STUDIES ON DNA-REPAIR IN AGING RAT NEURONS

A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

BY

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Studies on DNA-Repair In Aging Rat Neurons"** is entirely original work and was carried out by me in the department of Biochemistry, University of Hyderabad, under the supervision of Prof.K.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.

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on DNA-Repair In Aging Rat Neurons"** submitted by **Mr.T. Harikrishna** to the 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.

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CONTENTS

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List of Abbreviations

AP : Apurinic/Apyrimidinic

APE1 : AP endonuclease 1

AD : Alzheimer's disease

AT : Ataxia telangiectasia

ATP : Adenosine Triphosphate

BER : Base excision repair

BS : Bloom's syndrome

BuAdATP : N2- (p-n-butylanilino)-2'-deoxyadenosine-5'-triphosphate

BuPdGTP : N2- (p-n-butylphenyl)-2'-deoxyguanosine-5'-triphosphate

CAT : Catalase

CS : Cockayne Syndrome

dATP : 2'-deoxyadenosine 5'- triphosphate

dCTP : 2'-deoxycytidine 5'-triphosphate

ddTTP : 2',3'-Dideoxythymidine-5'-triphosphate

dGTP : 2' -deoxyguanosine 5'- triphosphate

DDW : Double distilled water

DMSO : Dimethyl sulphoxide

DNA : Deoxyribonucleic acid

DSB : Double strand break

DRPase : 5'-2-deoxyribose-5- phosphate lyase

DRP : 5'-2-deoxyribose-5- phosphate

DTT : Dthiothreitol

FA : Fanconi's anemia

FEN1 :'flap' structure specific endonuclease-1

GMP : Guanosine-5'-monophosphate

HGP : Hutchinson-Guilford progeria syndrome

HRR : Homologous recombination repair

IR	Ionizing radiation
Kda	Kilodalton
MMR	Mismatch repair
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
PAGE	Polyacrylamide gel electrophoresis
PCNA	Proliferating nuclear antigen
PD	Parkinson's disease
PMSF	Phenylmethyl sulfonyl Flouride
Pols	Polymerases
Pol a	DNA polymerase a(alpha)
Pol β	DNA polymerase P(beta)
Pol 5	DNA polymerase 5(delta)
Pol ε	DNA polymerase ε(epsilon)
Pol γ	DNA polymerase γ(gamma)
POPOP	2,2'-p–Phenylene-bis [5-henyloxazole]
PPO	2,5-Diphenyl-1, 3-Oxazole
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SSB	Single strand break
TCR	Transcription coupled repair
TTD	Trichothiodystrophy
TTP	thymidine 5'-triphosphate
UDS	Unscheduled DNA synthesis
WS	Werner syndrome
XP	Xeroderma pigmentosum
XRCC1	X-ray repair cross-complementing gene I

CHAPTER 1

INTRODUCTION

INTRODUCTION

Aging can be defined as the time related deterioration of physiological functions necessary for survival and fertility. Although usually assumed to start after maturity, most of the signs of the aging are not obvious until later in life. Aging changes are manifest at all levels of organization -molecular to organismic level and changes occurring after attaining reproductive maturity comprise the phenomenon of aging or senescence. However, fundamental molecular mechanisms involved in aging remains controversial and largely unproven and the major reason for this is the obvious complexity of the problem.

The average life span of humans has increased dramatically over the time, yet the maximum life span potential has remained approximately constant and is usually stated to be 90-100 years (Cutler, 1990). Benjamin Gompertz (1825) in the early 19th century first described an exponential increase in mortality with aging due to various causes, a phenomenon that is still seen today (Gompertz, 1825). The rapid advances in the medicine and biology has enabled control of infectious diseases responsible for the majority of deaths. Due to this progress in medicine and health care practice, average life span of humans has been on the increase during the past 100 years.

Powerful tools of molecular biology are now being applied by scientists to evaluate the leading hypotheses explaining aging process. This kind of work provides the scientific foundation to enhance the quality of life for people suffering the failings of age (Cristofalo, 1994)

First evolutionary arguments to explain the phenomenon of aging was given by August Weismann (1834–1914), the great German theorist and experimental biologist of the 19th century. His initial idea was that there exists a specific death-mechanism designed by natural selection to eliminate the old and worn-out members of a population. The purpose of

this programmed death of the old is to clean up the living space and to free up resources for younger generations.

THEORIES OF AGING

Many theories about the cause(s) of aging have been proposed over the years in an effort to adequately explain the phenotype of aged organisms. Modern technology and research techniques have allowed the researchers to test many of these theories of aging. Some theories have been proven to be related to aging where as others show high probability or possibility of affecting the aging, while others have been disproven. No single theory explains the various biochemical changes occurring during the process (Rattan and Clark, 1988; Gensler and Bernstein, 1981). In some cases, emphasis is given to some of the genetic factors as determinants of the process, while others give importance to random accumulation of damage such as that caused by mutations, errors, free radicals, in addition to genetic component (Smith, 1962; Warner, 1987; Rattan and Clark, 1988; Finch, 1990; Bernstein and Bernstein, 1991; Rao, 1993; Kanungo, 1994).

Every single hypothesis formulated on scientific basis, has its validity. There are programmed processes that contribute to the process of aging, and of course, the errors occurring during the lifetime of a cell speed up the process of aging.

Attempts have also been made to divide various theories of aging into two or three categories. Today such characterization has lost it's credibility since many of the theories, therefore the categories are not mutually exclusive. Only the emphasis on a particular aspect might vary. Therefore, a brief description of various theories/concepts/hypotheses to explain the possible mechanisms of aging phenomenon, is given below without any regard to the characterization since many of these concepts overlap with each other.

SOMATIC MUTATION THEORY

Somatic mutation theory is the most prominent among the theories that lay emphasis on stochastic factors and was proposed by Szilard (1959) which states that the accumulation of mutations during aging would result in decreased protein function thereby compromising the ability of cells to perform their respective function and ultimately to cell death. This theory arose from the observations that a sublethal dose of radiation shortens the lifespan of mammals.

Clark and Rubin, (1961) *in vivo* and Hoehn et al., (1975) *in vitro* showed that the effects of ionizing radiation on life shortening may be non specific and may occur due to radiation syndrome which is unrelated to natural aging. Post mitotic cell suffers from the validity of this theory as there is no means of measuring the range of mutations in these cells and the only way it has been measured is evaluating the mortality rate, which may be due to several other factors. The drawbacks of somatic mutation theory is that it cannot explain the ·nsitivity of germ cells of long lived species such as humans to ionizing radiation than those of mice and drosophila. Sinex, (1974), Strehler, (1964) and Walburg et al., (1966) have shown in Drosophila and mice respectively that acceleration of aging after exposure to ionizing radiation may be due to secondary effects, although at higher doses, the effects of mortality are evident.

ERROR CATASTROPHE

The error catastrophe theory proposes that random errors occur eventually in protein synthesizing machinery, that synthesize DNA or other template molecules and was proposed first by Orgel (1963). Errors occurring in proteins are lost by natural turnover and simply replaced with error free molecules. Errors in protein synthesizing machinery that introduce errors in molecules could result in rapid accumulation of error containing

molecules that would result in "error catastrophe" that would be incompatible with normal function and life. Studies by Linn et al., (1976); Murray and Holliday (1981); Krauss and Linn, (1982) have shown that various DNA Polymerases isolated from fibroblasts aging in culture are less faithful in copying synthetic polynucleotides than the polymerases isolated from early passage cells.

Contrary to this occurrence of errors in proteins that may cause aging, there are studies showing no changes in the primary structure of enzymes as a function of age (Kanungo and Gandhi, 1972; Patnaik and Kanungo, 1976; Srivastava, 1971). Although there are numerous reports of altered proteins in aging, no direct evidence of age dependent protein mis-synthesis has yet been reported. The altered proteins that occur in aging cells and tissues are supposed to be due to post-translational modifications such as oxidation and glycation (Kristal, 1992; Levin and Stadtman, 1996). The increase in altered proteins appear to be due to decreased clearance in older cells (Gracy et al., 1985).

CODON RESTRICTION THEORY

Fidelity and/or accuracy of mRNA message translation is impaired with aging due to cells inability to decode the triplet codons (bases) in mRNA molecules (Strehler et al., 1971). Degenerative tRNAs are the decoding molecules of the genetic code and the qualitative changes in the iso-acceptor tRNAs of amino acids may alter the rate of decoding of the message and this in turn affects translation.

This theory however does not explain the factors responsible for altering the gene expression for tRNAs, sythetases and those responsible for the post-translational modifications of tRNAs. The fundamental cause of aging remains elusive, as these changes are secondary in nature.

FREE RADICAL THEORY

The Free radical theory was initially proposed by Harman (1956,1981) which says that most aging changes are due to damage to the molecules by free radicals, which are atoms or molecules that contain an unpaired electron and are therefore highly reactive. Accumulation of age pigment-Lipofuscin with age due to damage directly affecting the Lipofuscin (Miquel et al., 1977) is often cited as supporting example for free radical theory of aging.

The production of free radicals is an unavoidable consequence of aerobic metabolism and the prominent members of Reactive oxygen species (ROS) metabolism such as catalase (CAT) and superoxide dismutase (SOD) protect cells against hydrogen peroxide and superoxide radicals respectively. ROS cause critical damage to chromosomes, resulting in mutations and translation errors, which is the basis for many cancers. Since most cancers are age related, it is suggested that relationship between cancer and ROS involve free radicals.

A direct test for free radical theory of aging would be extension of lifespan upon administration of antioxidants. Although a number of studies have been done, the results on balance are ambiguous. Comfort (1979); Halliwell and Gutterridge (1989) have shown that extension of average life span by antioxidants is minimal or absent in mammals, where as it has been demonstrated in lower organisms (Drosophila, nematodes, rotifers). Possibility of attempting to prolong life span by administrating antioxidants to complex organisms may fail as they are unable to access the area of ROS damage even in sufficient concentration. In general DNA damage hypothesis and free radical theory are compatible in that the most important target of free radical damage is DNA, and that other oxidatively damaged molecules are efficiently replaced as long as DNA is intact.

GENE REGULATION THEORY

According to "gene regulation theory" of aging, the depletion of certain "nuclear proteins or factors" responsible for keeping essential genes active leads to depressed functions of organs. Differentiation and growth occur by sequential activation and repression of certain genes unique to these phases. The products and byproducts of these genes after reaching critical levels and at specific time stimulate certain unique genes responsible for reproductive phase. Triggering of expression of these genes occurs by some product of genes (like hormones and other factors) that confer reproductive ability to organism. Failure to maintain a balance of factors and modulators results in the loss of reproductive ability and other functions. Thus decline in physiological functions may begin and lead to senescence (Kanungo, 1980).

NEUROENDOCRINE THEORY

Finch (1972) proposed that those functional decrements in neurons and their associated hormones are central to aging process. (McGeer and McGeer, 1975; Finch, 1978) evidenced a decrease in brain catacholamines and the reduction in catecholamine receptors in the corpus striatum and cerebellum of old rats (Greenberg and Weiss, 1978). Denckla (1975) showed that hypophysectomy in rodents followed by replacement of known hormones maintains or may extend lifespan. In addition, reductions in brain dopaminergic neurotransmission are more prominent in a short-lived rat strain (Cotzias et al., 1977). The mechanisms responsible for hormone receptor alterations during aging are unknown. Accumulation of DNA damage in non-dividing cells is likely to lead to reduced/altered gene expression. This may explain both the decreased population of hormone receptors and decline in general neuroendocrine function. This neuroendocrine theory of aging is compatible with the DNA damage hypothesis of aging.

IMMUNOLOGICAL THEORY

Walford (1969) proposed immunological theory based upon two main observations. First the functional capacity of the immune system declines with age, as evidenced by a decreased response of T cells to mitogens and reduced resistance to infectious diseases and increase in autoimmune phenomenon with age, such as increase in serum autoantibodies.

Organisms that share aspects of aging with higher organisms lack a complex immune system. This theory fails to interpret the aspects which distinguish between the immune system and the fundamental changes occurring in many types of cells and tissues and also the secondary effects mediated by aging altered immune system (Troen, 2003).

PROTEIN MODIFICATION

Changes in function are seen in addition to the changes in the steady state level of proteins with age. Aging is accompanied by decreased specific activity in many enzymes, altered heat stability, and increased carbonyl content of proteins (Levin and Stadtman, 1996). Kohn (1978) and Bjorkstein (1974) hypothesized that the accumulation of post translationally altered proteins could impair cellular, and ultimately, organ functions.

Some of such alterations could lead to increased functions at some sites and impaired functions at other sites the example being increased collagen crosslinking with age (Reiser et al., 1987). The cross-linking of macromolecules such as collagen, elastin, osteocalcin, and the eye lens protein crystallin could alter both the extracellular matrix and organ function. These covalent protein-protein interactions probably play a role in increased stiffness of vascular walls with aging (Troen, 2003).

TELOMERES AND AGING

During the past few years a new concept has emerged which again adds credibility to the theory of DNA damage and repair in explaining aging as well as cell replication and transformation of somatic cells into malignant cells. This hypothesis is based on results suggesting the non coding telomeric DNA (located at the tips of the eukaryotic chromosomes) may have a telling role in DNA replication (cell replication) and therefore in the phenomenon of cancer and aging. Telomeres are the physical ends of the chromosomes, which in mammals are composed of tandem repeats of TTAGGG (Meyne et al., 1989) and appear to stabilize the structure of chromosomes. Apart from providing stability to chromosomes, telomeres carry out another crucial function in replicating cells-the ability to allow the end of the linear DNA to be replicated completely without the loss of terminal bases at 5'- end. Such loss is predicted as a natural consequence derived from the properties of the replicative machinery of conventional semiconservative replication (Olovnikov, 1973). Von Zglinciki, (2000) suggested that the most important factor leading to the shortening of telomeres as the oxidative damage. The lost sequences of the telomere at each round of replication are synthesized again by the enzyme telomerase, a ribonucleoprotein (Blackburn, 1992).

Many of the cells in an adult animal are either quiescent or post mitotic and they proliferate rarely or not at all and their telomeres might not shorten significantly during the life of an individual. It is interesting that in certain human syndromes, characterized by features of premature aging viz., Progeria and Werner's syndrome, the average telomeric lengths are significantly shorter than in normal individuals, thus pointing out a relationship between average telomeric length and aging (Lansdorp, 2000).

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 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 upto certain age of an organism but thereafter it 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 the 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., 1979b), in skin cells of humans (Sutherland et al., 1980) and in lens epithelial cells from rat, rabbit, dog, cow, 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 in consistent with the lifespan of mice. Zahn et al.,(2000) showed in two mouse strains that the strain with shorter longevity, the damage increases and the repair deficiencies are drastically

deviating 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 cell populations, atleast 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 opinion of this lab that DNA-damage and repair theory occupies a central role in explaining the mechanisms of 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 the 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 stable molecule DNA. 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 case 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 heritable mutations is 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 only 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 37^0C loses about 10,000 purines and 500 pyrimidines from its DNA by spontaneous hydrolysis (Lindhal, 1977) and it should be promptly removed from the 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 to about 3% of 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 process in 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 -methylguanine, N -methyladenine and O -methylguanine (Barrows and Magee, 1982; Rydberg and Lindahl, 1982). The methylated bases are eventually converted to 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 (H2Q2).

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 and 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 skull. Even so UV induced damage is routinely used as model system with various tissues including brain (Rao, 1997).

Single strand breaks (SSB)

Single strand breaks are the most prevalent DNA-damage in mammalian cells. Single strand breaks may be formed from AP sites at alkaline pH, removal of modified base by suitable glycosylase in the initial step of Base excision repair. UV and ionizing radiations can cause SSB's by generation of free radicals directly or indirectly (Mullart et al., 1990). Single strand breaks 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, DSB and SSB. Important class of chemical modification in DNA is interstrand cross-links since they prevent strand separation needed for replication and also transcriptional process. 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 the endogenous factors and from such exogenous factors that can cross the blood brain barrier.

The frequency of occurrence of different DNA damages by various factors is summarized in the Table I.

TABLE 1: Estimated Rates of Occurrence of Endogenous DNA Damages in Mammalian Cells

Damage	Events per Day/cell	Reference
Depurination	12,000 13,920	Lindahl, 1977 Tice and Setlow, 1985
Depyrimidination	600 696	Lindahl, 1977 Tice and Setlow, 1985
Deamination	100-300	Lindhal and Nyberg, 1974 Tice and Setlow, 1985
Single-strand breaks (Including all types of Base damage Viz.. Oxidative damage, Adduct formation with reducing sugars, methyalation, Cross-links, a d so forth)	20,00-40,000	Saul and Ames, 1985
Double-strand break	8.8	Bernstein and Bernstein, 1991
Interstrand cross-link	8.0	Bernstein and Bernstein, 1991
DNA-protein cross-link	unknown	Bernstein and Bernstein, 1991

Bernstein C and Bernstein H (1981) "Aging, Sex, and DNA repair" Academic press Inc. San Diego, California.

DNA DAMAGE IN BRAIN

There are some studies to look into DNA damage in brain and these studies were conducted to measure 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; Kirkwood and Holliday, 1979; Hayflick, 1980; Gensler and Bernstein, 1981). Price et al., (1971) showed in mice that accumulation of SSB 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 age owing to the breaks. Murthy et al., (1976) observed more single strand regions in preparations with both isolated chromatin and DNA obtained from old cerebral cortex as compared to those from young. Mori and Goto (1982) using single strand specific S1 endonuclease assay showed that younger mice brain DNA contained only 2.0% single strand regions than mice aged 30 months. Interestingly they could not find any such age associated changes in other organs like liver, kidney, heart, and spleen.

