974, Gensler and Bernstein 1981, Bernstein and Bernstein 1991). The possible interrelationship between DNA repair potential and the aging process has been the subject of intense research and debate (Rao and Loeb 1992). Finally there seems to be a general consensus among scientists in the field that accumulation of DNA damage and a decrease in DNA repair capacity contribute to the phenomenon of aging and related disorders, including neurological disorders (Gorbunova *et al.* 2007, Subba Rao 2007, Schumacher *et al.* 2008, Vijg 2008).

The importance of DNA repair to the nervous system is most graphically illustrated by the neurological abnormalities observed in patients with hereditary DNA repair disorders. As discussed in the Chapter 1, most of the alterations in DNA are the result of continuous exposure of living organisms to DNA damaging agents like radiation, certain environmental components and products of cellular metabolism.

In mammals, cell types that do not divide like long-lived neurons, differentiated muscle cells, and the cell types that divide only slowly, accumulate DNA damage with age (Bernstein and Bernstein 1991, Rao 1997, Rao 2002, Nouspikel and Hanawalt 2002). It is likely that these cells may govern the rate of overall mammalian aging. Brain is composed of cells with a variety of developmental histories, functions and fates, and the capacity to repair DNA may be profoundly affected by developmental status of individual cells. The level of DNA repair is low in brain, endogenous damage accumulates, mRNA synthesis declines, and protein synthesis is reduced with age (Price *et al.* 1971, Chetsanga *et al.* 1977, Mori and Goto 1982, Zs-Nagy and Semsei 1984, Sajdel-Sulkowska 1985). Thus, for the brain, there appears to be a direct relationship between the accumulation of DNA damage and the important feature of aging. In contrast to non-dividing or slowly dividing cell populations, at least some types of rapidly dividing cell populations appear to cope with DNA damage by replacing lethally damaged cells through replication of undamaged ones. Examples include duodenum and colon epithelial cells and hemopoitic cells of bone marrow (Bernstein and Bernstein 1991). The last 50 years have seen great strides of advancement in the understanding of the various pathways of DNA repair both in prokaryotes and higher organisms including humans. These aspects have already been discussed in Chapter 1 of this thesis.

The results summarized in Chapter 3 provide important information that indeed damage to cellular DNA increases with age in both neurons and astrocytes of rat brain. Most of the damage seems to accumulate by the time the animal attains adulthood itself (6 months in the present case). There was of course, a further increase in damage between the ages of 6 and 24 months. These results demonstrated the fundamental premise of the "DNA damage and repair" theory of aging phenomenon that was proposed earlier by (Bernstein and Bernstein 1991) and supported by the work from this lab over the years.

The relationship between aging and DNA repair has a special significance in an organ like brain. DNA repair is affected in mammalian cells through four main different pathways (Rao 2002, Rao 2003b, Rao 2007). The base excision repair (BER) pathway, which is seen in almost all the tissues and responsible for repairing the simple alterations in DNA structure at the base level e.g., modifications of bases through methylation, ethylation, oxidation and spontaneous deamination or spontaneous loss of bases resulting in the formation of apurinic/apyrimidinic sites, leading to single strand breaks in DNA. This pathway is one of the most conserved and takes care of the minor but important damages that occur to bases in DNA.

From the above, clearly a post mitotic cell like a neuron would be an ideal model system to follow the extent of DNA damage and DNA repair with respect to the age of the animal.

The fact is that DNA damage accumulation in brain cells would be more realistic in view of the non dividing nature of the cells. Yet, the DNA repair assessment would also be valuable since these cells, although non-dividing, are metabolically very active, and it would be of immense value to see how DNA damage is handled during advancing age.

Uracil in genomic DNA mainly arises from deamination of cytosine residues and 8-hydroxyguanine (8-oxoG) is abundantly produced in DNA by ROS. BER is an important part of the brain to correct such type of DNA damage. The work presented Chapters 4 and 5 constitutes the overall efforts that have been going on in this lab to examine the link between DNA repair potential and aging with special reference to neurons.

The data suggest that accumulation of uracil in neurons and astrocytes is a gradual process with age while considerable amounts of 8-oxoG accumulate by adulthood, with a further increase in 8-oxoG in old neurons and astrocytes. Udg, Ogg1, Ape1 activities were decreased with age in both neurons and astrocytes. Basal DNA repair potential of brain appears to be at a low level and BER constitutes the main mode of DNA repair in the mammalian brain (Rao 2003b). Pol β, a predominant repair enzyme in neurons and a key factor in the BER pathway, declines with age (Waser *et al.* 1979, Prapurna and Rao 1997, Rao *et al.* 2000, Raji *et al.* 2002).

