Non-apoptotic DNA fragmentation: A molecular pointer of ageing

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Of the many theories proposed to explain the molecular mechanisms of ageing, the DNA-damage and DNA-repair theory has attracted much attention. There is considerable evidence, including the work done in this laboratory, to correlate accumulation of DNA-damage with the phenomenon of ageing. Accretion of DNA-damage with age may be due to increased DNA-damageing events or decreased DNA-repair capacity or both. It is suggested that non-apoptotic DNA-fragmentation in terms of single and double strand breaks, considered to be the end point to many a DNA-damage events, could be utilized as molecular parameter to follow the process of ageing not only in a post-mitotic cell like neuron but perhaps in other tissues as well.

AGEING may be broadly defined as genetically programmed deterioration of all physiological functions with age. Extensive research over the past several years on the molecular mechanism of this apparently inevitable phenomenon has led to postulation of many theories and hypotheses[1–4]. Among these, the theories that seek to explain the process of ageing at genetic level have naturally attracted maximum attention. One such theory is the DNA-damage and repair theory. It was Hart and Setlow[5] who first reported what appeared to be a correlation between the DNA-repair potential of a given organism and its longevity. Subsequently, Gensler and Bernstein[6] proposed that accumulation of DNA damage is the primary cause of ageing. This concept, although faced a turbulent period, continues to enjoy the prime attention of the scientists in the field. Indeed considerable data have accumulated in the past several years to demonstrate that decline in DNA-repair capacity and consequent accretion of DNA-damage plays a cardinal role not only in the phenomenon like ageing but also in diseases like cancer[5–9]. Excellent books and reviews have appeared on this subject in the recent past[3,7–13].

We, in our laboratory, have been looking at some of the biochemical changes that occur during ageing, particularly in brain tissue. During these investigations it became apparent to us that the amount of DNA-damage that occurs in an ageing neuron, and the ability of that neuron to repair such damage may be an important pointer to the maximum lifespan of that species. The tenet of this hypothesis is that since neurons do not possess the replicative capacity and that in almost all species of animals nerve cells are one of the earliest cells to be differentiated and, therefore, their lifespan being almost the same as that of the whole animal, the DNA-repair potential of a neuron should be a good reflection on the basal DNA-repair capacity of various tissues in the animal. Similarly, accumulation of DNA damage in a neuron may be a pointer to this process in the rest of the tissues as well. It is the purpose of this article to review, in brief, the types of damage that are known to accumulate in the DNA of cells with increasing age and to present the possibility that a quantitative assessment of such damage could be a useful molecular parameter to gauge the process of ageing.

DNA damage

Damage or alteration to the structure of DNA appears to be a universal phenomenon occurring both due to spontaneous endogenous metabolic events including errors in DNA replication[14,15] and oxidative reactions that take place in the cell as well as due to exogenous factors like radiation and mutagenic chemicals. Spontaneous damage can result in loss of bases, modification of bases, mismatched base pairs, strand breaks and also changes in the sequence of bases. Similar damage could also occur due to environmental factors which may result in cross links, strand breaks, formation of bulky adducts and oxidative damage. The number of ways in which the DNA can be damaged is summarized in Table 1 and Figure 1.

The types of DNA damage mentioned in Table 1 occur with varied frequencies and this information is summarized in Table 2. It is noteworthy that single strand breaks constitute a major form of DNA damage. This is because many other types of structural alterations in DNA molecule (depurination, depyrimidination, deamination, etc.) are likely to result in single strand breaks both in the absence or occurrence of DNA repair. If the rates of different damage events are added up, the overall rate of DNA damage events/cell/day

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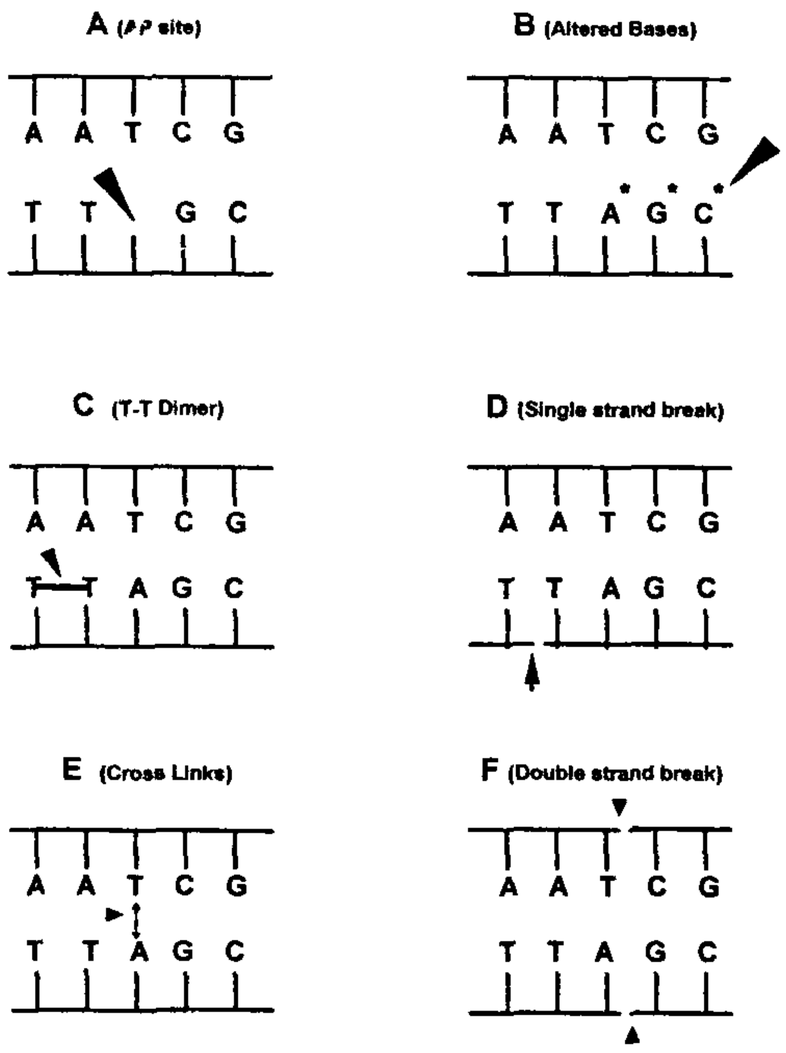

Figure 1. Major modes of DNA damage. *a*, Apurinic/apyrimidinic site (AP site); *b*, Altered/modified bases that may result in mismatches; *c*, Thymidine dimer, a major photoproduct formed due to UV irradiation (250–260 nm); *d*, Single-strand break, one of the most frequent consequential end points of various damages; *e*, Crosslinks formed between two strands of DNA (interstrand). Sometimes the crosslinks are between DNA and the surrounding proteins; *f*, Double-strand breaks – an occasional end point of oxidative/ionising radiation induced damage.