Tan et al., (1990) showed that steady state level of 7-methylguanine and 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, (1996) showed that the number of SSBs increase with age in both the cell types and in all the regions studied viz., cerebral cortex, cerebellum, hippocampus, hypothalamus and brain stem. Highest number of SSBs were seen in neurons and astrocytes of cerebral cortex of 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 of DNA damage in brain. Ono et al., 1976; Su et al., 1984; Mullart et al., 1990). The reasons for these discrepancies are not clear as of now.

Alterations with age at the genetic level were also observed by some workers. Studies by Kanungo and Thakur (1979); 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. 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 dramatic increase in the nucleosome spacing of the chromatin. All the above studies with the overwhelming literature point that with the advancement of age there is accumulation of DNA damage in brain.

DNA REPAIR

As a major defense against the environmental damage to cells, DNA-repair is present in almost all the organisms including bacteria, yeast, fish, amphibians, rodents and humans. 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., 2001).

The pathways involved in 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., 1991; Friedberg et al., 1995) There are three major DNA repair pathways to counteract the different types of DNA damages. 1) A simple reversal of damage 2) Recombinant repair including the endjoining 3) Excision repair including mismatch repair.

1) A simple reversal of damage

Direct reversal of the 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 formation 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 enzyme O^6-methyl guanine methyltransferase which removes methyl groups from O^6 -methyl guanine thus avoiding the mismatch formation since O^6-methyl guanine can pair with both C or T (Mitra and Kaina, 1993). In these modes of repair there is no cleavage of DNA strand but simply structural alterations are reversed *in situ* .

2) Recombinant type of repair

Both DNA double strand breaks 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 homologous recombination repair (HRR) or non-homologous end joining (NHEJ) the latter mode being less accurate.

3) Excision repair including mismatch repair

Excision repair pathway is the most predominant and perhaps universal one to maintain the genomic integrity. Essentially, the overall strategy in 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 constitutes 2 major subpathways-nucleotide excision repair (NER) and base excision repair (BER). The mismatch repair is generally considered as a part of NER.

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 cope with 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). Examples for NER are damage due to exposure to UV irradiation, adduct formation with a variety of compounds like cisplatin, psolaren, carcinogens like acetlyaminoflourine etc. Mechanisms of many of the steps of NER in eukaryotic cells is less known. The available evidence suggests that the overall process resembles that in *E. coli*, but there are many differences in detail.

NER differs from the BER in that excision patch is quite long in NER when compared to shorter patch in BER. For example, in the case of UV induced damage the 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, the NER is considered as 'long patch repair' while the BER is routinely considered as 'short patch repair'.

DNA mismatch repair (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. Both confer the genome a 100–1000 fold protection against mutations arising during DNA replication (Loeb, 1994), and both systems scan and repair 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 mismatch repair segregate with certain cancer predisposition syndromes hig. ights its essential role in mutation avoidance. Mutations in one of the human DNA MMR genes, hMSH-2, 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 in proliferative portions of oesophageal and intestinal epithelium (Leach et al., 1996; Wilson et al., 1995; Marra et al., 1996) and increases atleast 12 fold in proliferating cells (Marra et al., 1996).

Base excision repair (BER)

BER pathway consists essentially of 4 steps and can be divided into two sub pathways one concerned with 'short patch or single nucleotide replacing pathway' and the other 'long patch pathway' involving the insertion of upto 13 nucleotides (Fig 1). Step one of short patch pathway (left panel of Fig 1) consists of the recognition and cleavage of the altered base (A) from the deoxyribose phosphate moiety by an appropriate DNA-glycosylase. This enzyme also allows the AP endonuclease (APE1) to reach the site. (Fig. 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-oxy guanine 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 (Chakravarthi et al, 1991; Samson et al., 1991).

In the second step (Fig 1.2) DNA chain at 5'-side of the abasic site is cleaved by a major endonuclease APE1 specific for abasic site. APE1 is the major endonuclease in humans, also known as HAP1, APEX, 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 1st step, this enzyme, still bound to DNA, attracts and interacts with Pol p, which is involved in the next step in the repair pathway. The glycosylase dissociates from DNA at this point.

In third step, the 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 XRCCl and ligase III come off from the site leaving behind repaired DNA (Fig 1 left panel, B).

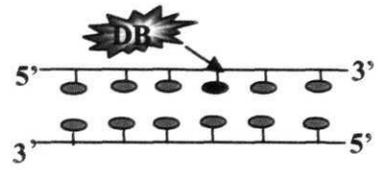
Figure 1

An outline of Pathways of Base Excision Repair (BER): On the left side is "Short patch" or Single nucleotide pathway and on the right side "Long patch" pathway

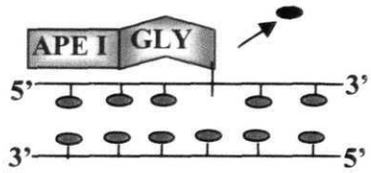
SHORT PATCH REPAIR

LONG PATCH REPAIR

A.



1.



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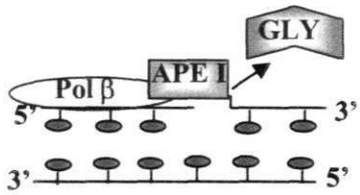

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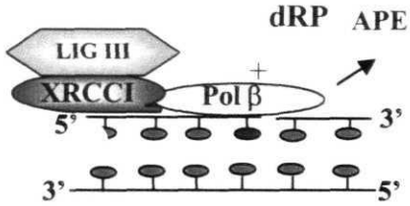

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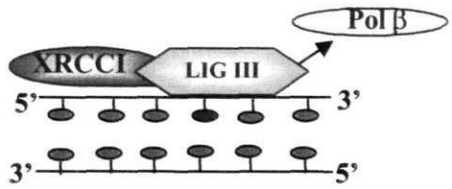
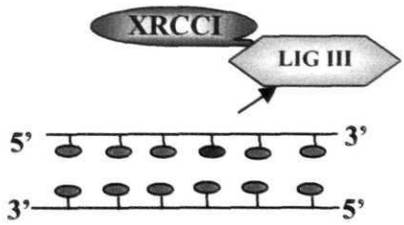
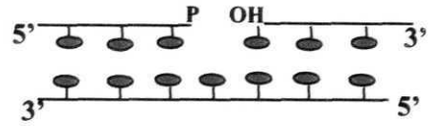


B.



5.



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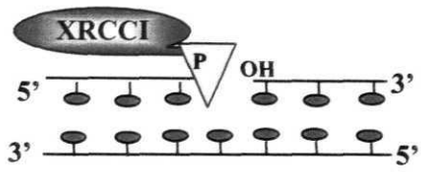
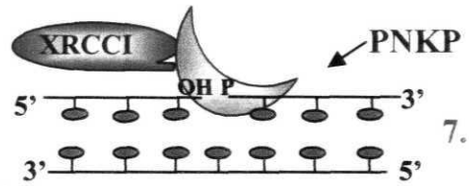


7.



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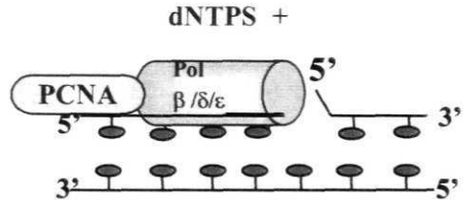

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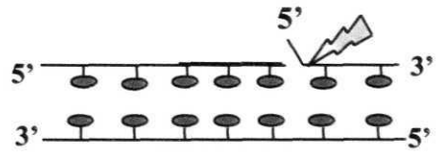
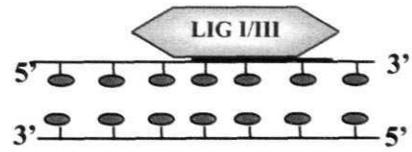


10.



Human XRCC 1 not only complexes with DNA-ligase III but also interacts with other core enzymes involved in BER and is therefore considered 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 closely 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 Fig 1 .The overall process of BER pathway 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 dRpase(5'-2-deoxyribose-5-phosphate lyase) activity of Pol β cannot act on the complex structure of the terminal sugar phosphate after the 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 8 or E, which then conduct strand displacement synthesis (Fortini et al., 1998; Stucki et al., 1998)

This long patch repair requires activator like proliferating nuclear antigen PCNA and a 'flap' structure specific endonuclease-1 (FEN1) activity to cut the flap like structure produced by the strand displacement type synthesis by Pol p (Wu et al., 1996; Klungland and Lindahl, 1997). Nealon et al., 1996 suggested that while Pol β is the major base excision repair 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 the 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; Walker and Bachelord, 1988; Mazzarello et al.,1992; Rao, 1993; Weng and Sirover, 1993).

Alexander (1967) first noticed that DNA-repair system is at a low key once cells are differentiated in to 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 Shultz, (1989) demonstrated through autoradiography, that the 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 damages if they occur at a low rate in these cells. Studies from this laboratory Subba Rao and Rao (1984) showed measurable levels of DNA Polymerase p, which is considered as 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 (Subba Rao and Rao, 1982; Subba Rao, 1990). Hanawalt et al., (1992) showed that not only significant DNA repair process occurs 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/Refl mRNA levels were shown to be particularly high in certain hypothalamic nuclei, as well as hippocampus and cerebellum (Wilson et al., 1996). APE/Refl 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 of fos and jun (Flaherty et al., 2001). Duguid et al., (1995) found that APE/Refl 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-oxo- G 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.

Le Doux 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 either in oligodendrocytes or microglial cells.

Studies have been conducted to examine DNA repair by mitochondria (LeDoux and Wilson, 2001; Bohr, 2001) The studies indicated that astrocytes have higher DNA repair capacity compared to other glial cell types. Chen et al., (2000) detected base excision repair of oxidative damage in mitochondrial extracts from adult rat brain, as well as from cultured cortical neurons and astrocytes. Mandavalli 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 damages to the DNA of brain would be due to endogenous factors and from such exogenous factors which can cross the blood brain barrier. There is ample amount of evidence, that base excision repair pathway would be the main guardian to ensure genomic stability in the highly metabolic organ brain. For example Pol p is the enzyme that takes part in the BER when compared to other polymerases found in the nuclei of the mammalian cells particularly in filling the single nucleotide gap (Wood RD, 1997; Wilson III DM and Thompson, 1997; Fortini et al., 1998). Waser et al., (1979) found that Pol β constitutes 90% DNA Polymerase activity in adult rat brain and it was concluded that Pol β is the repair enzyme. It thus can be expected that genomic maintenance in brain cells be largely taken c .e by Pol β dependent BER pathway. As outlined above, many proteins known to be participating in BER pathway are found in brain also (Rao, 2002). The status of BER in health and disease assumed great importance with the accumulating knowledge of several neurodegenerative diseases that appear in old age and their molecular link to the genomic stability (Martin, 1999; Sniden, 2001).

DNA POLYMERASE β

Replication of DNA is carried by enzymes called DNA Polymerases (Brautigam and Steitz, 1998). To day, the number of DNA Polymerases has increased to atleast 19 since the initial discovery of DNA Pol **a** (DNA-Polymerase a) in Eukaryotic cells in 1957. In the early 1970 Pols p and *y* (DNA Polymerase β and DNA-Polymerase y) were discovered,

leading to the simple concept that Pol a was the enzyme responsible for nuclear DNA replication, Pol β for DNA repair, Pol y for mitochondrial DNA replication. Later in 1980's Pol 8 (DNA-Polymerase δ) and Pol e (DNA-Polymerase e) 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, K, *X*, μ, p etc.,) and these polymerases show the interesting ability of copying the DNA strand with lesions 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 (E.C.2.7.7.7) 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, 1972; Chang, 1973; Matsukage et al., 1974; Wilson, 1990). The Pol β is expressed independent of the cell cycle stage (Zmudzdka et al., 1988), but the regulation of the enzyme is in a tissue specific fashion (Wilson, 1990). Pol β is composed of a single 39kda 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 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 recombinant enzyme is similar to the natural enzyme (Abotts et al., 1988). The recombinant enzyme has the same

template-primer specificity as the natural enzyme and has a reactive epitope for anti-p-Polymerase IgG.

Pol β is folded into distinct domains each associated with a specific functional activity. An 8kda amino terminus domain is connected to the 31kda 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; Casas Finet et al., 1992; Prasad et al., 1993; Prasad et al., 1994; Peirson et al., 1996). The 31 kda domain catalyzes nucleotidyl transferase reaction where as the 8kda 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 Polymerase β in the mammalian AP site BER pathway. Lack of DNA Pol β in DNA Pol β eficient cells or in the presence of neutralizing antibody, a reduction in DNA repair activity is seen which strongly suggests a role for DNA Pol β in BER pathway *in vivo* (Dianov et al., 1999). It was found that Pol β fills up 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 p 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 margin at

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 Kom, 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.

Polymerase p associates with other enzymes of the BER pathway such as DNA ligase I, AP endonucelase, and XRCC1-DNA ligase I. 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 p 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 Bohenhagen, 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 67kda S.cerevisiae homolog of mammalian Pol p encoded by nonessential Pol 4 gene has been implicated in double strand break repair. It probably utilizes a non homologous end-joining 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 the birth The increased apoptotic cell death observed in the developing central and pheriperal nervous system suggest that Pol p 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)

Xeroderma Pigmentosum is a DNA repair disorder related to the NER repair 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 (Hebra and Kaposi, 1984; Jung, 1986; Kraemer et al., 1987; Brougthon et al., 2002). Photosensitivity and the high cancer incidence observed in Xeroderma Pigmentosum patients are due to the defect in the NER pathway and the resulting genomic instability (Cleaver, 1968; Epstein et al., 1970; Day, 1975; Kraemer et al., 1994; Kraemer et al., 1994; Eveno et al., 1995; Brash, 1997; Kraemer et al, 1997). Most cases are symptomatic in childhood, except for an adult variant form. These symptoms include sun sensitivity, photophobia, and, in about 20% of the patients, neurological abnormalities (De Sanctis and Cacchione,1932; Kraemer et al., 1987; Vermeulen et al.,1994). Mutations in eight different genes have been reported in patients with Xeroderma Pigmentosum. These include genes involved in complementation groups XPA -XPG in the NER repair pathway.

The Xeroderma Pigmentosum variants are deficient in the Polymerase η that allows DNA replication through DNA lesions (Wood RD, 1991; Stefanini et al., 1993; Coin et al., 1999; Cleaver, 2000; Broughton et al., 2002).

COCKAYNE SYNDROME (CS)

Cockayne syndrome is a DNA repair disorder related to the transcription- coupled repair (TCR) repair pathway. It is a progressive neurological disorder characterized in infancy by growth failure, deficient neurological development, progressive retinal degeneration, and sensitivity to sunlight (Cockayne, 1936; Cockayne, 1946; Otsuka and Robbins, 1985). One of the hallmarks of Cockayne syndrome is pigmentary degeneration of the retina, first described by Cockayne in 1936. It also occurs in several types, depending upon the gene that is mutated (Patton et al., 1989; Proops et al., 1981). Type I, "the classical type," has an onset in the post natal period, whereas type II, "the severe type," occurs before birth and usually results in death by the age 6 or 7 years (Nance and Berry, 1992). The Unscheduled DNA synthesis (UDS) is normal in these patients, and there is lack of replicative DNA synthesis after the UV damage in CS cells similar to XP Cells (Lehman, 1987).

ATAXIA **TELANGIECTASIA** (AT)

It is also referred to as Louis-Bar syndrome. AT individuals have defective DNA repair to repair damage caused by ionizing radiation and bleomycin. It is an autosomal recessive genetic disorder that affects many systems of the body, particularly nervous system, immune system and skin. AT cells are abnormally sensitive to killing by ionizing radiation (IR). Patients with AT develop progressive ataxia resulting from atrophy of the cerebellum. The rapid degeneration results in many patients dependent upon wheel chairs before their teenage (Stankovic et al., 1998). Symptoms are seen in early childhood with

progressive cerebellar ataxia and later develop conjunctival telangiectases, other progressive neurologic degeneration, sinopulmonary infection, and malignancies. Dilation of blood vessels, in eye and skin (telangiectases) typically develop between 3 and 5 years of age.

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., 1973; Epstein et al., 1974).

WERNER SYNDROME (WS)

Mutations in the RECQL2 gene, encoding for a DNA helicase, are responsible for Werner syndrome. (Gray et al., 1997; Nehlin et al., 2000; Mohaghegh and Hickson, 2001; Shen J and Loeb, 2001). Werner syndrome 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 Werner syndrome 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 single strand breaks 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 fibrobiasts achieve approximately 60 population doublings in culture, while Werner syndrome cells usually achieve only about 20 population doublings (Faragher et al., 1993).

BLOOM'S SYNDROME (BS)

Bloom syndrome 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). Bloom syndrome is characterized by growth deficiency, variable degrees of immunodeficiency, and predisposition to cancers of many sites and types (German, 1995). Patients with this disease show a range of symptoms which include a small body size, sun-sensitive facial reddening, sub- or infertility, immunodeficiency and a predisposition to the full range of human cancers. 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)

Fanconi's anemia 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, hypersensitive

to cis-platinum (Fujiwara et al., 1987), DNA cross linking agents like mitomycin C (Fujiwara et al., 1977).