Recently, Harikrishna *et al* from our laboratory has shown that DNA gap repair activity declines in aging neurons, primarily because of the declining activities of Pol β and DNA ligase in aging rat neurons (Krishna *et al.* 2005). The overall BER potential of neurons initiated by uracil and 8-oxoG significantly declines with age.

Supplementing the neuronal extracts with pure Udg (in the case of Udg initiated BER), Ogg1 (in the case of 8-oxoG initiated BER), Ape1, Pol β and Ligase could not bring back the lost BER activity in adult and old neurons. However, supplementation of Pol β along with ligase has shown improvement of BER activity in adult and old neuronal extracts. Englander and Ma (2006) measured expression and activities of BER enzymes during rat brain ontogeny, i.e. during the physiologic transition from proliferative to the postmitotic differentiated state. A subset of BER enzymes (Ogg1, Ape1, Pol β) exhibited declining expression and excision activities.

In mice, an 85% decline in BER capacity was reported in brain nuclear extracts prepared from old mice as compared with 6-day-old mice (Intano *et al.* 2003). The extracts from these mice showed a decreased abundance of Pol β although addition of the purified polymerase did not restore BER activity. The levels of the other BER proteins (i.e. Ape1, XRCC1, Ligase I, and Ligase III) however, did not change relative to age.

In two separate studies, Pol β expression was found to remain generally high during brain development, with potential peaks at early and late stages of animal age (Shrivastaw *et al.* 1983, Subba Rao and Subba Rao 1984b). The BER pathway was studied in different mouse tissues (brain, liver, spleen and testis) at young (4 months) and old (24 months) ages by (Cabelof *et al.* 2002). In all the tissues tested, an age dependent decrease in the repair capacity was observed and this was found to be due to reduced levels of Pol β activity, protein and mRNA. Further, the studies have shown that both spontaneous and methyl methanesulfonate induced mutation frequency was very high in the old animal. All these findings underline the importance of the BER pathway and its key component Pol β, in maintaining the genomic integrity.

In rats, a consistent decrease in the level of Pol β mRNA has been observed during postnatal brain development (Nowak *et al.* 1990). Using northern and Western blotting, it was revealed that the mRNA and immunologically reactive molecules of DNA Pol β are reduced 30% and 20%, respectively, in old as compared with young rat brain (Rao *et al.* 2001). Surprisingly, activity gel assays and immunotitration experiments uncovered a 50% reduction in Pol β function in aging neurons. The more severe loss of activity relative to protein number was interpreted as an accumulation of catalytically inactive polymerase molecules in the rat brain with age.

It is well established now that dietary calorie restriction (CR) leads to extension of life span in many species, although the exact mechanism of this effect is still unknown. The emerging picture from the overall results of this study is described in Chapter 6. In the present study, we examined the effect of 40% CR imposed during a prolonged period of life span (from 6 months to 30 months) of rats on the activity of Pol β and DNA repair, in terms of short gap repair and template driven primer extension in cortical neurons. Pol β activity is very low at this late age. However, cortical neuronal extracts prepared from calorie restricted rats of 30 months age showed significantly higher Pol β protein levels and activity when compared to control 30 month old rats. Yet, one nucleotide gap repair in old control neurons and in CR neurons, which displayed a slightly better repair efficiency, could be visualized after supplementation of the extracts with T_4 DNA ligase, indicating that CR does not have a significant positive effect on ligase activity. No primer extension activity is seen either in CR or old control neuron extracts. These results indicate that the marked decline of BER in the brain of very old animals and that CR confers a significant beneficial effect in the case of Pol β. This might represent a critical step for senescence and neurological manifestation.

It is becoming increasingly apparent that DNA repair potential is intimately connected to diseases like cancer and a natural phenomenon like aging. In brain, it is likely that lowered BER may have an etiological connection with many neurodegenerative diseases often seen in aging populations.

In this regard, the present results showing that the reduced BER activity initiated at uracil and 8-oxoguanine in aging neurons can be improved *in vitro* by supplementing Pol β and Ligase could be of some significance. Admittedly, these studies are to be extended to an in vivo situation utilizing modern techniques and would be the challenge for future.