Table 1. Various types of DNA damage

Spontaneous damage	**Causative agents/event**
Mismatched base pairs	DNA-replication/repair
Alteration in the structure of base like Tautomeric shifts deamination	Cellular metabolism
Loss of bases (depurination, depyrimidination)	
Damage due to environmental factors	
Baseless sites	
Cross links	
Strand breaks, deletions	Alkylating agents and other
Alkylation and bulky adducts	damage inducing chemicals
Oxidative damage	Radiation, free radicals

would approximate 60,000. This is an enormous onslaught on the structural integrity of genomic apparatus.

DNA repair

Just as there are a multitude of ways by which DNA molecule can be structurally altered, there are also different types of DNA-repair mechanisms that organisms have developed during the course of evolution. A detailed discussion of these mechanisms is beyond the

Table 2. Approximate frequencies of occurrence of various DNA damage in mammalian cells*

Type of damage	Events per day/cell	Reference no.
Depurination	10,000	34
Depyrimidination	500	35
Deamination	100–300	36
Base damages (including all types of base damage, viz. oxidative damage, adduct formation with reducing sugars, methylation, crosslinks, and so forth)	10,000	27
Single-strand breaks	20000–40000	37
Interstrand crosslinks	8	10
(Double-strand breaks	9	10
DNA-protein crosslinks	Unknown	10

*The rates are calculated on the basis of spontaneous (endogenous) damageing events and therefore could be actually much higher depending on the dietary composition and style of living.

scope of this article. Several comprehensive reviews on the subject have appeared. In brief, DNA repair can be divided into two classes: those that simply reverse the damage without involving the breakage of phophodiester bond (for example, monomerization of pyrimidine dimers by light, removal of methyl groups and simple rejoining of strand breaks). The other and perhaps a major category of DNA-repair process is nucleotide and base excision repair including the mismatch repair. Except for the initial differences, these two processes actually converge to become a common pathway involving the removal of the damaged portion of DNA and resynthesis of that patch of DNA (Figure 2). However, it is also known that the base excision repair involves a short patch resynthesis, while in the nucleotide excision repair the resynthesized patch is a long one. There is yet a third type of DNA repair, the recombinational repair, the details of which are less understood.

The first step in nucleotide excision repair pathway seems to be the recognition and incision of the damaged or altered site by an incision endonuclease (Figure 2). From the plethora of endonucleases found in different organisms, it appears that these enzymes have varied specificity[16]. The second step is the excision of the damaged region, which may include some adjacent nucleotides as well, by an exonuclease. However, in the case of UV-induced damage the incision seems to be precisely at 6 bases 3' to the damage and 22 bases 5' to the damage, thus releasing a 29 nucleotide fragment[17]. The third step involves the filling up of the gap by a DNA-polymerase using the other strand as template and finally the sealing of the gap by DNA-ligase. In the case of base excision repair, firstly the baseless site formed either spontaneously or by the action of a DNA-glycosylase would be recognized by an apurinic/apyrimidinic endonuclease. This is followed by the

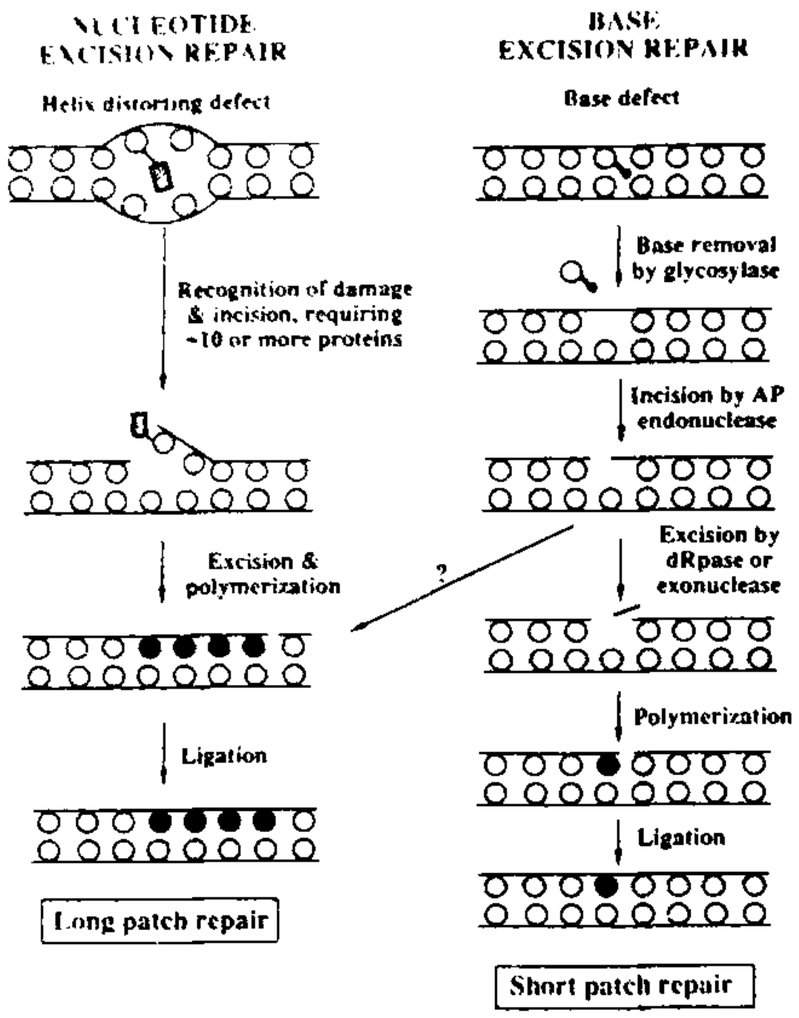

Figure 2. Pathways of excision repair: On the left and right sides are the nucleotide and base excision repair pathways, respectively. This diagram is courtesy of Larry H Thompson, Lawrence Livermore National Laboratory, Livermore, CA, USA.

action of a deoxyphosphodiesterase to create a single nucleotide gap[18]. The subsequent steps are similar to those of nucleotide excision repair.