ALZHEIMER'S **DISEASE** (AD)

AD is a degenerative disorder of the central nervous system in humans. It 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 suggesting reduced repair of some types of DNA damage (Ionizing radiation, DNA damaging alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine etc.,), a decline in gene expression in the brain, and cellular degeneration in a specific region of the brain. Sensitivities of DNA to ionizing radiation was shown from a series of unrelated AD individuals where majority of them showed significant greater sensitivity than cells from age matched control donors (Kidson and Chen, 1986). Scudiero et al., (1986) reported that AD cells showed small but statistically significant hypersensitivity to the DNA damaging alkylating agent MNNG (N-methyl-N'-nitro-N-nitrosoguanidine). At present AD cells were reported to be defective in repair of X-ray damages by some investigators (Kidson and Chen, 1986; Robbins et al., 1983a; Robbins et al., 1985). But there are reports contradicting the above studies (Smith et al., 1987; Smith and Itzhaki, 1989)

PARKINSON'S DISEASE (PD)

PD is characterized by progressive degeneration of the central nervous system in elderly. Both AD and PD are sporadic disorders. In PD, Neurons in the substantia nigra, basal forebrain, and locus ceruleus are progressively lost. Robbins et al., (1983a) 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.

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 (Childs, 1975).The great abundance of human genetic variations raises the possibility that certain mutations will effect genes concerned with longevity. Although we know of no single mutation that lengthens maximum life span, it is apparent that a number of mutations shorten life. Whether or not any of these life shortening mutation reflect 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 Ataxia talengeitasia, Cockaynes syndrome, Werner's Syndrome. Neurodegeneration is seen in Ataxia talengeictasia, Cockaynes syndrome, Xeroderma pigmentosum, Huntingtons disease, Parkinsons disease, Alzheimers disease.

SCOPE OF THE PRESENT STUDY

The present study constitutes the continued efforts from 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 of course on the brain cells.

1. To day several DNA Polymerases performing various functions contributing to the overall DNA-replication and maintenance of its structural integrity are known. Some of these polymerases are discovered in only in recent past and it is likely that this number

might increase in future. However in a post mitotic cell like neuron, there are reports to indicate that Pol p is the only polymerase almost exclusively present. Studies from our laboratory have also revealed that Pol β is the most predominant DNA Polymerase in the rat whole brain. We have now undertaken to examine the DNA Polymerase activities in extracts of neuronal and astroglial cells fractions from the rat cerebral cortex at three different ages. The results showed that the while Pol β is the most predominant DNA Polymerase in these brain cells at all the post-natal ages, some activity attributable to Pols α,δ,ε is also present. These results are presented in the chapter 3.

2. The relationship between DNA repair and phenomenon of brain aging has been subject of study in this laboratory for the past several years and (BER) accounts for the main DNA-repair mechanism in brain cells and Pol β being a main player of that pathway. Earlier results from this lab pointed out that with the advancement of age, not only the levels of Pol p come down but also there is accumulation of the catalytically incompetent Pol β molecules. In order to show that the Pol β dependent DNA-repair is the one that is compromised in brain cells during aging, we have taken up a more *in vivo* relevant functional assay for Pol β activity. We have therefore, chosen a simple and 'easy to work with' model for measuring the Pol β activity in aging neuronal extracts. The results of primer extension activity with age indicate that the activity of Pol β in brain cells is compromised with age and that this deficit can be corrected *in vitro* by addition of pure recombinant rat liver Pol β under appropriate condition. The above studies also indicated the presence of 3'-5'exonuclease activity (proof reading activity) in neuronal extracts which was found to be facilitating the extension activity Pol p particularly with respect to primers with a mismatched base at the 3'-end. We have

carried out the exonuclease activity assays in order to know the status of 3'-5' exonuclease activity in brain cells with advancing age of the animal since this activity could become a constraint for proper functioning of Pol p. These results are presented in chapter 4.

3. It is reported that Pol β acts most efficiently in filling up a gap of single nucleotide and can also fill up upto six nucleotides in a relatively processive manner. Therefore, investigations were continued to examine the gap filling repair activity in aging neuronal extracts with an appropriate model oligo substrate. The results indicate that the gap filling activity is very low in adult and old neuronal extracts. Supplementing these neuronal extracts with recombinant Pol p restored the gap repair activity predominantly by slow distributive strand displacement manner. These results are presented in chapter 5.
4. In chapter 6 all the results presented in earlier chapters have been discussed in the light of the exi ing information.

Objectives of the study

1. To study base excision repair pathway with a synthetic oligo model system in Young, Adult, and Old neurons.

2. To Examine the ways and means to bring back the lost activity (if any) in aging neurons to normal level.

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 5 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, Bovine serum albumin, Adenosine Triphosphate (ATP), Leupeptin, Pepstatin, Phenylmethyl sulfonyl Flouride (PMSF), Dithiothreitol (DTT), Sephadex G-50, Trypsin Type V-S from bovine pancreas, Trypsin inhibitor type II from soyabean, Sigmacote, 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 thymidine 5'- triphosphate(TTP) were purchased from pharmacia Fine chemicals, Uppsala, Sweden. Poly (dA). Oligo (dT) $_{12\text{-}18}$ and Poly (dA.dT) were purchased from Midland Certified Reagent Co. (Midland, TX, U.S.A.). Monoclonal antibody, SJK 132-20 against human DNA Polymerase a was obtained from PL-Biochemicals, Wisconsin, U.S.A. Dimethyl sulphoxide (DMSO) was from Sisco Research Laboratory (Bombay, India). ddTTP was from Boehringer Mannheim, Germany. The nucleotide analogs, N2- (p-n-butylphenyl)-2'-deoxyguanosine-5'-triphosphate (BuPdGTP) and N2- (p-n-butylanilino)-2'-deoxyadenosine-5'-triphosphate (BuAdATP), were generous gifts from Dr George Wright (Dept. of Pharmacology, University of Massachusetts Medical School, Worcester, MA, U.S.A.). (H)-TTP (specific activity 78 Ci/mmol) was purchased from New England Nuclear, USA. Radiolabeled (^{32}P –α-dCTP; ^{32}-P -γ -ATP) were purchased from BARC (Bombay, India),

E.Coli DNA Polymerase I (Pol 1), Ficoll 400 were purchased from Amersham Pharmacia Biotech, Uppasala, Sweden. Calf thymus terminal transferase was purchased from Roche Applied Science. Restriction endonuclease HinP1 was purchased from New England Biolabs. 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 were supplied by BangaloreGenei, Bangalore, India. 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 rat cerebral cortex of different ages were prepared essentially as standardized in this laboratory (Usha 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 were separated from cerebral

cortex and grey matter was sliced into very small pieces and incubated at 37°C for one hour in the 0.1% trypsin. Grey matter from young was incubated in isolation medium at 37°C for 30minutes. 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 minute. The remaining procedure was carried out at 0-4°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, 48μm. The tissue was placed on 105uM 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 760 x g for 15 minutes. The supernatant th obtained was discarded and the crude cell rich pellet which consisted of both neurons and astrocytes was suspended in 20ml(10ml 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.

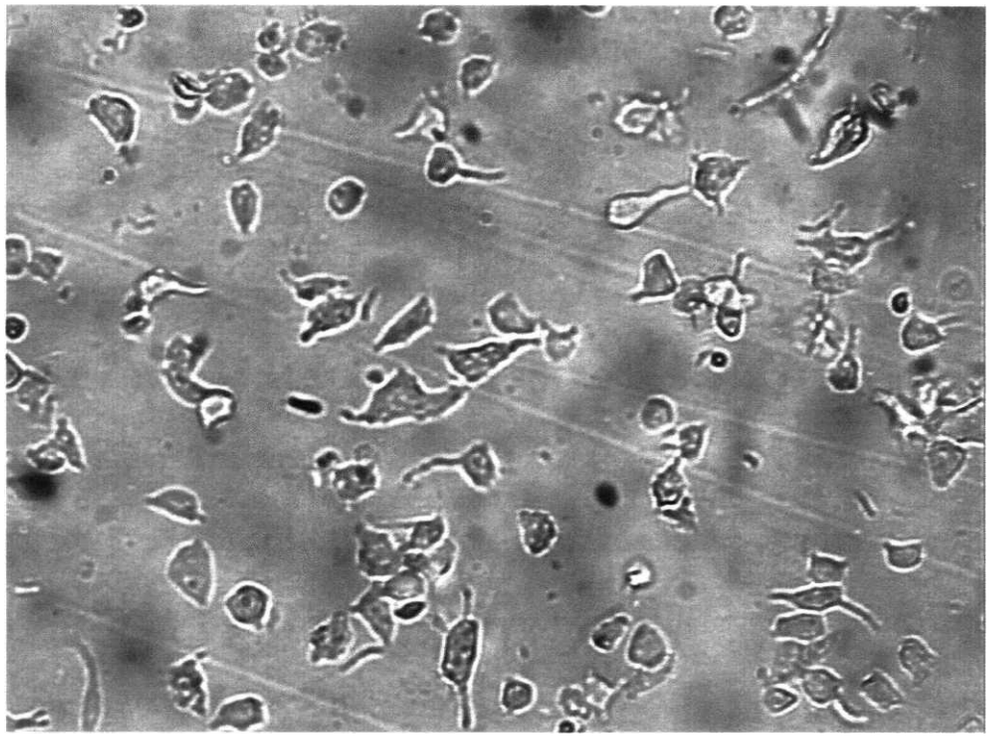
The crude neuronal pellet was suspended in 5 ml of isolation medium and was loaded onto discontinuous Ficoll gradients for further purification. The supernatant was diluted in a ratio of 1:1.125 with isolation medium and centrifuged at 1100 x g for 10 minutes. Supernatant was discarded and the astrocyte rich pellet obtained was suspended in 5ml of isolation medium and was loaded onto discontinuous ficoll gradients for further purification. Ficoll gradients were prepared in 50ml Polycarbonate tubes from the bottom

up, of 5ml each of 28%, 22%, 10% ficoll (w/v) in the medium. The neuronal and astroglial cell suspension was loaded onto the 10% ficoll and centrifiiged at 7800 x g 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 gradient and then washed with 5ml of medium without ficoll three times at 1500 x g for 10 minutes and then in 5ml of phosphate buffered saline (1X PBS pH 7.4) thrice at 1500 x g 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 (Figure 2).

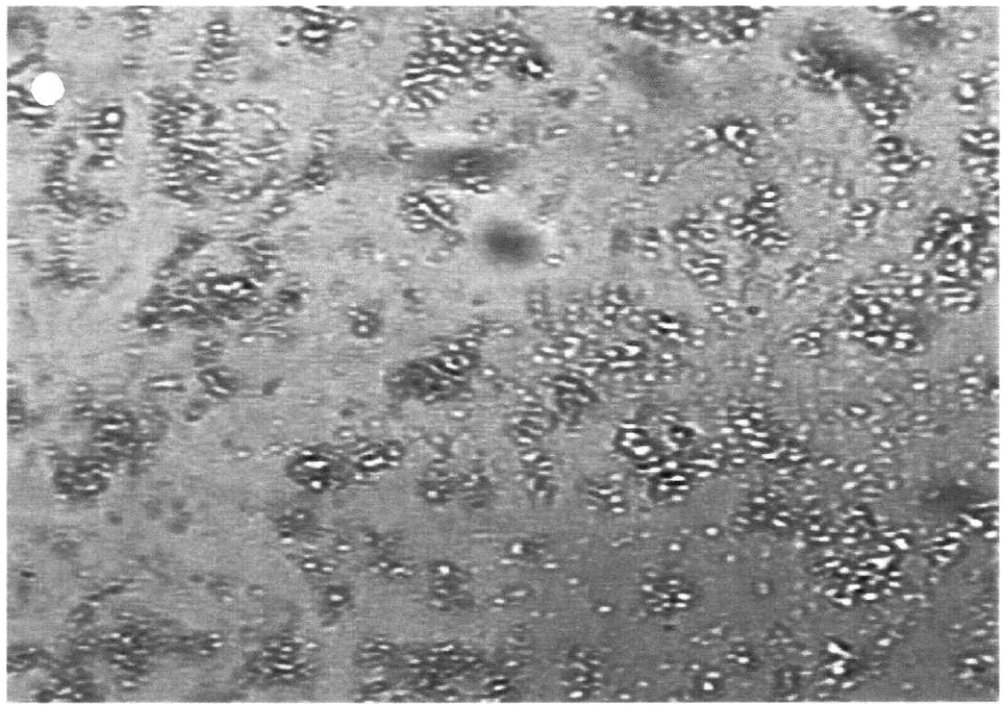
Figure 2

Morphology of the Neurons and Astrocytes isolated from 'Young' (5 days postnatal) rat brain cerebral cortex by Ficoll gradient (28% ficoll). Image taken at 40X magnification from an inverted microscope

NEURONS



ASTROCYTES



Preparation of DNA **Polymerase enzyme extract**

The final preparation of the cells was suspended at a concentration of 10 million cells/ml in extraction medium. The extraction medium consists of 20 mM Tris pH 7.5, 0.1 mM dithiothreitol, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 0.1 mM PMSF (just before use), 5 mM P-mercaptoethanol, 1 mM $MgCl_2$, 1 μg/ml Leupeptin and 1 μg/ml Pepstatin A (both just before use) and 0.5 M KCl and sonicated for 5 seconds, 3 times with the setting at 5 in a Branson sonifler. The suspension was kept at 0-4°C for 30 min and then centrifuged at 100,000 xg for 1 hour in a Beckman Ultracentrifuge and the clear supernatant was used as the source for DNA Polymerases and exonuclease activity. Protein concentration was estimated by the method of Bradford (1976)

DNA Polymerase assay

The activity of total DNA Polymerase was assayed according to the procedure of Prapurna and Rao (1996).

Total DNA Polymerase assay

The reaction mixture contained in a final volume of 50μl, 40 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 1mM P-mercaptoethanol, 4 mM ATP, 100μM each dATP, dGTP, dTTP, 25μM dCTP, 5 ug `Activated' calf thymus DNA, 1μCi α-^{32}P-dCTP (4000Ci/mmol) and cell extract as enzyme source (10 μg protein). The incubation was carried out at 37 C for 20 minutes. 200 ug of Calf thymus DNA and BSA were added as carriers and the reaction was stopped with 1M perchloric acid: 10 mM tetra sodium pyrophosphate. The samples were kept on ice for 5 minutes and centrifuged at 3000 x g for 10 min. The supernatant was aspirated carefully and the precipitate was dissolved in 0.5 ml of 0.2 M NaOH. A 2 ml volume of stop solution was again added and centrifuged after 10 min. The alkali

solubilization step and reprecipitation with stop solution was repeated. The whole solution along with the precipitate was transferred to 2.5 cm glass fiber filters (Schleicher & Schuell) and washed three times with ice-cold stop solution and twice with 95% ethanol. The washed filters were dried by keeping in oven at 40° C for 20 min or keeping in a hood overnight. The dried filters were then taken in toluene-based scintillation fluid containing 5g PPO and 0.5g POPOP per litre having 0.1% Triton-X- 100 and the radioactivity was counted in a Wallac 1409 counter. The specific activity is expressed as picomoles of dCMP incorporated into acid insoluble fraction /mg protein/ hour.

DNA Polymerase assay with Poly (dA). Oligo (dT) $_{12-18}$ or Poly (dA.dT) as **template primer**

The reaction mixture with Poly (dA). Oligo (dT) $_{12-18}$ or Poly (dA.dT) as template primer contained in a final volume of 50 µl, 40 mM Tris-HCl pH 7.5, 0.8 mM $MgCl_2$, 5 µg of bovine serum albumin, 2% glycerol, 2 mM dithiothreitol, 50 uM dATP (when Poly dA.dT was the substrate), 25 uM TTP, 1 µCi α-^{32}P-TTP (4000Ci/mmol) and enzyme (20 ug protein). The incubation was carried out at 37^0C for 20 minutes. The rest of the procedure is carried out as that of the total DNA Polymerase assay. The specific activity was expressed as picomole of TMP incorporated into acid insoluble fraction/mg protein/hour.

Different DNA Polymerase levels in Neuronal and Astroglial fractions

The reaction mixture contained either 'Activated DNA' or Poly (dA). oligo $(dT)_{12-18}$ as substrates and the components of the reaction mixture are same as that of total DNA Polymerase assay with 'Activated DNA' or with Poly (dA). oligo (dT) $_{12-18}$. When 'Activated' DNA is the substrate inhibitors were used at two different concentrations in polymerase assay and the inhibitors are BuAdGTP, BuAdATP, ddTTP and Pol **a** specific monoclonal antibody-SJK-132-20. BuAdGTP is used at two concentrations of 1 and

200μM, for BuAdATP 1 and 100μM, for ddTTP 50uM and 1mM and Pol a specific monoclonal antibody-SJK-132-20 is used at a concentration of 1μg.

With Poly (dA). Oligo (dT) $_{12\text{-}18}$ the same procedure is followed except that the concentration of BuAdGTP used were 10μM and 100μM and those for BuAdATP were 15 and 200μM respectively.

Concentration of the enzyme taken was 20μg. The extracts were preincubated with the inhibitor for 10 min on ice prior to the start of the reaction. After the preincubation the reaction was carried out at 37^0C for 20 minutes. The rest of the procedure is carried out as that of the total DNA Polymerase assay. The specific activity was expressed as picomole of dCMP/TMP incorporated into acid insoluble fraction/mg protein/hour. Statistical analysis of the data was done using Sigma Plot 2000 software.

Primer extension assays

A model substrate to measure the Pol β activity in neuronal extracts of rats of different ages is designed as follows.