Summary and Conclusions

1. The hypothesis that "decreased DNA repair capacity in the brain is at least one of the major biochemical markers associated with advancing age and deterioration of brain function" has been tested in this investigation.
2. DNA SSBs and DSBs were studied in isolated neuronal and astroglial cell fractions from the rat cerebral cortex at three different ages of 'Young' (7 days postnatal), 'Adult' (6months), 'Old' (≥ 2 years).
3. The alkaline version of the comet assay showed that neurons from adult and old rats accumulate 4.2 and 5.1 fold more SSBs as compared to young neurons, respectively. Similarly, astrocytes from adult and old rats accumulate 3.6 and 4.2 fold more SSBs as compared to young astrocytes, respectively.
4. The neutral version of the comet assay showed that neurons from adult and old rats accumulate 6.2 and 7.4 fold more DSBs as compared to young neurons, respectively. Similarly, astrocytes from adult and old rats accumulate 5.3 and 6.1 fold more DSBs as compared to young astrocytes, respectively.
5. The modified alkaline comet assay showed that in neuronal DNA the content of uracil between the young and adult ages has increased 3.3 fold and between young and old ages by 6.48 fold. Similarly, adult and old astrocytes accumulate 3.28 and 6.58 fold more damage, respectively, when compared to young astrocytes.

6. The Udg activity in neuronal extracts of adult and old rats has decreased significantly when compare to young rats. However, the decrease seen in adult and old astroglial extracts are of a lesser order, although statistically significant.

7. The Ape1 activity in neuronal and astroglial extracts of adult and old rats has decreased significantly when compared to young rats.

8. With a synthetic oligoduplex DNA containing uracil, the overall BER activity declined in adult and old brain neuronal extracts. BER activity of young neuronal extracts was inhibited by neutralizing polyclonal DNA Pol β antibody. However, aphidicolin had no effect, indicating the involvement of Pol β in uracil initiated BER.

9. Supplementation of adult and old neuronal extracts with pure Udg, Ape1, Pol β and Ligase independently could not enhance BER activity. On the other hand, supplementation with Pol β and Ligase together improved BER activity. These results are taken to suggest that overall BER activity is adversely affected primarily due to a deficiency of Pol β and Ligase in aging neurons.

10. The expression of both Pol β and Ligase III enzymes was markedly reduced by adult age itself, with further reduction reaching the lowest level by old age, whereas the level of APE1 decreased gradually with age in neurons.

11. The modified alkaline comet assay showed that in neuronal DNA the content of 8-oxoG between the young and adult ages has increased 5.23 fold and between young and old ages by 7.43 fold. Similarly, adult and old astrocytes accumulate 3.23 and 4.94 fold more damage respectively, when compared to young astrocytes.

12. The Ogg1 activity in neuronal extracts of adult and old rats decreased significantly when compare to young rats. However, the decrease seen in adult and old astroglial extracts are of lesser order to that seen for Pol β, although statistically significant.

13. With a synthetic oligoduplex DNA containing 8-oxoG, the overall BER activity declined in adult and old brain neuronal extracts. Supplementation of adult and old neuronal extracts with Ogg1, Ape1, Pol β and Ligase independently did not show any beneficial effect, whereas supplementation with Pol β together with Ligase improved BER activity. This result is taken to indicate that the 8-oxo G driven BER pathway is markedly decreased in aging neurons and that partial restoration of the activity could be achieved through supplementation of the extracts with Pol β and DNA ligase.

14. The effect of 40% calorie restriction (CR) imposed during an extended period of life span (from 6 months to 30 months) of rats on the activity of Pol β and DNA short gap repair and template driven primer extension was studied.

15. Cortical neuronal extracts prepared from CR rats showed significantly higher Pol β activity and protein levels when compared to control 30 month old rats.

16. Single nucleotide gap repair exhibiting a slightly improved efficiency in CR neurons, could be visualized after supplementation of the extracts with T4 DNA ligase. No significant primer extension activity was seen either in CR or old control neurons.

17. The present results demonstrating the accumulation of SSBs, DSBs, uracil and 8-oxoG, decreased expression of BER enzymes and reduced BER activity initiated by uracil and 8-oxoguanine in aging neurons, reveal important parameters in the pathogenesis of aging in neurons. Thus, improved BER activity following the supplementation of neuronal extracts with Pol β and Ligase could have far reaching consequences in the area of therapeutic possibilities.

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