DNA damage can accumulate in ageing cells for the following reasons: 1) The frequency of events damageing the DNA may be higher than that of the DNA-repair capacity. 2) The DNA repair efficiency may be decreased in an age-dependent and programmed manner, leading to an inadequate repair of the damage. 3) A combination of both 1 and 2. As already mentioned, one of the major forms of DNA damage is a single strand break while double strand breaks seem to accumulate at a much lesser rate probably because double strand breaks beyond a critical level could be extremely lethal and such cells would have died and got eliminated from the system. The result of these strand breaks would be a fragmented DNA which can be detected with reasonable ease by agarose gel electrophoresis.

There is convincing evidence today to indicate that DNA repair capacity does decrease with advancement of age[15,19]. In our laboratory, the usefulness of isolated neurons from the cerebral cortex of the rat brain was examined as a model system to study DNA repair without resorting to the use of hydroxyurea to inhibit the possible replicative DNA synthesis that might be going on simultaneously in a given cell system[20]. The results indicated that neurons obtained from adult (6 months) and old (> 540 days) animals offer a good model system to measure the UV-induced unscheduled DNA synthesis (UDS) without any interference of DNA-replicative

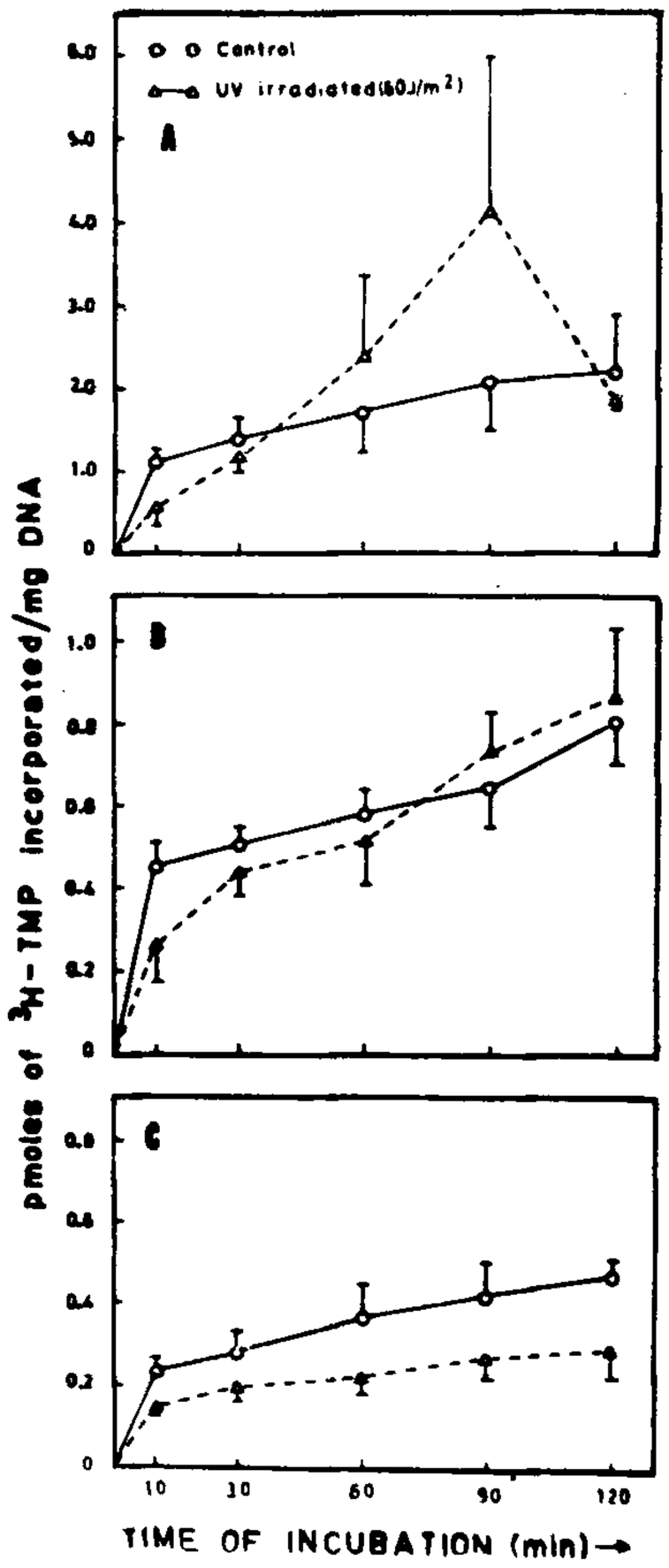

Figure 3. Time course of the incorporation of ^{3}H-TMP into DNA of nuclei of cerebral cortex of rats of different ages: effect of UV irradiation (254 nm). *a*, young (10 days), *b*, adult (6 months), *c*, old (> 2 years). For other details see text and ref. 21 from which the figure is reproduced.

Table 3. UV-induced unscheduled DNA synthesis in neurons of rat brain at different ages

Age	Control	20 J/m^2	Ratio UV/control	40 J/m^2	Ratio UV/control
Young (1 day)	61 ± 2 (8)	71 ± 6 (8)	1.16	91 ± 21 (3)	1.49
Adult (6 months)	28 ± 3 (8)	35 ± 2 (8)	1.25	33 ± 2 (4)	1.18
Old (> 540 days)	29 ± 3 (8)	30 ± 4 (8)	1.03	34 ± 3 (3)	1.17

Values are expressed as mean of DPM × 10^{-3} of ^{3}H-thymidine incorporated/mg DNA ± SD. Number in parentheses indicates the number of experiments carried out. For other details see ref. 20 from which data are reproduced.

synthesis. These investigations revealed that while the spontaneous UDS in old neurons remains unchanged as compared to that of the adult, the response of ageing neurons, in contrast to the young and adult neurons or splenic lymphocytes of any age, to a mutagenic challenge like UV light is markedly limited (Table 3). It is suggested that, it is this lack of responsiveness of DNA repair against a given damage that leads to general metabolic deterioration and senescence. The effect of UV irradiation on the DNA repair/synthesis along with RNA synthesis in nuclei isolated from cerebral cortex of rats of different ages was also studied[21]. Time course studies revealed a temporal relationship between the recoveries of DNA repair and RNA synthesis, overcoming the initial inhibition due to UV, in young nuclei. However, no such correlation could be seen in adult and old nuclei in which DNA repair recovery was very low (Figure 3). These results also demonstrate an age-dependent decrease in DNA-repair potential.