5' - c g c g a t c g g t a g c G –3	(14 mer-oligo 1)
3 - g c g c t a g c c a t c g C g t t a c c g - 5	(21 mer-C- oligo 2)
5 - c g c g a t c g g t a g c G - 3	(14 mer- oligo 1)
3 - g c g c t a g c c a t c g T g t t a c c g - 5	(21 mer -T- oligo 3)
5 - c g c g a t c g g t a g c G - 3	(14 mer- oligo 1)
3 - g c g c t a g c c a t c g A g t t a c c g – 5'	(21 mer -A - oligo 4)
5 - c g c g a t c g g t a g c G - 3	(14 mer- oligo 1)
3 ' - g c g c t a g c c a t c g G g t t a c c g - 5	(21 mer –G- oligo 5)

Oligo Duplexes Used for the Gap repair assays

1 Gap Oligo duplex:

(14 mer) ^{32}P 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' (17 mer)
3'-gc 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' (32 mer)

4 Gap Oligo duplex:

(14 mer) ^{32}P 5'- c g a g c c a t g g c c g c ——— t t t t t g c g g t g c c - 3' (14 mer)
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' (32 mer)

5'-End labeling of 14-mer with T4 Polynucleotide Kinase (T4PNK)

5'- end of the 14 mer (Primer) is radioactively labeled with γ-^{32}P-ATP. In a final reaction volume of 5µl, 2 picomoles of the 14-mer (oligo-1) was 5'-kinased using equimolar γ-^{32}P-ATP (specific activity, 5000 Ci/mmol) and T4-Polynucleotide kinase (2.5 units/ picomole of substrate). The reaction was carried out for 40 minutes at 37° C in the buffer to a final concentration (70 mM Tris Hcl, pH 7.6, 10 mM $MgCl_2$ and 5 mM dithiothreitol). The reaction is stopped by heating at 70°C for lOminutes. Each µl of the substrate contains 400 femtomoles of the 5'end labeled 14-mer.

5'-End phosphorylation of Oliogodeoxynucleotide with unlabeled ATP

5'- end of the 14 mer (Primer) is phophorylated with unlabeled ATP. In a final reaction volume of 5 µl, 1 picomoles of the 14-mer (oligo-1) was 5'-kinased using 1mM ATP and T4-Polynucleotide kinase (2.5 units / picomole of substrate). The reaction was carried at 37°C for 10 min and the reaction is stopped by heating at 70°C for 10 min. Each µl of the substrate contains 400 femtomoles of the 5'end phosphorylated 14-mer.

3'-End labeling of 14-mer with Terminal transferase (TdT)

The 3' end of the 14 mer is radioactively labeled using α-^{32}P-dCTP and Terminal transferase enzyme. The final reaction volume of 5µl contained 1 picomoles of the deoxyoligonucleotide, 1 picomoles of a- P-dCTP, 1X reaction buffer (200mM potassium cocadylate, 25mM Tris-Hcl, Bovine serum albumin 0.25mg/ml pH 6.6 at 25°C), 1 mM $CoCl_2$ and 1 unit of terminal transferase. Incubation was carried at 37^0C for 10 min and the reaction is stopped by heating at 70°C for 10 min. Each µl of the substrate contains 400 femtomoles of the 3'end labeled 14-mer.

Annealing of Oligodeoxynucleotides

The 5'-^{32}P-kinased or 3'- end labeled 14-mer was hybridized with each of the four different 21-mers (oligos 2 to 5) in equimolar concentrations of 14mer and 21-mer in the reaction mixture containing 50 mM NaCl and 5 mM $MgCl_2$. The annealing reaction was carried out at 70°C for 10 minutes and gradually cooling to room temperature.

For annealin£ he substrates in the gap repair experiments, 32 mer (Template) was annealed to the 5'-end labeled 14 mer and either 17mer or 14 mer as the downstream primer. Equimolar concentrations of the oligos were annealed in the reaction mixture containing 50 mM NaCl and 5 mM $MgCl_2$. The annealing reaction was carried out at 70°C for 10 minutes and gradually cooling to room temperature.

DNA Polymerase assay with synthetic oligodeoxy duplexes

The procedure for the DNA Polymerase assay using synthetic oligodeoxyduplexes is essentially similar to that of DNA Polymerase assay except that the substrate used is 400 nm of Oligoduplex template primer (unlabeled 14-mer hybridized to four different 21-mers) but with one of the four dNTPS labeled (usually dCTP). Specific activity was expressed as the

femtomoles of the radioactive nucleotide incorporated into acid insoluble fraction/mg protein/hour.

Assay of the mismatch removal and primer Extension (exo-extension) activity of neuronal extracts.

Primer Extension assays was carried as follows. The final reaction volume of 30ul contained 20mM HEPES pH 7.5, lmM $MgCl_2$, 0.1 mM DTT, O.lmg/ml bovine serum albumin, 2% glycerol and 20 uM of all the four dNTPS. 400 femtomoles of the hybridized oligo duplex either with correctly matched (G-C) or with mismatched base pair (G-A, G-G, G-T) or single stranded 5'-end labeled 14-mer/21-mer were incubated with 5μg of neuronal protein extracts at 37^0C for 20minutes or at different time intervals of 1,3,5,10,20 minutes and the reaction is stopped by heating at 70°C for 10 minutes.

Single step reaction

Single step reaction essentially consists of incubating the 400 femtomoles of 5'- P-kinased primer (14mer) hybridized to four different 21 mers in the reaction buffer containing 20mM HEPES pH 7.5, lmM $MgCl_2$, 0.1 mM DTT, O.lmg/ml Bovine serum albumin, 2% glycerol and 20 uM of all the four dNTPS in a final reaction volume of 30μl with 5 μg of Young, adult and old neuronal extracts alone or supplemented with pure recombinant rat liver DNA Polymerase p or pure calf thymus α-Polymerase or E.coli Polymerase I.The reaction was carried at 37°C for 20minutes and stopped by heating at 70°C for 10 minutes.

Two step reaction

The two step reaction is divided into two steps. The first step consisted of final reaction volume of 10μl and the reaction buffer to the final concentration of 20mM HEPES pH 7.5, 1mM $MgCl_2$, 0.1 mM DTT, O.lmg/ml Bovine serum albumin, 2% glycerol without

dNTPS. 400 femtomoles of the hybridized oligo duplex was incubated with 5 μg of the neuronal protein extracts at 37^0C for 10minutes and then stopped by heating at 70°C for 10 minutes. The second step consisted of final reaction volume of 30μl and the reaction buffer to the final concentration of 20mM HEPES pH 7.5, 1mM $MgCl_2$, 0.1 mM DTT, O.lmg/ml bovine serum albumin, 2% glycerol and dNTPS were added to a final concentration of 20μM. Pure recombinant rat liver β-Pol (2.5 units)was added and incubated further for 20 minutes at 37^0C and then the reaction was stopped by heating at 70°C for 10 minutes. Two step reactions with Mncl2 were essentially carried out as outlined above with lmM $MnCl_2$ instead of 1mM $MgCl_2$. lmM $MnCl_2$ is used in the second step of the two step reaction only.

Three step reaction

The final reaction volume of the three step reaction is 40 ul. Half the reaction product of the two step reaction as outlined above (15μl) was taken and the restriction digestion w. carried out in a reaction volume of 40 ul with lOunits of restriction endonuclease HinPI in the 1x reaction buffer (5 mM NaCl, 1 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol (pH 7.9 at 25°C). The incubation was carried for 6 hours at 37°C and then stopped by addition of 1ul of 0.5M EDTA.

3'-5' exonuclease assay with neuronal extracts

Exonuclease assays was carried exactly similar to that of the primer extension assays. The final reaction volume of 30μl contained 20mM HEPES pH 7.5, lmM $MgCl_2$, 0.1 mM DTT, 0.1mg/ml Bovine serum albumin, 2% glycerol and with or without 20 uM of dATP, dGTP, dTTP, dCTP. 400 femtomole of the hybridized oligo duplex either with 573' ^{32}P end labeled correctly matched (G-C) or with mismatched base pair(G-T) or single

stranded 5'/3'-end labeled 14-mer/21-mer was incubated with 5ug of neuronal protein extracts at 37^0C for 10minutes or at different time intervals of 1,3,5,10,20 minutes. The reaction was stopped by heating at 70°C for 10 minutes.

Sephadex G-50 Column purification of labeled Oligoduplex

Sephadex G-50 columns were prepared in 1ml tips by clogging the tip ends with glass wool. The columns were packed by slowly pouring the slurry of the Sephadex G-50 presoaked in water overnight. The columns were washed twice with water before loading the radiolabeled exoextended products.

Sephadex G-50 columns were used to separate out the excess and unbound p -y-ATP from the labeled substrate. Before loading on to the columns the samples were made upto 100ul with double distilled water and eluted with double distilled water. The first 300ul void volume is discarded and the fractions from 301-900 ul (600µl) were pooled and freeze dried under vacuum. The dried samples were reconstituted with 6ul of DDW and 6X loading dye ٦.002gms of bromophenol blue/ml formamide) in the ratio of DDW to dye of 2:1 ratio. Samples were denatured at 85°C for 5minutes and then immediately cooled on ice.

Assessing the Chain length on 20% PAGE-UREA (7M Urea) sequencing gel

20% Polyacrylamide denaturing Urea gel was poured between glass plates (35 x 45mm). 14.25 gms Acrylamide, 0.750 gms Bisacrylamide, 31.5gms Urea were dissolved in 15ml of 1X TBE (90mM Tris-borate EDTA buffer, pH 8.3) and made upto 75ml.The solution was filtered through cotton and 140ul 10% APS, 14µl of TEMED were added and slowly poured between the glass plates with the help of a syringe. The gel was pre run at 3000v in 1X TBE for 30 minutes by loading the 6X loading dye (0.02gms bromophenol blue in lml formamide).

The denatured samples were loaded onto the gel along with markers and electrophoresed in 1X TBE, at 3000v for 5 hours. The gel was then exposed to the X-ray film for autoradiography. Under these conditions the oligos could be separated on a single base difference.

CHAPTER 3

DNA Polymer as e activities in neuronal and Astroglial cell fractions in aging

DNA Polymerase activities in neuronal and astroglial cell fractions in aging

Introduction

DNA Polymerases are important components of replisome that are template directed machines catalyzing phosphoryl transfer reactions. They are also involved in recombination, repair. There is gradual increase in the discovery of number of polymerases, and today there are atleast 19 Polymerases. Since both DNA replication and repair are primary importance to cells, nature has created safety mechanisms by employing different Pols (Polymerases) for similar functional tasks. Many of the Pols contain other functional subunits and in addition to polymerizing subunit, often shows proof reading 3'5'-exonuclease. Substrates for DNA Polymerases vary from single nucleotide gaps to kilobase size gaps and from relatively simple gapped structures to complex replication forks in which two strands need to be replicated simultaneously.

Pol α was discovered in 1957 and it is localized in the nucleus. It lacks proof reading exonuclease activity and this fact created suspicion about its function in DNA replication. It is now thought to be involved in lagging strand synthesis (So and Downey, 1988; Waga and Stillman, 1994). Additional role for Pol a primase has been found in the checkpoint that couples S-phase to mitosis (D'Urso et al., 1995) Its role in DNA repair in yeast has not been identified although it may function in the repair of UV damage in Xenopus oocytes (Saxena et al., 1990; Oda et al., 1996).

Pol y and β were discovered in early 1970's. Pol β is small, highly conserved molecule and is essentially considered as a major DNA repair enzyme and has been extensively characterized enzymatically and by X-ray crystallography (Arnold et al., 1995;

Mullen and Wilson, 1997). Pol β has no proof reading exonuclease activity and is distributive in its catalytic function although short gaps with 5' phosphate are filled processively (Singal and Wilson, 1993). The Pol β has been proposed to function in DNA repair reactions involving filling in of very short gaps (Mullen and Wilson, 1997).

Pol y is involved in mitochondrial DNA replication and it has both 5'-3' and 3'-5' exonuclease activities (Graves, 1998).

Pol 8 was identified initially as a proof reading DNA Polymerase in mammalian cells (Lee et al., 1980; Hindges and Hubscher, 1997). It has tightly associated, intrinsic 3'-5' exonuclease activity and along with its auxiliary protein, proliferating cell nuclear antigen (PCNA) carries out replication of DNA processively (Tan et al., 1986; Prelich et al., 1987). Common characteristics of Pol δ are an active proofreading 3'-5' exonuclease activity, a low polymerase processivity in the absence of PCNA, sensitivity of the enzyme to aphidicolin, resistance to Pol a specific drug N2- (p -n-butylphenyl)-2'-deoxyguanosine 5-triphosphate (BuPhdGTP). These properties have been often applied to distinguish Pol δ from Pol a and Pol ε (Burgers et al., 1990).

Pol s which is previously known as DNA Polymerase II and the first report of it with proof reading activity was from yeast S.cerevisiae and was known as Polymerase B (Wintersberger and Wintersberger, 1970). It is distinguished from Pol δ because of its high processivity in the absence of PCNA (Burgers, 1991).

Novel Polymerases such as Pol ζ, Pol η, and Pol ι, Pol K and Rev 1 are involved in tranlesion DNA synthesis (Woodgate, 1999).

DNA Pols appear to possess a common catalytic active site. A two metal ion catalyses the phosporyl transfer mechanism which guarantees the incorporation of the

appropriate deoxynucleoside triphosphate base. Differences in the various aspects of structural architecture of the DNA Pols shows that the active site of the DNA Pol is conserved through evolution, where as the structure of the surface of the molecules might differ considerably (Foiani et al., 1997, Steitz, 1999).

Much of the information about the enzymology of DNA repair has originated from prokaryotic systems particularly from studies with E.Coli (Freidberg, 1985). Biochemical reconstitution studies often show specificity for certain repair enzymes in the recognition of the DNA damage and the incision steps of DNA repair, there is often little or no specificity for the enzymes required for the resynthesis steps. For example, gap filling during *in vitro* nucleotide excision repair can be carried out by Pol 8 or Pol ε holoenzyme (Aboussekhra et al., 1995) Pol δ or Pol ε also appear to be required for mismatch repair in eukaryotes. Some PCNA mutants of S.cerevisiae show defects in mismatch repair (Jhonson et al., 1996; Umar et al., 1996; Essenberg et al., 1997)

DNA Polymerase β, a smallest amongst the major DNA Polymerases was shown to be primarily involved in DNA repair. The mechanistic basis for the participation of Pol p in repair has been studied in more detail than that for Pol 8 or Pol e. In a post mitotic cell like neuron where replicative activity is absent the major DNA Polymerase activity found, is attributed to DNA Pol β (Waser et al., 1979). Evidence points out that Pol 8 as the primary DNA Polymerase for most DNA repair pathways, with Pol ε able to substitute for Pol 8 in the nucleotide excision repair pathway. Pol β is delimited to base excision repair.

Over the years the studies from this laboratory also revealed that Pol p is the most predominant DNA Polymerase in rat brain (Rao, 1997). Using whole brain extracts and specific inhibitors to the various DNA Polymerases like aphidicolin and 2'3'-

dideoxythymidine 5'-triphosphate to distinguish Pol a (aphidicolin sensitive) and Pol β (ddTTP sensitive), and monoclonal antibody to the Pol a, it was shown that the most predominant activity was that of Pol (3, while some activities can be contributed to the Pols a and 5/E (Prapurna and Rao, 1997).

Brain consists of heterogeneous populations of cells with two major types- neurons and astroglia with different characteristics.Therefore we have taken up the present study to examine DNA Polymerase activities in extracts of isolated neuronal and astroglial cell fractions from the rat cerebral cortex at three different ages. Rat brain neurons and astrocytes isolated from three age groups were designated as 'Young' (5 days postnatal), 'Adult' (6 months) and 'Old' (>2 years).

Methods

Preparation of neuronal and astroglial cell fractions from the rat cerebral cortex from 'Young', 'Adult' and 'Old' neuronal and astroglial cell enriched fractions were essentially prepared by the method of Usha Rani et al., (1983). DNA Polymerase assays and DNA Polymerase assay using inhibitors was carried out as described in Materials and Method, Chapter 2.

Results and Discussion

The relative proportions of DNA Polymerase a, (3 and δ/ε activities in isolated neuronal and astroglial cell fractions from developing, adult and aging rat brain cerebral cortex, was examined through a protocol that takes advantage of the reported differential sensitivities of different DNA Polymerases towards certain inhibitors like butylphenyl and butylanilino nucleotide analogs, 2', 3'-dideoxythymidine triphosphate (ddTTP), monoclonal antibody of human a Polymerase and the use of two template primers as substrates.

The concentrations of inhibitors used were arrived at after careful examination of the sensitivities exhibited to various inhibitors by Polymerases a, 5 and p and the I 05 (the amount of analog that gave 50% inhibition of the Polymerase activity) values reported by Lee et al., (1985) with respect to a given template primer. The reported sensitivity of β and 8 Polymerases towards ddTTP (were also taken into account (Dresler and Kimbro, 1987; Wahl et al, 1986). Accordingly, the Polymerase activity (with 'activated DNA' as substrate) inhibited by 1 μM BuPdGTP (N2- (p–n-butylphenyl)-2'deoxyguanosine-5'-triphosphate) or BuAdATP (N2- (p–n-butylanilino)-2'deoxyadenosine-5'-triphosphate) was considered as the activity of Polymerase a, whereas, at higher concentrations the additional reduction in activity is taken to be associated with 8 or e Polymerase. Similarly, the reduction in activity with 50 μM ddTTP was taken to be p Polymerase and further inhibition noticed at 1 mM level of ddTTP was attributed to 8 or E Polymerase. Polymerase a activity was considered to be the activity inhibited by the DNA Polymerase a specific monoclonal antibody, SJK 132-20. With Poly (dA). oligo (dT) $_{12\text{-}18}$ as substrate, the same procedure was followed, except that the two concentrations of BuPdGTP used were 10 and 100 μM and those for BuAdATP were 15 and 200 μM, respectively.