Hence measurement of the end point of either decreased DNA-repair capacity or increased DNA-damage may be used as a molecular pointer for the process of ageing. For example, a precise net end point of the above two processes would be the number of single strand breaks and double strand breaks at any given point of time during the lifespan of any given species. These end points should reflect on the status of the genomic stability or otherwise. We have, therefore, made attempts to assess the number of single and double strand breaks in the rat neuronal DNA at different ages[22].

Two biochemical strategies were adopted by us to assess the single and double strand breaks in neuronal DNA. After isolation, the cells were permeabilized and subjected to a nick translation type of incubation using *E. coli* DNA polymerase I with one of the four dNTPs used in the incubation medium being radioactive. Since *E. coli* pol I is known to add nucleotides at the 3′-OH of a single strand break using the other strand as template[23], the amount of radioactivity incorporated into DNA would be proportional to the number of single strand breaks in the DNA provided the number of nucleotides added at each 3′-OH group are not too variable. Under the standardized conditions, it has been found that about 1500 nucleotides are added at each of 3′-OH group representing a single intrastrand break.

In a similar fashion the property of terminal transferase enzyme to add deoxynucleotides to the 3′-termini of DNA without any need for template direction and the enzyme's preference for 3′-termini of duplex DNA[24] was utilized for assessing the double strand breaks in DNA. Under the optimal conditions, about 50 nucleotides are added at each 3′-termini of a blunt end. From this the number of double strand breaks in the DNA of permeabilized cells can be calculated from the amount of radioactive nucleotides incorporated. The strategy used in this technique is depicted in Figure 4. Since each

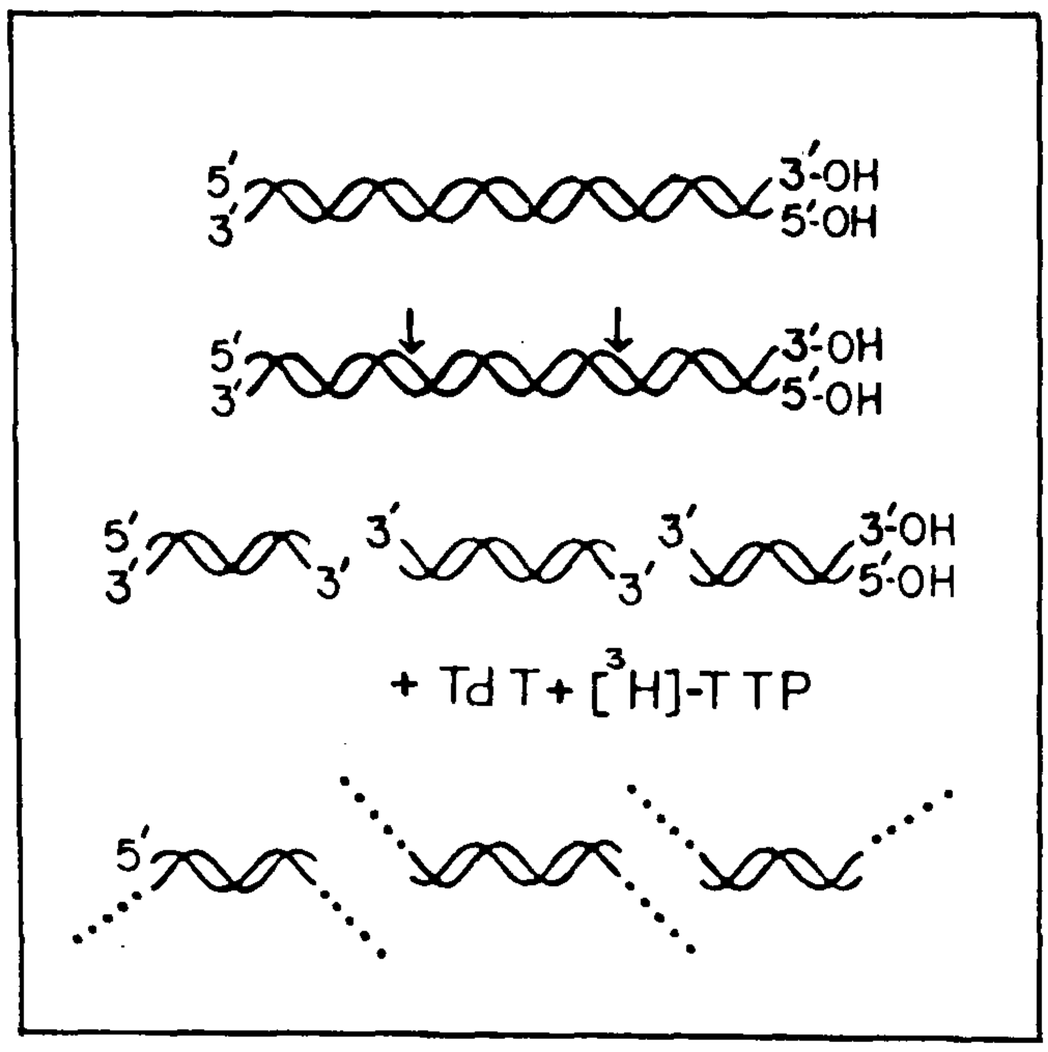

Figure 4. Strategy for calculating the number of double strand breaks (DSB) present in genomic DNA using terminal transferase activity. At the top is shown an intact double-stranded linear DNA molecule. Such a molecule would have two terminal 3′-OH groups. Supposing that two DSB occurred in that molecule as shown by arrows, the result would be three pieces of double-stranded DNA with the generation of four more terminal 3′-OH groups. The number of such terminal 3′-OH ends can be assessed by labelling those ends with a radioactive nucleotide using terminal transferase assay[24]. As can be seen, the DSB that are present in the DNA sample would be equivalent to half the number of terminal 3′-OH groups minus one.

neuron has 6 picograms of DNA, one can actually calculate the number of single and double strand breaks in each cell using this strategy.

Figure 5 shows the mobility pattern of DNA isolated from brain cells of different ages when subjected to agarose gel electrophoresis under neutral and alkaline conditions. The mobility profile on the neutral gel is expected to show the extent of double strand breaks while that on the alkaline gel gives an idea about total fragmentation including both single and double strand breaks. It is clear, although only qualitative in nature, that in rat brain neuron both double strand and single strand breaks increase with age.