Table 2 shows the DNA Polymerase activities in isolated neuronal and astroglial cells from rat cerebral cortex of Young (5 days postnatal), Adult (6 months) and Old (>2 years). 'Activated DNA', Poly (dA). oligo (dT) $_{12\text{-}18}$ and Poly (dA.dT) were used as three different template primers to measure the polymerase activity. It can be seen from Table 2, the activity with 'activated DNA', both in neuronal and astroglial fractions, undergoes a significant decrease by the time the animal reaches adult stages of life. There is considerable variation in the activities from one animal to the other. However the pattern of changes with

age are quite consistent. In case of the synthetic oligos the activity in either type of cells is far less when compared to activated DNA. Also no age dependent changes were observed in both neuronal and astroglial cells when synthetic substrates Poly (dA). oligo $(dT)_{12-18}$ and Poly(dA.dT) were used. It is possible that the low activities with these synthetic substrates shows that these substrates are not the preferred ones for Pol p. It was shown by Syvaoja et al.,(1990) that Pol 5 and E are active towards these substrates and Pol ε prefers a template primer such as Poly (dA). Oligo$(dT)_{12-18}$ that contains long stretches of single stranded Poly(dA). Conversely, Polymerase 5 exhibit equal preference towards 'activated DNA and Poly (dA).Oligo$(dT)_{12-18}$ (Crute et al.,1986; Wahl et al.,1986). However, the activity of Pol 5 on Poly(dA).Oligo$(dT)_{12-18}$ is highly influenced by the inter primer nucleotide distance in the substrate and the presence and the presence of PCNA (Crute, 1986; Syvoaja et al., 1990). It is therefore taken that this low but steady activity is due to Polymerase 5/s present in the cell extracts.

Table 3 and 4 shows the extent of inhibition exerted by the various inhibitors used with 'activated DNA' as substrate. The activity without any inhibitor (control) is taken as 100 and other values are relative to the control. The Polymerase activity in young neurons inhibited by 1 uM BuPdGTP is 35%. This activity was therefore attributed to Polymerase a. This assumption appeared to be reasonable since the monoclonal antibody to human a Polymerase also exerted inhibition to a similar extent. However, the activity was inhibited by 71% when the concentration of BuPdGTP was raised to 200 uM. The rise in inhibition by 36% was thought to be due to the inhibition of Polymerase 8 and/or Polymerase e. A similar rationale applies to our interpretation of data with the other nucleotide analog inhibitor, BuAdATP. When ddTTP was used at a concentration of 50 uM level, Polymerase

activity was inhibited by 50% and the inhibition went up 78% when the concentration of ddTTP was raised to 1 mM. The percentage of activity inhibited by 50 uM ddTTP was attributed to Polymerase β activity and the additional inhibition at 1 mM level was taken as that of 5 and /or e Polymerase activity.

Table 4 shows the range of inhibitions exerted by the same inhibitors with astroglial extracts. The results indicate that a relatively higher levels of Pol a and lower levels of Pol δ/ε in astroglia than in neurons which is a notable difference between these types of cells.

Table 5 shows the calculated relative percentages of different DNA Polymerases, out of the total Polymerase activity. It is shown in the table the value obtained for each of the inhibitor used with respect to calculating the percentage of a given polymerase and the average is taken to minimize the error. It can be seen that in both types of cells and at all the three ages studied, Pol β appears to be the predominant polymerase, which confirms earlier reported observations. Polymerase activity is attributable other than Pol β is due to Pol 5/e activity which is closely behind the Pol β activity in neurons whereas Pol a is the second predominant polymerase in astroglia. It can be seen from the table 5 that the relative percentages of Pol β in young, adult and old neurons are 50, 43 and 42 respectively. Where as the values for Pol a in young, adult and old neuronal cells the values are 33, 20 and 21. For Pol 5/e, the values read 34, 32 and 42. In astroglia, the relative abundance of Pol β at young, adult and old ages is 67,50, and 53 respectively; for Pol a in the same order 47, 24, and 35; for δ/ε, the figures are 25, 19 and 16.

Tables 6, 7 the results of similar experiments with Poly (dA). oligo $(dT)_{12-18}$. as template primer are shown. Firstly, the basal activity itself is very low with this substrate (see Table 2). Compared to activated DNA as substrate the pattern of results with various inhibitors, in both neurons and astroglia, is similar. The results from the tables 6,7 showed striking difference between the two cell types in that, the neurons showed higher percentage of Polymerases δ/ε activity even in aging brain and astroglia showed a more sustained Pol a activity. When ddTTPS was used as the inhibitor, the inhibition is less potent in astroglia with the synthetic substrate leading to lower percentages of Pol β and Pol 5/s (compare the data in Table 5 and 8). Hence it appeared that this synthetic primer might be more useful to distinguish various DNA Polymerase activities with pure proteins (Syvaoja et al., 1990) rather than with crude extracts. Nonetheless, the data do indicate the presence of activity that is attributable to Polymerases δ/ε throughout the lifespan.

Table 8 shows the relative percentages of Pol β in young, adult and old neurons. Relative pe..entages for Pol p in young adult and old neuronal cells are 41, 41 and 27 respectively. Where as the values for Pol a in young, adult and old neuronal cells the values are 39, 20and 21. For Pol δ/ε, the values are 20, 11 and 30. In astroglia, the relative abundance of Pol β at young, adult and old ages is 37,36, and 39 respectively; for Pol a in the same order 31 24, and 24; for 5/e, the figures are 16, 15 and 9.

The percentage calculations for each type of nuclear DNA Polymerase is based on the extent of inhibition exerted by various known inhibitors for these Polymerases and with two different substrates, the activated DNA and Poly (dA). oligo $(dT)_{12-18}$. It may be argued that this is an oversimplification of a complex situation since the inhibitors used in this study are not absolutely specific and the calculated percent distribution of the polymerases

is only approximate. In spite of this limitation, these data do give sufficient hint regarding the relative abundance of the various DNA Polymerase activities in two major cell types of rodent brain as sufficient care was taken in choosing the two different concentrations of the inhibitors.

Table 2: DNA Polymerase activity in rat neuronal and astroglial cells of different ages with three different Template-Primers.

	Substrate and cell Fraction used	AGE YOUNG	ADULT	OLD
I.	'Activated **DNA'**			
	Neurons	2023±1076	719±541*	568±412*
	Astroglia	1471±550	822±512*	694±652*
II.	**Poly(dA).Oligo(dT)$_{12-18}$**			
	Neurons	18.4±3.9	17.7±3.2	17.7±3.6
	Astroglia	37.6±18.6	33.0+14.9	31.2±11.8
III.	Poly dA-dT			
	Neurons	16.7±3.0	18.0±4.8	19.1±3.6
	Astroglia	35.9±16.5	35.7±14.9	34.5±12.0

Values are averages ± S.D. and expressed as picomoles of the radioactive deoxynucleotide Incorporated into the acid insoluble fraction in 1hr/mg protein.

*These values are significantly different ($p<0.001$ for neurons and 0.02 for astroglia respectively) from the corresponding value at 'Young'.

Table 3: Effect of various inhibitors on the activity of DNA Polymerases in extracts of neuronal cells isolated from rat brain of different ages using *Calf Thymus 'Activated DNA'* as Template-Primer.

Inhibitor concentration	Activity (%) at different ages		
	YOUNG	ADULT	OLD
Control	100	100	100
+BuPdGTP, 1µM	65.0±4.8	77.3±12.2	82.2±3.2
+ BuPdGTP, 200µM	28.7±7.4	42.1±0.6	33.5±15.0
+BuAdATP, 1µM	68.6±2.7	92.3±2.2	78.5±12.4
+ BuAdATP, 200µM	31.1*±4.7	49.5±7.5	27.3±9.4
+ddTTP 50µM	50.0±14.6	57.4±1.6	57.6±13.0
+ddTTP, 1mM	22.3±5.5	37.3±6.0	31.3±8.4
+SKJ132-20 ab, 1 µg	68.4±1.3	72.1 ±6.2	77.5*±3.7

Activity expressed as in Table 2The control value is taken as 100 and the other values in the presence of various inhibitors are expressed average ±SD. The values are from three different experiments except in two cases marked with an asterisk.

Table 4: Effect of various Inhibitors on the activity of DNA Polymerases in extracts of astroglial cells isolated from rat brain of different ages using *Calf Thymus "Activated" DNA* as Template-Primer

Inhibitor concentration	Activity (%) at different ages		
	YOUNG	ADULT	OLD
Control	100	100	100
+BuPdGTP, 1µM	57.7±7.4	80.8±7.0	58.6±11.3
+ BuPdGTP, 200µM	24.5±2.0	60.2±4.2	44.7±4.2
+BuAdATP, 1 µM	49.8±1.0	70.8±7.66	79.4±2.9
+ BuAdATP 200µM	19.7±2.2	46.3±10.5	79.4±2.9
+ddTTP, 50µM	33.6±6.2	49.7±6.2	47.2±7.6
+ddTTP, 1mM	21.8±1.4	39.4±5.8	33.5±5.2
+SKJ132-20 ab, 1µg	50.9±13.1	76.1±8.5	57.6±4.5

Activity expressed as in Table 2.The control value is taken as 100 and the other values in the presence of various inhibitors are expressed average ±SD. The values are from three different experiments.

Table 5: Percentage of different DNA Polymerases present in neuronal and astroglial cell fractions isolated from rat brain of different Ages using *calf thymus `activated' DNA* as Template-Primer

	NEURONS									***ASTROGUA***								
	a			**δ / ε**			P			a			5 / E			P		
	Young	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**
BuPdATP	35	23	18	**36**	35	49	**NS**	**NS**	**NS**	42	19	41	33	21	14	**NS**	**NS**	**NS**
BuAdGTP	31	8	22	38	42	51	**NS**	**NS**	**NS**	50	29	21	30	24	20	**NS**	**NS**	**NS**
ddTTP	**NS**	**NS**	**NS**	28	20	27	50	43	42	**NS**	**NS**	**NS**	12	10	14	66	50	53
SJK132-20	32	28	22	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	49	24	42	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**
Average	**33**	**20**	**21**	**34**	**33**	**42**	**50**	**43**	**42**	**47**	**24**	35	**25**	**19**	**16**	**66**	**50**	**53**

These data are recalculated, as explained in text, from the values in tables 3 and 4. NS = not sensitive

Table 6: Effect of various Inhibitors on the activity of DNA Polymerases in extracts of neuronal cells isolated from rat brain of different ages using *Poly(dA).Oligo(dT)$_{12-18}$* as Template-Primer

Inhibitors concentration	Activity (%) at different ages		
	YOUNG	ADULT	OLD
Control	100	100	100
+BuP dGTP, 10μM	61.9±3.9	78.9±1.6	77.5±1.5
+BuP dGTP, 100μM	43.4±2.5	68.3±3.6	43.5±0.9
+BuAdATP, 15 μM	64.1±2.4	82.5±1.8	81.7±4.6
+BuAdATP, 200μM	48.6±3.5	70.7±3.3	48.9±2.7
+ddTTP, 50μM	59.5±5.2	59.3±3.7	73.1±1.3
+ddTTP, 1mM	35.0±4.4	50.5±1.4	50.1±3.8
+SKJ132-20 ab, 1μg	56.6±11.5	78.2±4.6	76.4±7.8

Activity expressed as in Table 2.The control value is taken as 100 and the other values in the presence of various inhibitors are expressed average ±SD. The values are from three different experiments.

Table 7: Effect of various Inhibitors on the activity of DNA Polymerases in extracts of astroglial cells isolated from rat brain of different ages using *Poly(dA).Oligo(dT)*$_{12-18}$ as Template-Primer

	Activity (%) at different ages		
Inhibitors concentration	**YOUNG**	**ADULT**	**OLD**
Control	100	100	100
+BuPdGTP, 10μM	73.0±0.4	80.2±0.6	73.3±1.2
+ BuP dGTP, 100μM	59.8±1.3	61.3±1.3	61.2±5.1
+BuAdATP, 15 μM	65.1±1.4	71.1±2.6	78.3±1.2
+BuAdATP, 200μM	49.9±0.6	58.4±2.5	70.4±0.4
+ddTTP, 50μM	63.4±0.8	63.9±1.1	61.2±2.3
+ddTTP, 1mM	44.5±1.2	50.8±2.4	53.3±1.0
+SKJ132-20 ab, 1μg	68.8±8.4	76.6±1.3	75.7±0.2

Activity expressed as in Table 2The control value is taken as 100 and the other values in the presence of various inhibitors are expressed average ±SD. The values are from three different experiments.

Table 8: Percentage of different DNA Polymerases present in neuronal and astroglial cell fractions isolated from rat brain of different Ages using Poly(dA).Oligo$(dT)_{12-18}$ template- primer

	NEURONS									***ASTROGLIA***								
	a			6 / E			β			*a*			δ / ε			P		
	Young	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**
BuPdATP	38	21	22	19	11	34	**NS**	**NS**	**NS**	27	20	27	13	19	12	**NS**	**NS**	**NS**
BuAdGTP	36	18	18	16	12	33	**NS**	**NS**	**NS**	35	29	22	15	13	8	**NS**	**NS**	**NS**
DdTTP	**NS**	**NS**	**NS**	24	9	23	41	41	27	**NS**	**NS**	**NS**	19	13	8	37	36	39
SJK132-20	43	22	24	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	31	23	24	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**
Average	**39**	**20**	**21**	**20**	**11**	**30**	**40**	**41**	**27**	**31**	**24**	**24**	16	**15**	**9**	**37**	**36**	**39**

These data are recalculated, as explained in text, from the values in tables 6 and 7. NS = not sensitive

DNA Pol β is the predominant polymerase in the adult brain. Traces of other polymerase activity was suspected to be the a type (Waser et al, 1979; Shrivastaw et al, 1983; Subrahmanyam and Rao, 1988; Rao, 1997).These observations appeared logical since only DNA repair but not replication is required in the post mitotic tissue like brain and the Pol p is considered to be predominant repair enzyme (Wood and Shivji, 1997; Hubscher, 2000). Neuronal cells, once differentiated, do not replicate, but they show high metabolic activity and the gene expression in these cells is 2 to 3 times more as compared to the cells in rest of the body (Chaudhari and Hahn, 1983; Tobin, 1994). Also the genomic DNA in brain cells is shown to suffer various types of damage due to endogenous sources (Rao, 1997). Abasic sites and many types of base modification alterations in DNA can be repaired in brain cells through Base Excision Repair pathway (BER) and it is well established now that Pol p is the enzyme that participates in BER. There is now considerable evidence that even in adult brain, some progenitor stem cells are present capable of differentiating into mature neurons (Van Praag et al, 2002). It is possible that a small portion of the different polymerase activities noticed in this study could have come from these maturing neurons. It is not however, clear what are the *in vivo* stimuli that would induce this differentiation and that too in the specific area of brain used in this study viz., the cerebral cortex. The present results may largely reflect the situation in terminally differentiated neurons which are essentially incapable of dividing.

CHAPTER 4

Primer extension and Exonuclease (Proof Reading) activities in Aging Rat Neurons And Restoration of lost primer extension activity *in vitro* by DNA Polymerase β

Primer extension and Exonuclease (Proof Reading) activities in Aging Rat Neurons And Restoration of lost primer extension activity *in vitro* by DNA Polymerase β

Introduction

Out of many DNA repair pathways Base excision repair (BER) pathway repairs genomic DNA of damaged nucleotides and abasic sites arising from a variety of exogenous and endogenous sources (Friedberg et al., 1995; Lindahl, 1982; Samson, 1992; Barnes et al., 1993; Seeberg et al., 1995; Barzilay and Hickson, 1995; Lindahl, 1992). The damaged lesions due to a spontaneous and enzymatic removal of base residues, creating abasic sites are repaired by BER and these lesions are processed to the abasic site or apurinic/apyrimidinic (AP) site alike in both prokaryotic (Seeberg et al., 1995) and mammalian cells (Lindahl, 1992) sharing a number of similar features including the enzymatic steps. The short excision gap intermediates produced in double stranded DNA after removal of the AP site distinguishes BER from other DNA repair pathways. DNA adducts/lesions repaired by BER pathway are generally limited to single nucleotide modifications that are less bulky than those repaired by nucleotide excision repair pathway (Van Houten, 1990; Sancar, 1996). Brain being highly active organ and in view of the generally protected situation of the brain (including the blood brain barrier) the main damage to the genomic DNA of the brain comes from the small changes arising due to variety of changes in the bases of the DNA due to spontaneous deamination of bases, oxidative damage by free radicals etc., Accumulation of spontaneous DNA damage and mutations has been hypothesized to contribute to the process of aging and because the BER

is the pathway that largely ameliorates such damage(Lindhal, 2000) plays an important role in aging.

There is now evidence to suggest that Pol β plays an important role in BER in mammalian cells (Wilson, 1998; Wood, 1996). It is also shown that most of the polymerase activity in the adult brain is of DNA polymerase β (Waser et al., 1979; Subrahmanyam and Rao, 1988). It can be seen that pol β is likely to have a prominent role in maintaining the structural integrity of DNA in brain cells. With the advent of several neurodegenerative diseases making their appearance in old age and accumulating knowledge of their molecular link to genomic stability (Martin 1999; Sniden 2001) the status of BER in health and disease assumed great importance. DNA Polymerase p (39Kda), one of the smallest of many known cellular polymerases with no associated proof reading activity, shown to be primarily involved in DNA-repair (Waser et al., 1979; Wilson, 1998). The relationship between the phenomenon of brain aging and its DNA-repair potential has been subject of study in this laboratory for the past several years. pol p is an important component of BER machinery. Further, earlier systemic study from this lab through Western, Northern and Activity gels assays in young, adult and old rat brain showed that, with the advancement of age not only the levels of pol p go down in brain but there is also accumulation of catalytically incompetent pol β molecules (Rao et al., 1994).

This study is taken up to examine the DNA-repair activity actually catalyzed by pol p, is the one that is compromised in brain cells during aging, We have taken up a more *in vivo* relevant functional assay for Pol β activity.

For this we have designed a study to examine the age related capacity of rat neurons to extend a primer, with or without a correctly matched base pair at its end, in a synthetic oligodeoxynucleotide duplex.

Methods

DNA polymerase assay

DNA polymerase assay was carried out using the unlabeled hybridized oligodeoxyduplexes with one of the dNTPS being radioactively labeled in young, adult and old neuronal extracts. Equal concentrations of the Primer (14mer) and Templates (21 mer) are annealed in the presence of 50mM Nacl and 5 mM $Mgcl_2$ in a volume of 10 ul and incubation is carried at 70°C for lOminutes and gradually cooled to room temperature. The polymerase assay is done as mentioned in chapter -2.

Primer exo-extension assays

Extension/excision of the primer with oligodeoxyduplexes formed by annealing a 5'-kinased 14-mer to four different 21-mers to form either correctly *(G-C)* or mismatched (G-A, G-T, G-G) base pair was carried out using rat neuronal extracts from different age groups. The Primer extension assays were divided into different steps and supplementation of pure enzymes was carried out as described in Materials and methods -Chapter 2.

Single step reaction

Single step reaction essentially consists of incubating the 400 femtomoles of 5'- ^{3} P-kinased primer (14mer) hybridized to four different 21 mers in the reaction buffer. The details of the reaction conditions are as described in materials and methods chapter 2.

Two step reaction:

The two step reaction consisted of final reaction volume of 10µl and the reaction buffer to the final concentration of 20mM HEPES pH 7.5, lmM $MgCl_2$, 0.1 mM DTT, O.lmg/ml Bovine serum albumin, 2% glycerol without dNTPS. 400 femtomole of the hybridized oligo duplex was incubated with 5µg of the neuronal protein extracts at 37^0C for 10minutes and then stopped by heating at 70°C for 10 minutes. The second step consisted of final reaction volume of 30µl and the reaction buffer to the final concentration of 20mM HEPES pH 7.5, lmM $MgCl_2$, 0.1 mM DTT, O.lmg/ml Bovine serum albumin, 2% glycerol and dNTPS were added to a final concentration of 20µM. Pure recombinant rat liver β-Pol (2.5 units) incubated further for 20 minutes at 37^0C and then the reaction was stopped by heating at 70°C for 10 minutes. Two step reactions with $Mncl_2$ were essentially carried out as outlined above with 1mM $MnCl_2$ instead of lmM $MgCl_2$. lmM $MnCl_2$ is used in the second step of the two step reaction only.

Three step reaction:

The final reaction volume of the three step reaction is 40 u!. Half the reaction product of the two step reaction as outlined above (15µl) was taken and the restriction digestion was carried out in a reaction volume of 40 µl with lOunits of restriction endonuclease HinPI in the 1x reaction buffer (5 mM NaCl, 1 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol (pH 7.9 at 25°C). The incubation was carried out for 6 hours at 37°C and then stopped by addition of 1 µl of 0.5M EDTA.

Exonuclease assays

Proof reading activity of the rat neuronal extracts from different ages was examined both in the presence and absence of dTNPs. Both 3' and 5' end labeled oligoduplexes were used to study the nature of the exonuclease activity in rat neuronal extracts. The procedure carried out is described in material and methods of Chapter 2

Purification of the labeled exo **extended product**

The exo-extension reaction products were purified by passing through the Sephadex G-50 columns as described in materials and methods chapter 2.

20% PAGE sequencing Urea **gel and** Autoradigraphy:

The reaction products were run on 20% polyacrylamide gels in the presence of 7M urea and the gel is run in 90mM Tris-Borate EDTA buffer pH 8.3 at 3000V.The details are as described in chapter 2.

Results and Discussion

The synthetic oligoduplexes used in this investigation offer a simple and 'easy-to-work with' model and the oligo template-primer model used in the present study offers an advantage. Both the primer extension activity and removal of the mismatched base at the 3'-end of the primer can be followed simultaneously. The substrates may not exactly represent the type of substrate that β-pol would encounter during BER but nevertheless serves as a good model to follow the proofreading and overall polymerase (including that of β-pol) activities.

DNA polymerase activity in neuronal extracts with the hybridized oligo duplex as template primer was measured essentially as standard polymerase assay as described in chapter 2 using 400 nmol of oligoduplex template primers. The results of detectable

template primer dependent incorporation of the label into acid insoluble fraction are shown in Table 9. Incorporation of the label into acid insoluble fraction with the template primers was noticed in both correctly (G-C) and three mismatched (G-T, G-G, G-A)primers. The activity was lower in the case of mismatches, and decreased with age signifying the limitation of this activity with advancement of age.

Having made sure that the synthetic oligoduplex model system is good enough for assessing the polymerase activity, combined/simultaneous activity of polymerization and excision(exo-extension) was examined next.

In the exo-extension reaction, the labeled primer was used in the reaction mixture with or without dNTPs (the details of the assay are given in chapter 2). The results of exoextension activity by the neuronal extracts in the absence of dNTPS is shown in Figure 3.The results indicate that in the absence of dNTPs, the 14-mer is excised apparently in a step wise manner from 3'-end both in correctly matched duplex (G-C) and incorrectly matched duplex (G-T) in all the age groups. No extension of the primer is seen. The excision activity appears to be of generalized nature lacking any absolute specificity since all the four primers, including the correctly matched one, are excised to shorter lengths in a sequential manner.

However, when dNTPs are present (20μM) in the reaction mixture, extension by one nucleotide is seen in correctly matched (G-C) duplex that too only in young neuronal extracts. The extension was not observed in the 'Adult' and 'Old' neuronal extracts. On the other hand, excision activity was observed in all the cases and at all the ages of the neuronal extracts. These results are shown in Figure 4.

It was earlier shown Wang and Korn (1982) that substituting Mn^{++} in place of Mg^{++} can enhance the processivity of the β-pol activity. In the present study when Mg^{++} in the

reaction mixture is replace by Mn^{++}, primer extension occurred significantly both in correctly and mismatched duplexes but only in young neuronal extracts and thus there was age dependent decrease in primer extension activity (Figure 5).The results indicating age-dependent decrease in the primer extension activity could perhaps be attributed to the decrease in the expression of β-pol gene and more importantly due to the accumulation of catalytically inactive β-pol molecules in aging rat brain as was observed earlier in our laboratory (Rao et al., 1994).

We have therefore undertaken *in vitro* studies in order to examine the possible restoration of the lost primer extension activity in the neuronal extracts by supplementing with pure DNA polymerases. The neuronal extracts were supplemented with either calf thymus a polymerase or E.coli Polymerase I or Rat liver recombinant polymerase p.

DNA polymerase a is involved mainly in the replication of the DNA and it has no proof reading 3'-5' exonuclease activity. Figure 6 shows that supplementing pure calf thymus α-polymerase to neuronal extracts, could extend the 14-mer in the case of correctly paired duplex (G–C). Towards the mismatched primers, the activity was very low confirming the known inability of DNA polymerase a to extend mismatched primers (Perrino and Loeb, 1990) probably because of the absence of proof-reading 3'–5' exonuclease activity in the enzyme and also perhaps, in the present case, the inappropriateness of oligoduplex substrate.

Figure 7 shows a typical autoradiogram of primer extension when the neuronal extracts were supplemented with *E.coli* pol 1. This enzyme, either alone (lanes 13-16) or together with neuronal extracts was able to achieve extension of the 14-primer either mismatched or correctly matched, with considerable facility. This is not unexpected since

E.coli pol I possesses the proof reading 3'-5' exonuclease activity in itself. It is observed that the primer extension profiles observed with this enzyme are quite inconsistent and no set pattern could be discerned. Although there was extension of the 14-mer to 21-mer at all ages and by the enzyme alone, there was no consistency except perhaps in the 'young' extracts. Mixing of 'young' extracts with 'adult' or 'old' along with E.coli pol I also gave erratic results (data not shown). It is taken that the oligoduplex template primer used in these experiments is not a preferred substrate for this enzyme. Therefore further experiments with this enzyme were not taken up.

Addition of pure rat liver p-pol to neuronal extracts (Figure 8) restored the extension activity significantly and with a pattern. It was seen that what ever the extension activity found was consistent in different experiments with a number of characteristic features. It is seen that p-pol either itself or in the presence of neuronal extracts could not extend the mismatched primers efficiently whereas the correctly matched primer (G–C) was extended with good facility. Preference for the mismatches was seen for G-T and the addition of nucleotides to the primer is a slow and distributive process with a number of products of intermediate lengths. Efficient extension is seen in young neuronal extracts supplemented with P-pol than adult and old extracts. Extension was complete to a length of 21 nucleotides in young extracts, at least to some extent even in the case of mismatched primers where as in case of adult and old extracts the extension was seen upto 18 or 19 nucleotides. It can be seen from the present results that there are factors which facilitates the primer extension by the P-pol, the levels of which are different in young and adult/old extracts. One such factor could be the 3'–5'exonuclease activity degrading the primer to shorter lengths and in the

process removing the mismatch. Furthermore it is also possible that the young extracts may have a factor, which contributes to the processivity of the P-pol catalysis.

We have designed a two step reaction for enhancing the processivity of P-pol aided primer extension. The two step reaction consisted of preincubating oligoduplex with neuronal extracts for 10 min both in the absence of dNTPs and p-pol. The second step consisted of supplementing rat liver P-pol along with dNTPs. The results showed that preincubation of oligo substrate with neuronal extracts improved the P-pol's ability to extend the primer significantly even with mismatched base pair. These results are shown in Figure 9. It can be seen from the results that the efficient primer extension towards a linear primer template depends on the removal of the mismatch at the 3' end of the primer and also on the factors that contribute to the processivity of the nucleotide addition.

In order to examine the above possibilities, extension of the pol p supplemented neuronal extracts is tested in the presence of GMP, a known inhibitor for 3'-5' exonuclease activity (Pialek and Grosse, 1993). The results are shown in Figure lO.The extension of the mismatched primers(G-T,G-A, and G-G) particularly in terms of number of nucleotides added was inhibited. The maximum chain length of 18 nucleotides with spots at lower lengths also being present. The results were taken to suggest that significant extension by supplemented p pol occurs only after the removal of mismatch at the 3' end of the primer. The maximum chain length of 19 nucleotides seen in case of correctly matched duplex(G-C) is still shorter by two nucleotides of the maximum achievable length of 21. This may be due to the high concentration of GMP as compared to the other nucleotides which would be affecting the processivity of nucleotide addition.

In the next set of experiments, the effect of replacing Mg^{++} with Mn^{++} in the reaction mixture was tested. The reaction was carried out both in single step and two step reactions. Earlier Wang and Korn (1982) showed that substituting Mn^{++} instead of Mg^{++} can enhance the processivity of the p-pol activity. The single step reaction mixture consisted of 400 femtomoles of oligoduplexes as susbtrate, neuronal extracts, 2.5 units of P-pol, and the reaction containing 20uM dNTPs and 1mM Mn^{++}. The Substitution of Mn^{++} significantly improved the processivity of the nucleotide addition. In the single step reaction (Figure 11), the major product seen is of 21 nucleotide length. However, this differential trend had disappeared once the reaction was carried out in two steps-allowing the exonuclease to act first. The major product is 21-mer in all the cases and in good measure(Figure 12).These results thus suggest that under appropriate conditions, p-pol can restore the DNA repair / synthetic activity of the neuronal cells irrespective of the of the animal from which these extracts are made.

In the continued efforts to further improve the primer extension activity in neuronal extracts, it has been found that increasing the concentration of dNTPs to 100 uM along with Mn^{++} in the buffer is useful. The results are shown in Figure 13.There was considerable improvement in the primer extension activity both in case of properly matched primer(G-C) was distinctly more than that with mismatched primers(G-T,G-A, and G-G).Further this improved activity is seen only with 'young' neuronal extracts but not with the extracts from 'adult' and 'old' animals. It is also seen that there is considerable excision of the primer resulting in a number of radioactive spots corresponding to shorter lengths indicating exonuclease activity in the extracts. In view of the improvement of extension activity found

at least in 'young' neurons, these conditions were used in subsequent experiments concerned primer extension.

Figure 14 shows the results of a 2 step reaction carried out with the difference that Mn^{++} has replaced Mg^{++} and in the second step dNTPs were used at 100uM level. As can be seen, the primer extension activity is quite significant at all ages and in all cases including the mismatched duplexes. These results are taken to indicate that for proper extension activity by pol β removal of the mismatched base is a facilitative requirement.

The above results indicated that the primer extension activity with a oligoduplex DNA substrate is low in neuronal extracts obtained from young rat brain and almost undetectable in adult and old brain neuronal extracts. Supplementation of DNA polymerase β (pol p) extented correctly matched primer but not that of mismatched primers efficiently. But Preincubating of the oligoduplex substrate with neuronal extracts in the absence of dNTPs and replacing of Mg++ with Mn++ in the reaction mixture during the second step addition of pol β along with 100uM dNTPs, resulted in good extension of all the primers including the mismatches, at all the ages. These observations were strongly indicative of a exonuclease activity during preincubation affecting the removal of mismatched base at the 3'end of the primer, should it be present.

Table 9: DNA polymerase activity in rat neuronal extracts of different ages with the four synthetic oligo duplex molecules as template primers

	Age		
Base pair at primer 3'-end	YOUNG	ADULT	OLD
G-T	37.0±13.1	24.2 ±6.8*	22.5 ±17.7*
G-A	27.3 ±11.3	21.5 ±8.6*	9.3 ±6.5***
G-G	25.2 ±14.6	18.4 ±8.0	6.8+5.5***
G-C	51.3 ±34.3	33.6 ±15.6*	26.0 ±9.1***

The results are the average ± S.D. of 12 independent experiments. The activities are expressed as femtomoles of radioactive deoxynucleotide incorporated into acid insoluble fraction. p value <0.01.

*These values are significantly lower than those corresponding young group and **, those of corresponding adult group, at a *P* value <0.01.

Figure 3

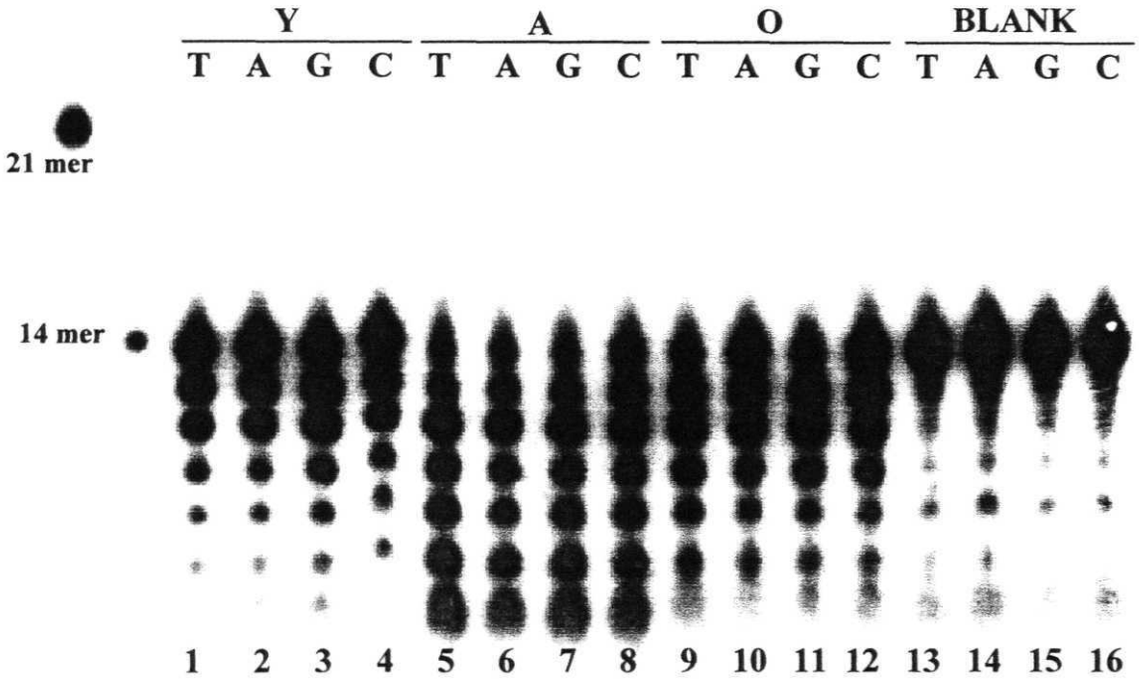
Exo-extension activities of rat brain neuronal extracts with correctly matched and mismatched oligo duplex template primers in the absence of dNTPs.