Data regarding the precise number of single and double strand breaks as assessed by the two biochemical strategies mentioned above are shown in Table 4. Two points are clearly indicated from these data. Firstly, both single and double strand breaks accumulate in neuronal DNA with age. There are about 7,400 single strand breaks in the DNA of old neuron as against 3000 in young neuron. Similarly, the number of double-strand breaks in old neuronal DNA was 600, whereas young neuronal DNA contained only 156. What is more important, however, is the susceptibility of genomic DNA to MNNG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, an

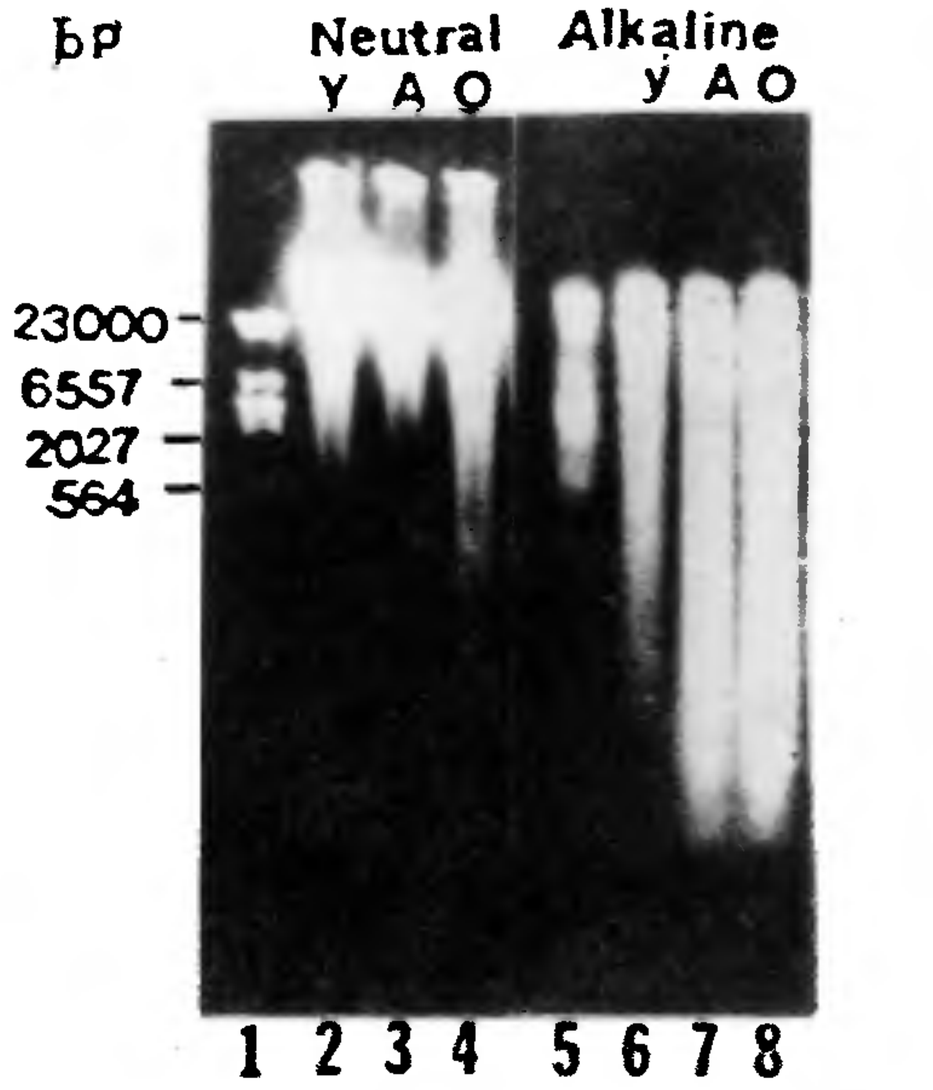

Figure 5. Alkaline and neutral 0.5% agarose gel electrophoresis for observation of neuronal DNA fragmentation due to SSB and DSB. The bands were visualized by ethidium bromide staining. Lane 1 (from left): Lambda DNA *Hin*dIII digest. Lanes 2–4: Young, adult and old neuronal DNA. Lane 5: Same DNA standard as in Lane 1. Lanes 6–8: Young, adult and old neuronal DNA run under alkaline conditions. Neutral gels were run in 0.5 × tris-borate-EDTA buffer at 30 volts for 4 h while alkaline gels were run in a buffer consisting of 30 mM NaOH and 2 mM EDTA.

alkylating mutagenic compound) and higher concentration of glutamate (excitotoxic). When the cells were treated with either of these compounds prior to the estimation of strand breaks, there was significant increase in the strand breaks. Further, the absolute increase in the number of strand breaks, either single or double (Table 4) was much higher in old and adult neurons as compared to the young. For example, glutamate treatment for 18 h resulted in the addition of 1680 double-strand breaks in old neuronal DNA (Table 4) while the same treatment resulted in 924 breaks in young neuronal DNA. In other words, these two substances were able to induce further fragmentation of DNA irrespective of the age of the neuron but it was apparent that genomic DNA from old neurons was more susceptible.

The strategy adopted for measuring single strand breaks was also used to assess the number of apurinic/apyrimidinic (AP) and alkali labile sites in neuronal DNA at different ages. The DNA isolated from neurons was subjected to nick translation type of incubation and the single strand breaks were calculated from the incorporation of radioactive nucleotides as in the case of permeabilized cells. The results are shown in Table 5.

Table 4. Estimation of single (SSB) and double (DSB) strand breaks in permeabilized neurons of different ages by Nick translation and terminal transferase assays

	Control	MNNG (50 μM)	Glutamate (1 mM)
Single strand breaks			
Young	3.0	5.4	14.0
Viability (%)	92	28	25
Adult	4.6	15.0	24.0
Viability (%)	94	24	28
Old	4.6	19.0	25.0
Viability (%)	90	26	25
Double strand breaks			
Young	156	360	1080
Viability (%)	92	28	25
Adult	276	960	1680
Viability	94	24	28
Old	600	2700	2280
Viability	90	26	25

Values of SSB are per neuronal cell ($\times 10^3$), while in the case of DSB they are the actual number per neuronal cell. Viability was judged by trypan blue exclusion test. Young, 4 days old; adult, 6 months; old > 2 years. For other details see ref. 22 from which data are taken.

Table 5. Estimation of apurinic/alkali labile sites in neuronal DNA of ageing rat brain

	Young	Adult	Old
Control	2.4	3.6	4.7
Alkali-treated DNA	2.8	3.8	5.4
Depurinated and alkali-treated DNA	7.4	9.4	11.6

The values are number of SSB in thousands per neuron. Isolated DNA was subjected to nick translation assay with and without prior to alkali treatment alone or combined with depurination. Alkali treatment of DNA was done by mixing the DNA samples with one tenth volume of 1 N NaOH and incubated for 20 min at 37°C. Depurination was achieved by adding equal volume of 10 mM sodium citrate buffer, pH 4.5 to the DNA sample and then heating at 70°C for 10 min.