A typical autoradiogram is shown and the same pattern of results is seen in 3 different experiments. Lanes 1-4 contain neuronal extracts from young brain (Y, 5 days postnatal), Lanes 5-8 contain neuronal extracts from adult brain (A, 6 months) Lanes 9-12 contain neuronal extracts from old brain (O,> 2 years). Lanes 13-16 are without any neuronal extracts (Enzyme blanks).

Protein concentration in neuronal extracts is adjusted to be the same in all the tubes. Details regarding the incubation conditions and identification of the products through sequencing gel electrophoresis are given in chapter 2.The mobilities in standard 21-mer and 14 mer are shown.

The base in 21-mer corresponding to that at the 3'-end of the primer is shown on the top. Since the primer 14 -mer has G at the 3' end, for the neuronal extracts prepared from animals at each age (young-Y, adult-A and old-O) the first three tubes represent mismatches G-T, G-A and G-G (lanes l-3,5-7,and 9-11 respectively). The fourth tube (lanes 4,8,and 12) represents a correctly matched duplex G-C.

Equal amounts of the product were loaded for the sequencing gel electrophoresis.



5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

5' - c g c g a t c g g t a g c G - 3 ' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

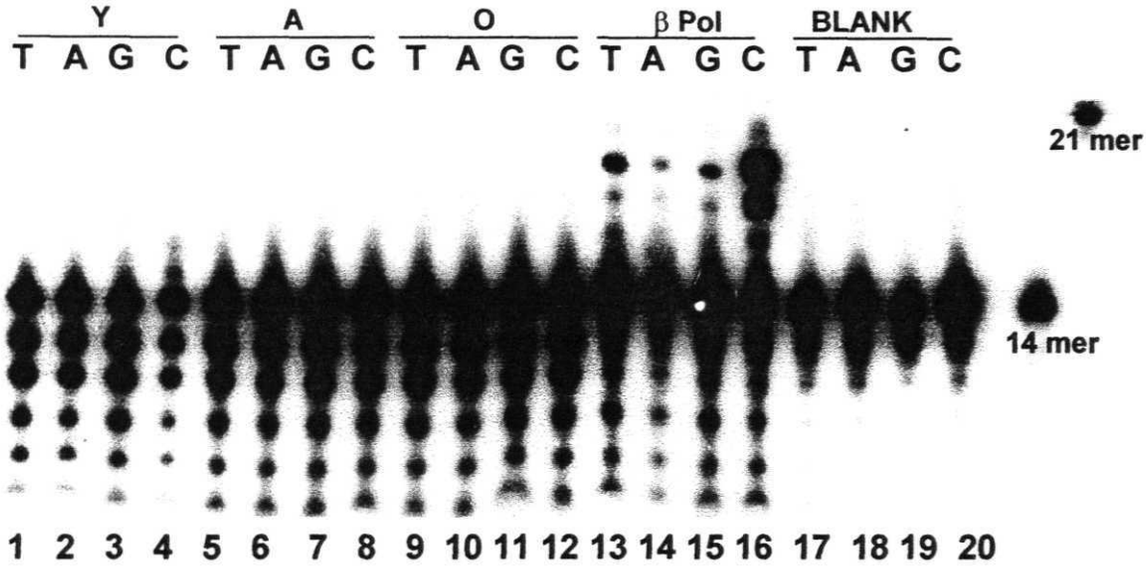
5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g A g t t a c c g - 5' (21 mer -A- oligo 4)

5 ' - c g c g a t c g g t a g c G - 3 ' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g G g t t a c c g - 5' (21 mer -G- oligo 5)

Figure 4

Exo Extension activities of rat brain neuronal extracts with correctly matched and mismatched oligo duplex template primers in the presence of 20μM dNTPs.

A typical autoradiogram is shown and the same pattern of results is seen in 4 different experiments. Lanes 1-4 contain neuronal extracts from young brain (Y, 5 days postnatal), Lanes 5-8 contain neuronal extracts from adult brain (A, 6months) Lanes 9-12 contain neuronal extracts from old brain (O,≥ 2 years). Lanes 13-16 are with added pol β (2.5 units). Lanes 17-20 are without any neuronal extracts (Enzyme blanks). Details regarding the incubation conditions and identification of the products through sequencing gel electrophoresis are given in chapter 2.The mobilities of standard 21-mer and 14 mer are shown. Other notations are similar to that of Figure 3.



5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

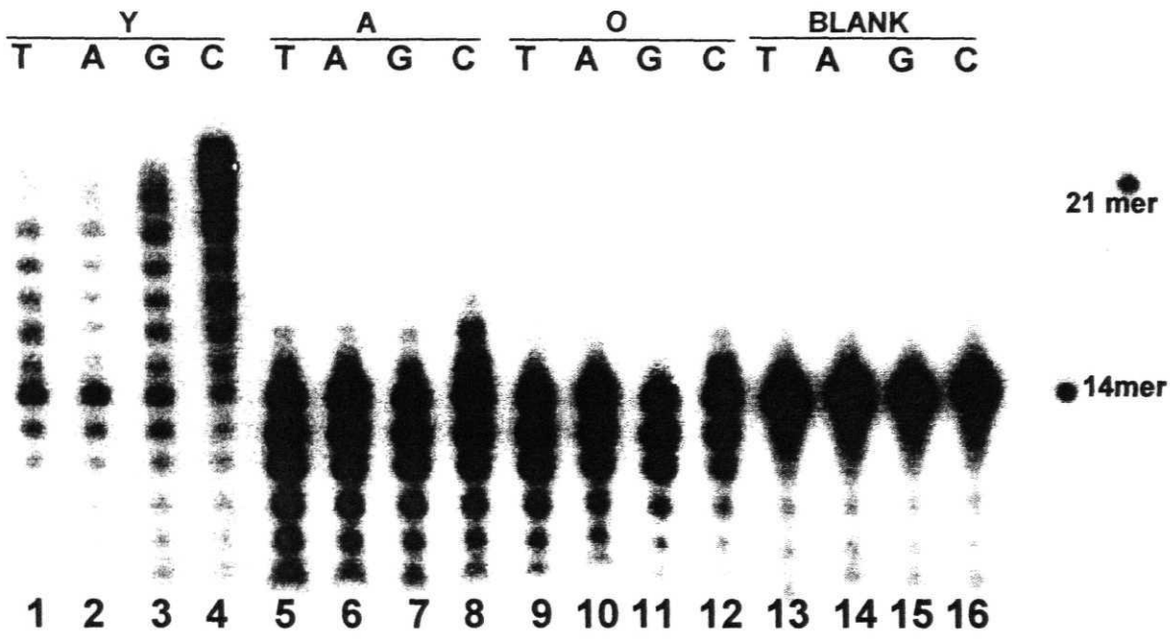
5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g A g t t a c c g - 5' (21 mer -A- oligo 4)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g G g t t a c c g - 5' (21 mer -G- oligo 5)

Figure 5

Effect of replacing 1mM Mg^{++} with Mn^{++} on the exo extension activities of rat brain neuronal extracts with correctly matched and mismatched oligo duplex template primers in the presence of 20uM dNTPs.

A typical autoradiogram is shown and the same pattern of results is seen in 3 different experiments. Other notations are similar to that of figure 3.



5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

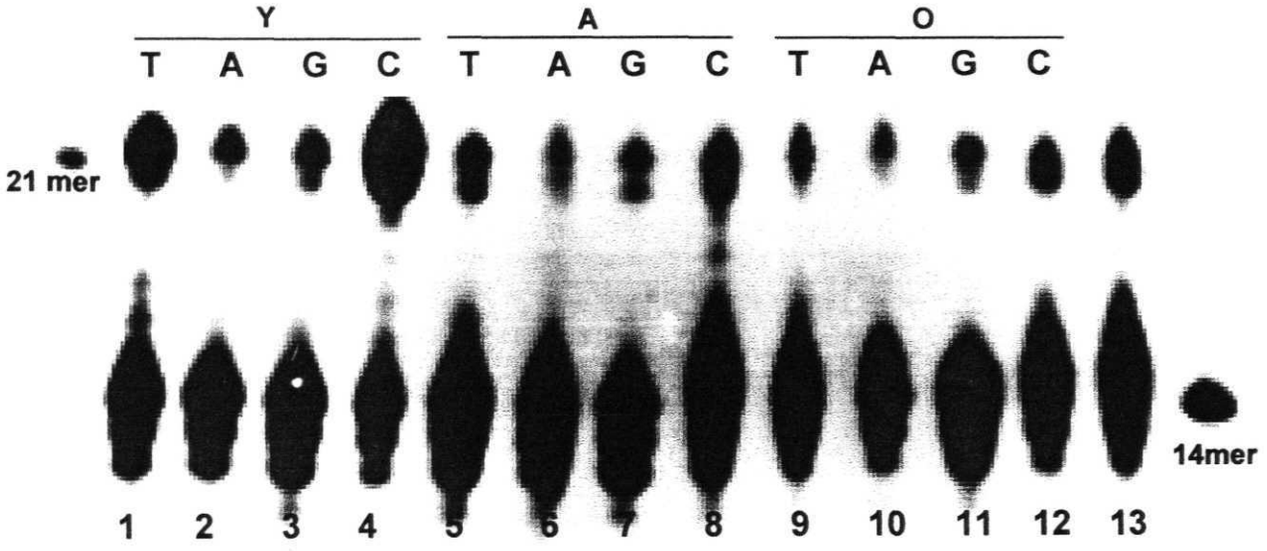
5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g A g t t a c c g - 5' (21 mer -A- oligo 4)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g G g t t a c c g - 5' (21 mer -G- oligo 5)

Figure 6

Exo-extension activity when neuronal extracts (young-Y, adult-A and old-O) supplemented with 0. 25 units of calf thymus a-poiymerase.

One unit of polymerase activity is equivalent to 1 n mole of total nucleotides incorporated into acid insoluble fraction in 1 hr. at 37° C with activated DNA as substrate. A typical autoradiogram is shown and the same pattern of results is seen in 2 different experiments. Other notations are similar to Figure 3



5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

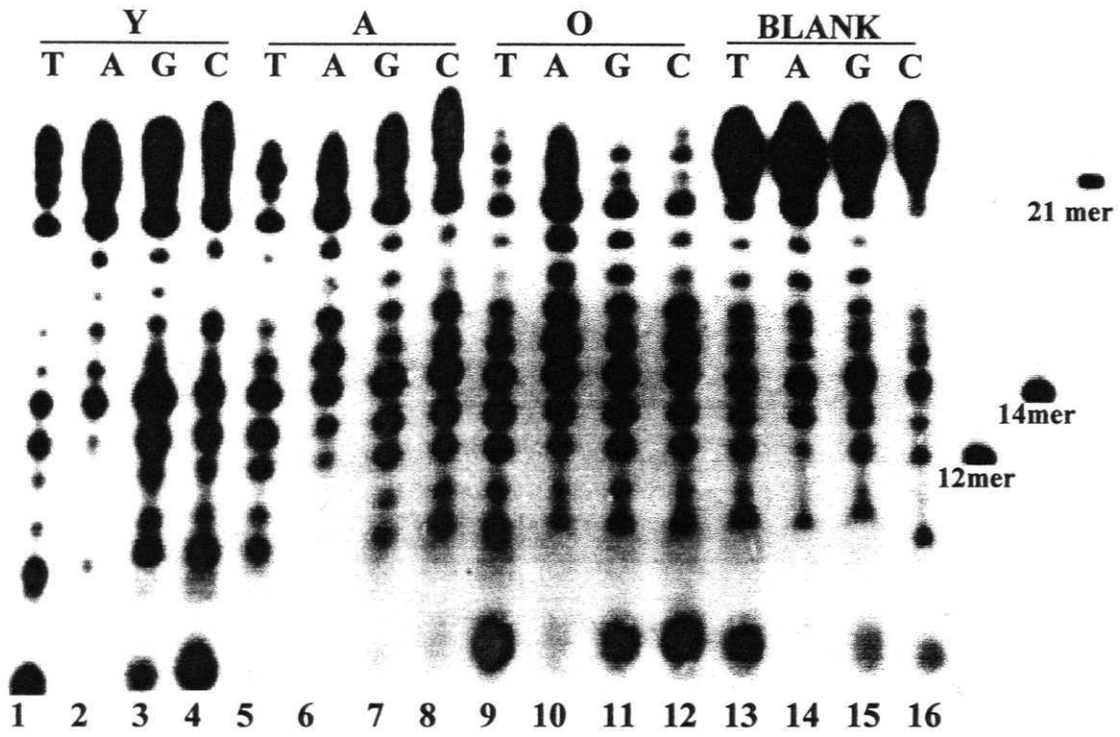
5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g A g t t a c c g - 5' (21 mer -A- oligo 4)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g G g t t a c c g - 5' (21 mer -G- oligo 5)

Figure 7

Exo-extension activity of *E. coll* polymerase I alone (Lanes 13-16) or when added to neuronal extracts (young-Y, adult-A and old-O) with the four different oligo duplex template primers.

2 units of the *E. coli* polymerase I and 10 μg of neuronal extract were used in all cases. A typical autoradiogram is shown. Other details and notations are as in Fig 3.



5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

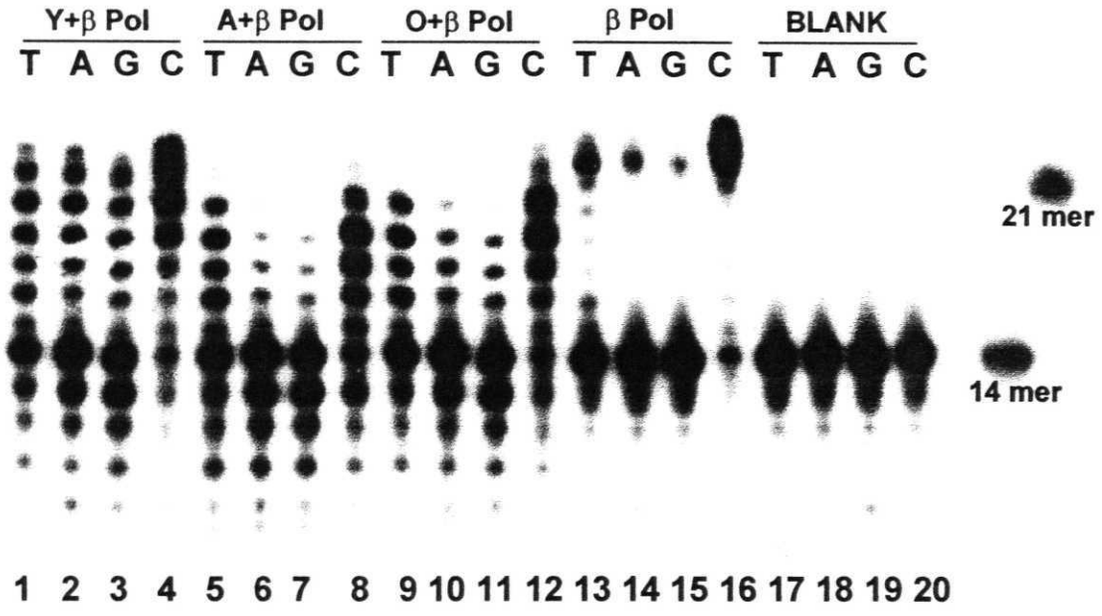
5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g A g t t a c c g - 5' (21 mer -A- oligo 4)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g G g t t a c c g - 5' (21 mer -G- oligo 5)

Figure 8

Exo-extension activity in rat brain neuronal extracts of different ages when supplemented with rat liver pol (3 with four different oligoduplex template primers

(Lanes 1-4,Y, young; lanes 5-8,A, adult and lanes 9-12,O, old). 2.5 units of the purified pol **p** and 10μg of neuronal extract protein were used in all cases. One unit of polymerase activity is equivalent to 1nmole of total nucleotides incorporated into acid insoluble fraction in lh at 37°C. Lanes 13-16, pol β alone without neuronal extracts and lanes 17-20,no enzyme controls. A typical autoradiogram is shown and the same pattern of results is seen in 3 different experiments.



5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

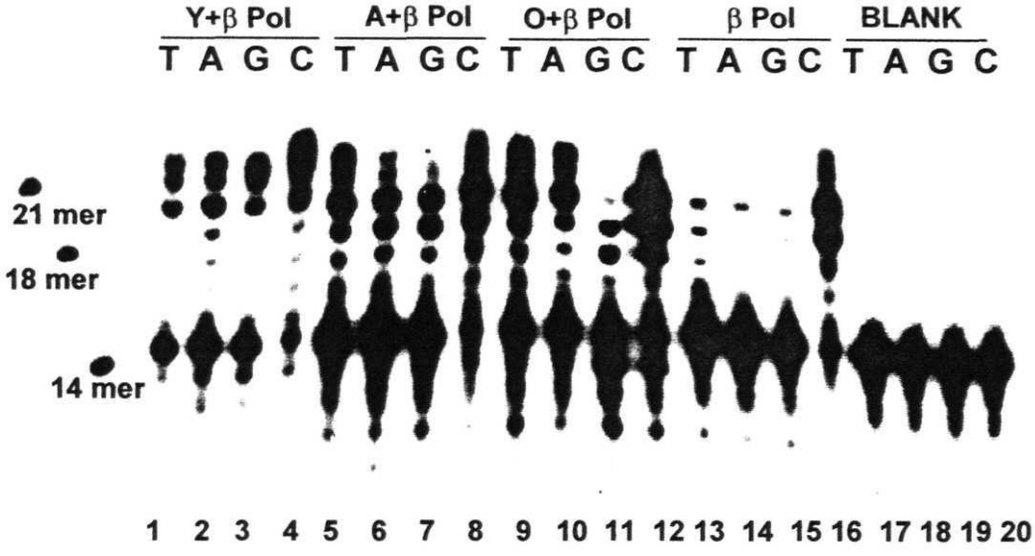
5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g A g t t a c c g - 5' (21 mer -A- oligo 4)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g G g t t a c c g - 5' (21 mer -G- oligo 5)

Figure 9

Two-step reaction to assess the effect of prior incubation of the oligo duplex template primers **with the neuronal extracts (young-Y, adult -A, and old-O) on the primer elongation achieved by pol β in the subsequent step.**

A typical autoradiogram is shown and the same pattern of results is seen in 3 different experiments. During the first step, the four oligo duplexes were incubated with neuronal extracts for 10 min at 37°C in the absence of any exogenous polymerase and dNTPs. The reaction was stopped by heating at 70°C for 10 min. In the second step 20 μM dNTPs and pol **p** (2.5 units) were added and the incubation at 37°C was continued for 20 min. For rest of the details and notations please see Fig. 3 and chapter 2.