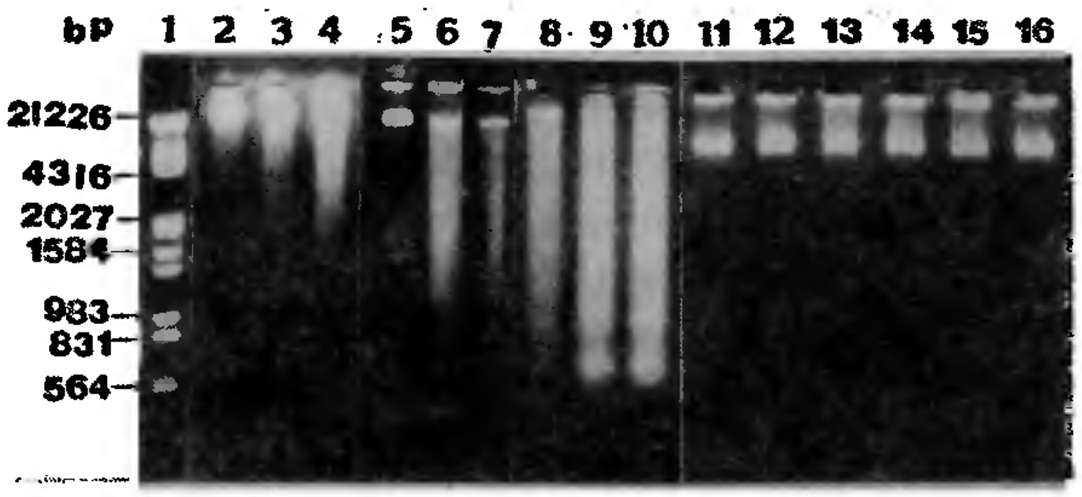

Figure 6. DNA fragmentation in ageing neurons. Lanes 2–4: DNA from young, adult and old neurons. Lanes 5–7 and 8–10: DNA from young, adult and old neurons treated with 1 mM glutamate for 6 and 18 h, respectively. Lanes 11–16: DNA from young, adult and old neurons incubated with glutamate for 18 h along with 1 μg/ml cycloheximide (11–13) or 100 μM aurin tricarboxylic acid (14–16). Figure is taken from ref. 22.

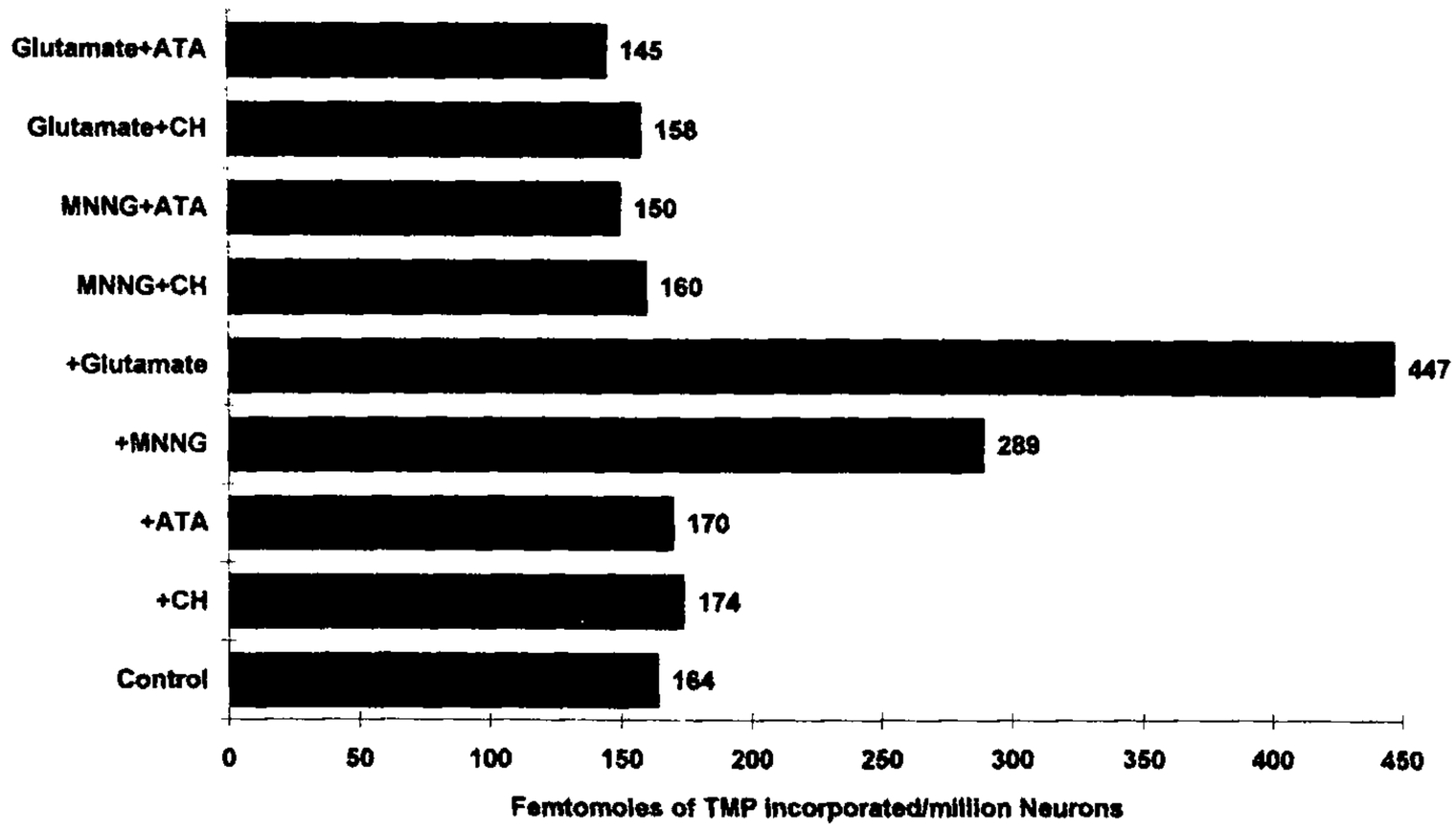

Figure 7. Effect of cycloheximide and aurin tricarboxylic acid on biochemical assessment of DSB by terminal transferase assay in permeabilized neurons of old brain. Values represent femtomoles of ^{3}H-TMP incorporated/million cells. For other details see ref. 22 from which the figure is reproduced.

DNA isolated from the cortical neurons of the brain of young adult and old rats showed increasing single strand breaks with age as observed in earlier experiments. When the isolated DNA was alkali treated, the number of single strand breaks further increased in all the three ages, indicating the conversion of alkali labile sites into single strand breaks. This also increased with age. If the DNA was first depurinated and then alkali treated, there was further enhancement in the single strand breaks, with the trend of increasing numbers with age remaining the same. Thus, these experiments once again demonstrated that both the alkali labile sites and AP sites increase in neuronal DNA with increasing age.

The DNA fragmentation noticed in the neurons of 'normally' ageing rats and further enhancement of this fragmentation by MNNG and glutamate could be due to the activation of apoptosis, the suicidal programme involving gene expression and protein synthesis[25], leading to cell death. This hypothesis was tested in our laboratory by subjecting the DNA isolated from lysed cells to 1.5% agarose electrophoresis. The mobility patterns of DNA are shown in Figure 6. Some degradation of DNA is seen even in untreated cells and as expected, this was higher in DNA of old neurons. If the cells are treated with 1 mM glutamate for 18 h prior to the lysing of the cells and electrophoresis of the DNA fragments, a typical nucleosomal DNA ladder, characteristic of apoptosis is seen. That glutamate induces apoptosis is also borne out by the fact that when cells are treated simultaneously with either cycloheximide (a protein synthesis inhibitor) or with aurin tricarboxylic acid (a nuclease inhibitor) then no nucleosomal DNA ladder is seen (Figure 6). Similar results were obtained with 50 μM MNNG. These results were further substantiated by terminal transferase assay in which the incorporation of radioactive nucleotides into DNA of permeabilized cells was followed in a similar set of experiments (Figure 7). Furthermore, morphological observation of cells stained with acridine orange after treatment with glutamate or MNNG showed a higher uptake of the dye indicating hypercondensation of the chromatin, once again a characteristic feature of apoptosis. These results are taken to indicate that both glutamate and MNNG induce apoptosis in neurons irrespective of the age of neurons and that in the neurons of 'normally' ageing rats, the DNA fragmentation noticed may not be due to apoptosis[22]. While it is possible that apoptosis may come into operation when DNA damage reaches a critical level, apoptosis *per se* does not seem to be the cause of DNA fragmentation observed in the neurons of 'normally' ageing rats.

Regional distribution of strand breaks in ageing rat brain

The single and double strand breaks were also assessed in DNA of neurons isolated from different regions of the brain of rats of different ages[26]. The data are shown in Figures 8 and 9. A steady increase in both types of breaks was observed with advancement of age in all the brain regions studied. However, the number of SSB encountered in the cerebral cortex was the highest as compared to those of other regions. Among the other regions, hippocampus showed higher number of breaks. With respect to double strand breaks, however, all the regions studied showed equal number of double strand breaks and the age-dependent increase was clearly seen. Thus, cerebral cortex and hippocampus appear to be the most vulnerable regions for accumulating SSB with age, while for DSB, all the regions are equally vulnerable[26].

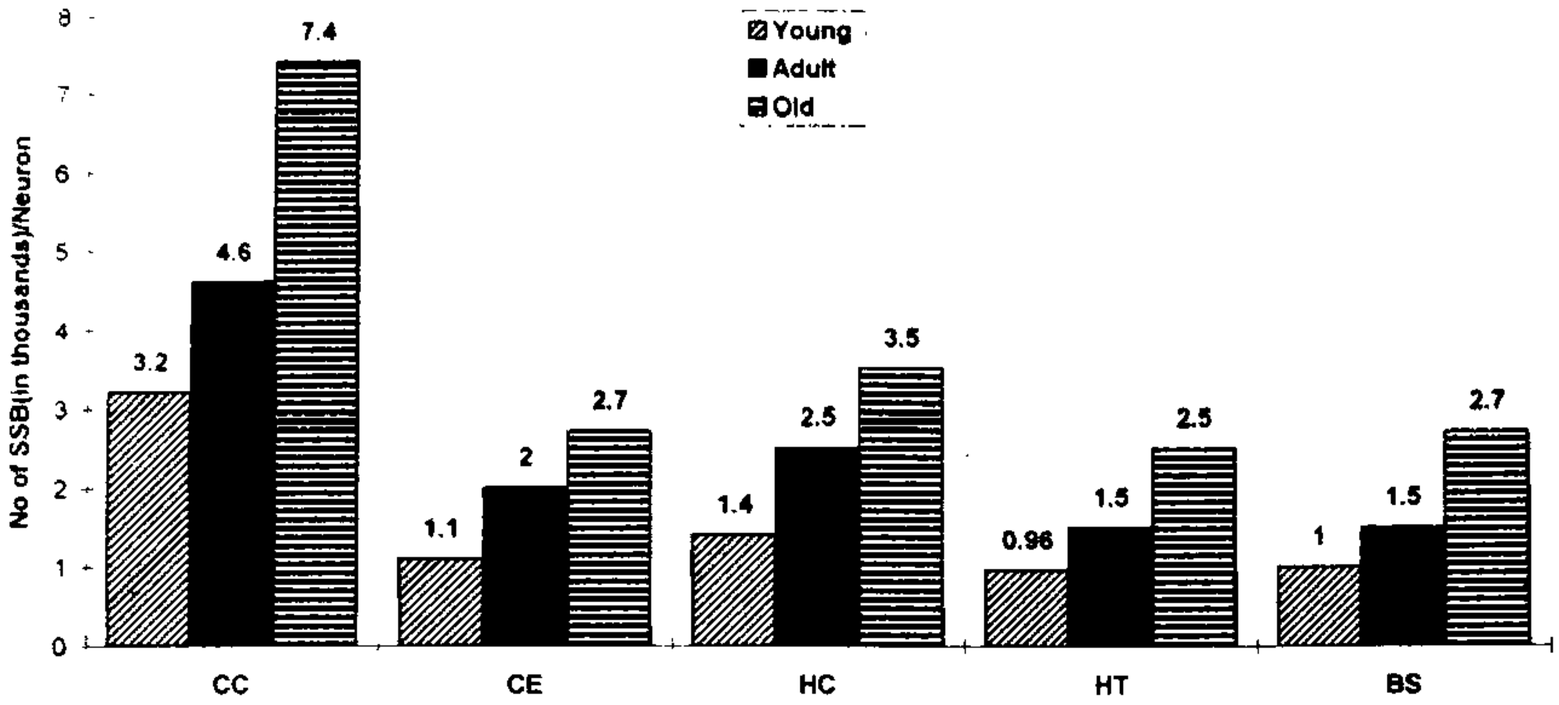

Figure 8. DNA single-strand breaks per neuron isolated from different regions of the brains of young, adult and aged rats. Values are in thousands. CC, cerebral cortex; CE, cerebellum; HC, hippocampus; HT, hypothalamus; BS, brain stem. For other details see Table 4 and ref. 26 from which the figure is taken.

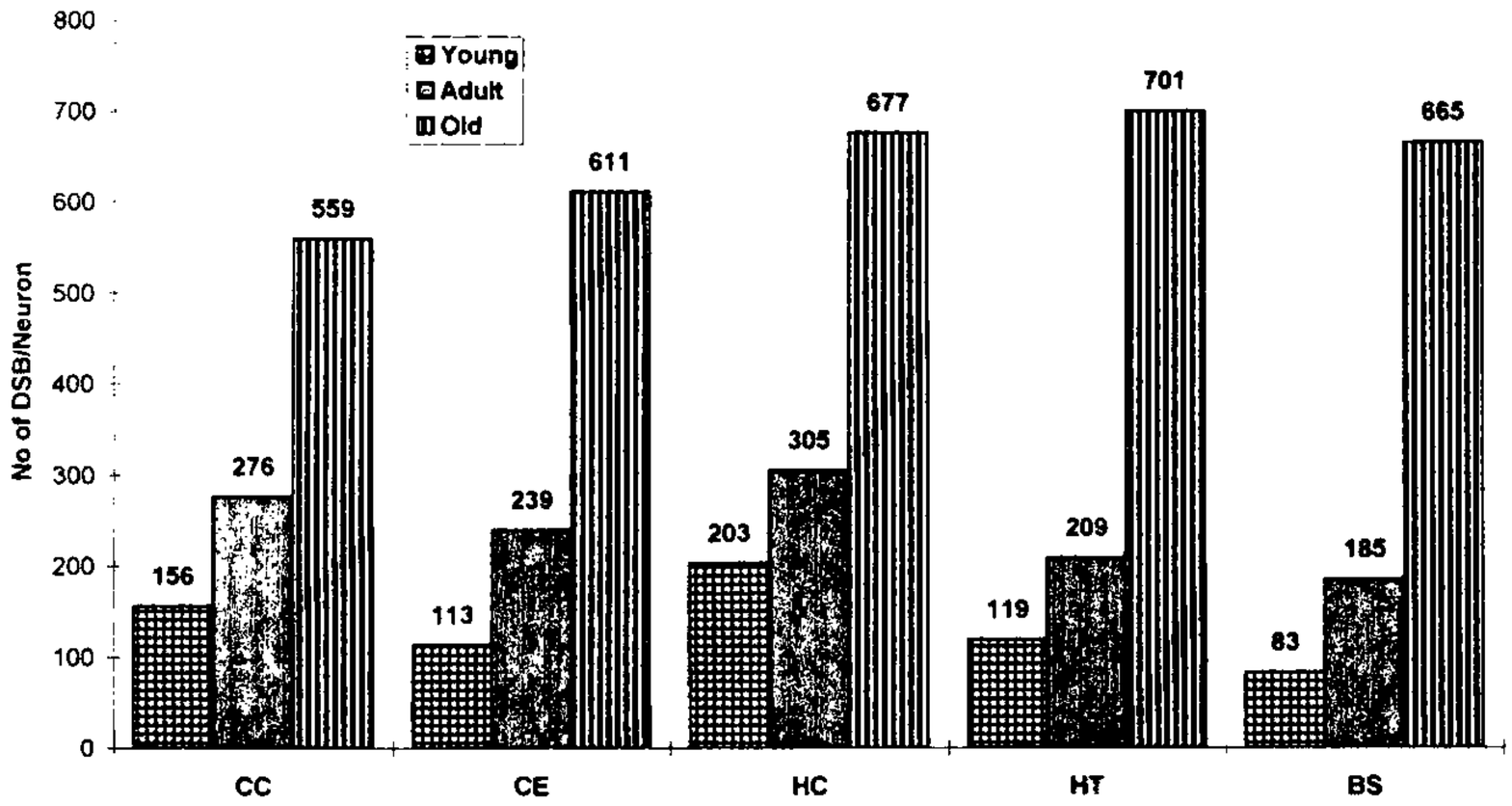

Figure 9. Number of DNA double-strand breaks per neuron isolated from different regions of young, adult and old rat brain. For details see Figure 8, Table 4 and ref. 26 from which this figure is taken.

Ever since the DNA-damage theory was originally conceived and supported subsequently, voluminous literature has accumulated to suggest correlative evidence that chromosomal aberrations and DNA damage increase with age[6,10,15]. Mitochondrial DNA (mtDNA), like the nuclear DNA, is also shown to be subjected to damage and in fact, mtDNA is found to be more extensively damaged during ageing than the nuclear DNA[27]. This is primarily of oxidative type resulting in high levels of 8-OH dG[28]. In another study, Linnane and coworkers[29] have shown a common deletion in mtDNA which is more prevalent in tissues of older people.

Considerable evidence is available to indicate that DNA-repair capacity decreases with age which may atleast in part account for the accumulated DNA damage. In a recent investigation, Wei *et al.*[19] demonstrated a steady decrease in UV-damaged DNA-repair in human peripheral lymphocytes with age. Repair of UV damage in telomere regions has also been shown to decrease with age in human fibroblasts[30]. DNA-repair deficiency is also seen in human syndromes like Cockayne syndrome, Werner syndrome and Down syndrome which exhibit symptoms of accelerated ageing[9,31,32]. Indeed the gene responsible for Werner's syndrome has been cloned recently and the protein is found to have a DNA helicase activity[33].

Work done in this laboratory over the years, a part of which is reviewed here, substantiates the DNA-damage and DNA-repair theory of ageing particularly with respect to a post-mitotic cell like neuron. One of the most important end-points of either increased damage or decreased repair is the strand break resulting in fragmented DNA. Since this end-point can be assessed with reasonable accuracy, the strand break accumulation in a

non-dividing cell like neuron may be used as a molecular pointer for the ageing process, and perhaps for the critical point of damage at which stage the cell may actually activate its apoptotic phenomenon. Towards this end, the number of double strand breaks, which are more lethal, may be a more useful pointer than the number of single strand breaks.

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