5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

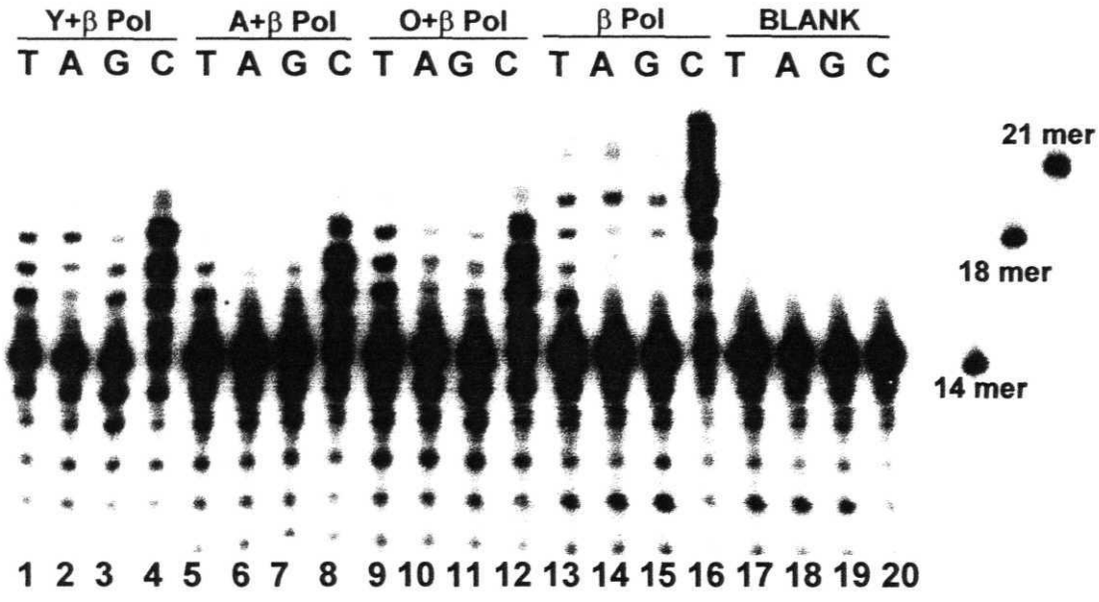
5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g A g t t a c c g - 5' (21 mer -A- oligo 4)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g G g t t a c c g - 5' (21 mer -G- oligo 5)

Figure 10

Effect of 5 mM GMP on the exo-extension activity of rat brain neuronal extracts (young-Y, adult -A, and old-O) supplemented with rat liver pol β (2.5 units) or of pol β (lanes 12-16) alone with the four oligo duplexes as template primers.

Notations are the same as in earlier figures. A typical autoradiogram is shown and the same pattern of results is seen in 2 different experiments. Note the inhibition of the extension activity, particularly of the mismatched primers.



5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

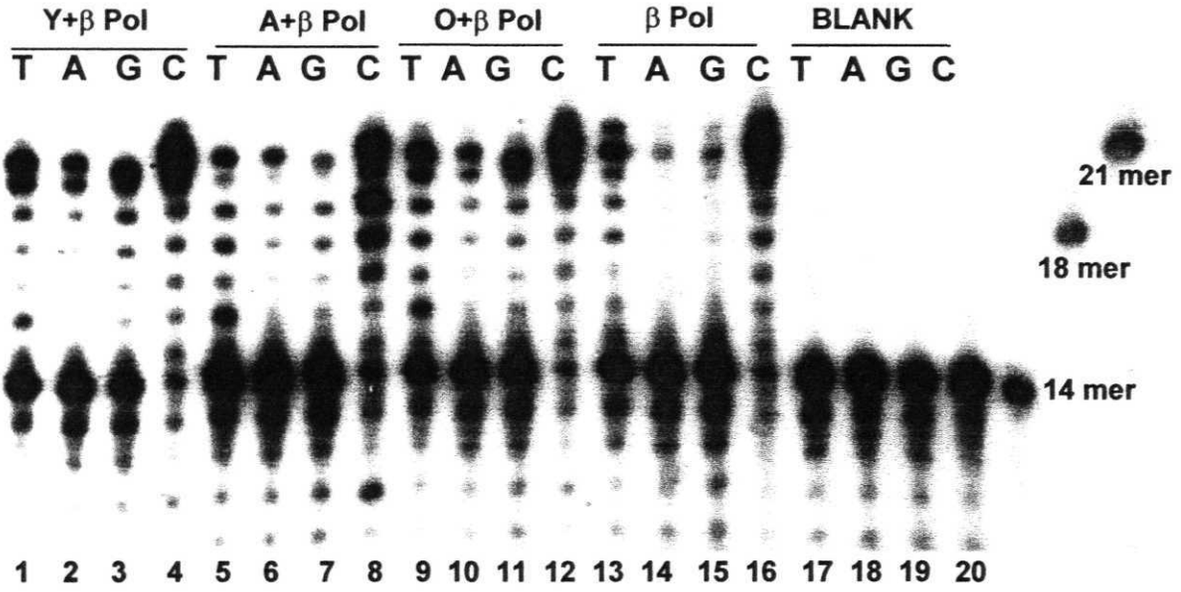
5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g A g t t a c c g - 5' (21 mer -A- oligo 4)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' -g c g c t a g c c a t c g G g t t a c c g - 5' (21 mer -G- oligo 5)

Figure 11

Effect of 1 mM Mn^{++} in place of Mg^{++} in the reaction mixture on the exo-extension activity (single step) of rat brain neuronal extracts (young-Y, adult -A, and old-O), supplemented with 2.5 units of rat liver **pol** (3 **or pol β alone (no neuronal extract).**

A typical autoradiogram is shown and the same pattern of results is seen in 3 different experiments. Notations same as in earlier figures. Note the improved extension activity and the processivity of the extension.



5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

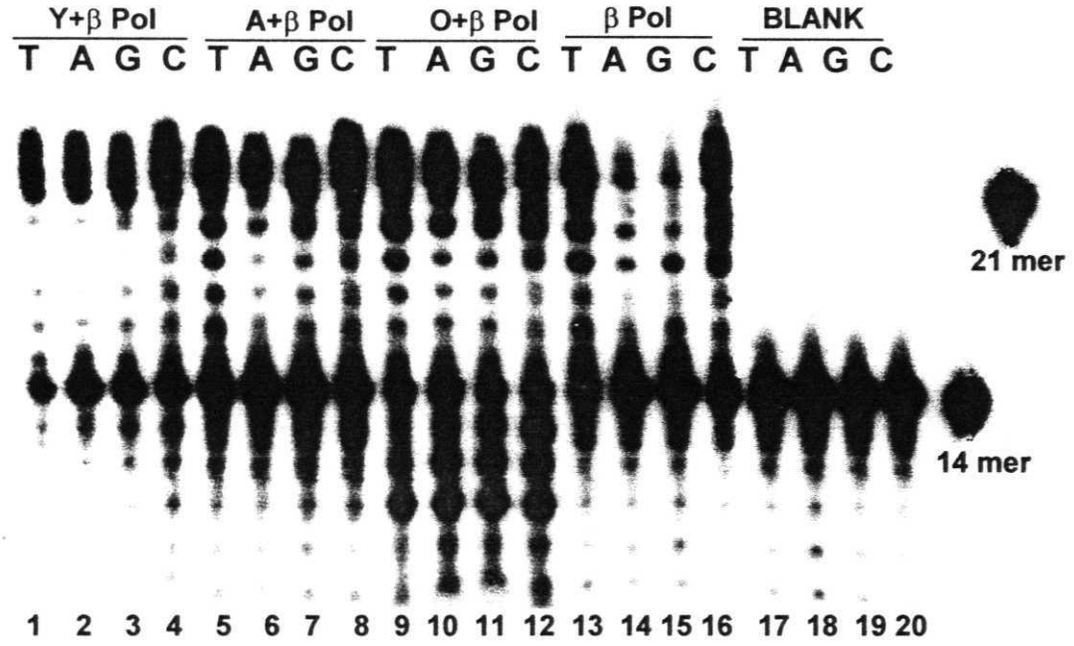
5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g A g t t a c c g - 5' (21 mer -A- oligo 4)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g G g t t a c c g - 5' (21 mer -G- oligo 5)

Figure 12

Effect of 1 mM Mn^{++} in place of Mg^{++} in the second step of the two-step reaction to assess the exo-extension activity of rat brain neuronal extracts (young-Y, adult -A, and old-O), supplemented with 2.5 units of rat liver pol β.

For details regarding the two step reaction, please see Fig. 9 and chapter 2. A typical autoradiogram is shown and the same pattern of results is seen in 2 different experiments. Notations are as in earlier figures. Note the marked improvement in the extension activity at all ages with product being the predicted 21 -mer.



5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

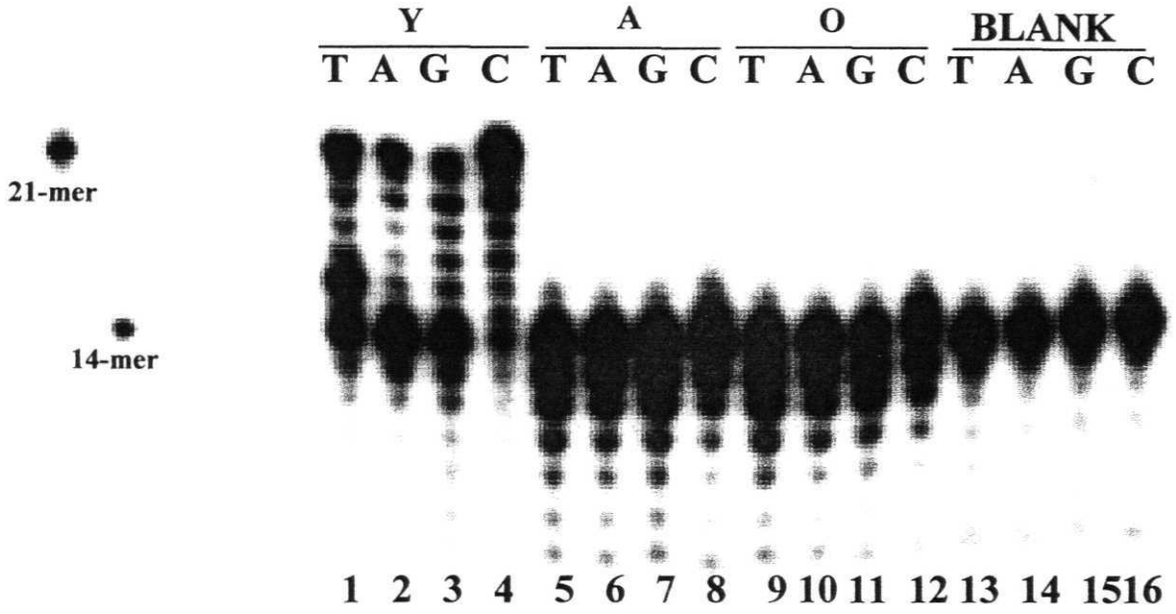
5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g A g t t a c c g - 5' (21 mer -A- oligo 4)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g G g t t a c c g - 5' (21 mer -G- oligo 5)

Figure 13

Primer extension / exonuclease activity in neuronai extracts prepared from young, adult and old rat brains.

The reaction mixture contained 100μM dNTPs and 1 mM Mn^{++} replacing the usual Mg^{++}. Incubation time 20 min. The autoradiogram shown is a typical one. The same pattern of results is seen in 2 different experiments.



5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

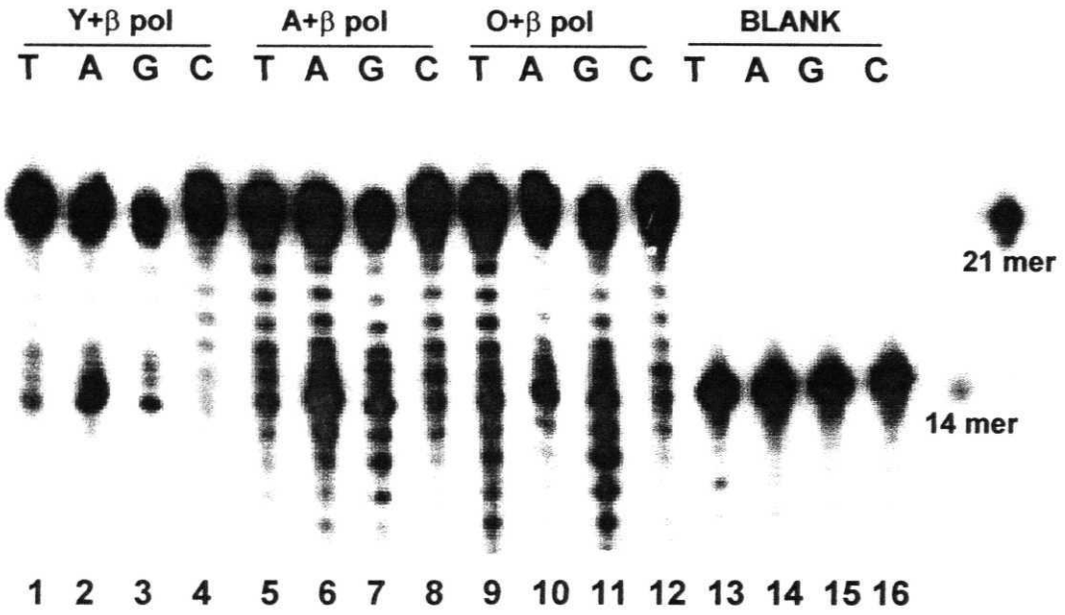
5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g A g t t a c c g - 5' (21 mer -A- oligo 4)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g G g t t a c c g - 5' (21 mer -G- oligo 5)

Figure 14

Two-step reaction to assess the effect of prior incubation of the oligo duplex template primers with **the neuronal extracts, on** the primer **elongation achieved by recombinant pol β in the subsequent step.**

In the second step, Mn^{++} replaced Mg^{++} and dNTPs were at 100 μM concentration. Y+ pol β- Neuronal extracts prepared from 'young' animals plus pure pol p; A+ pol β- Neuronal extracts prepared from 'Adult' animals plus pure pol P; O+ pol β- Neuronal extracts prepared from 'Old' animals plus pure pol p. All other notations are as in Fig 3. The amount of recombinant pol **p** added in the second step was 2.5 units. The autoradiogram shown is a typical one. The same pattern of results is seen in 2 different experiments.



5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g A g t t a c c g - 5' (21 mer -A- oligo 4)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g G g t t a c c g - 5' (21 mer -G- oligo 5)

It was, however became necessary to establish that pol β aided extension of a primer with a 3' mismatch will occur optimally only after the removal of the mismatch and that such proof reading activity is indeed present in neuronal extracts. Moreover, it is also important to know the status of such proof reading activity in brain cells with respect to the age of the animal since this activity could become a constraint for pol β function.

For this we have adopted a 3 step reaction strategy to examine directly whether the predicted oligo (12 mer) is obtained after the restriction digestion of the extended product of the step 2 mentioned above with HinPl. The restriction digestion is carried in a reaction volume of 40 ul with lOunits of HinPI with the 10x reaction buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9 at 25°C). The incubation is carried at 37°C for 6 hours and then stopped by adding 1ul of 0.5M EDTA. The products were identified by gel electrophoresis followed by autoradiography(chapter 2 for details)

The strategy behind the third step of HinPl digestion is as follows. If a correctly matched oligo template-primer (G-C) is the substrate then proof reading activity is not required for the primer during the first incubation. In the second Pol β catalyzes the extension of the primer (14 mer) using the other strand (21-mer) as template and the extended product would be obtained as follows.

^{32}P-5'-C G C G A T C G G T A G C G 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'

If the mismatched template is formed by annealing template with either T or A or G at the 14th position from 3' end then the oligoduplex formed would have a mismatched base at the 3' end of the primer. The extended product of this mismatched primer (for example in the

case of a G-T mismatch) the following duplex would be the product after the two step reaction.

^{32}P-5'-C G C G A T C G G T A G C G C A A T G G C –3'
3'-G C G C T A G C C A T C G T G T T A C C G-5'

If the above products, either from correctly matched and mismatched duplex substrates were, treated with restriction endonuclease HinP1, with a specificity towards tetranucleotide base sequence 5'-G C G C-3' a labeled 12-mer would be seen on the sequencing gel because of the cleavage of the upper strand at the site shown by arrow. Which means that when there was no mismatched base at 3 end of the primer or even if it was there but not removed before the extension occurred, a labeled 12-mer will be seen in the autoradiogram. On the other hand, if the extension of the primer occurred after the removal of the mismatched base at 3'end of the primer the following duplex would have formed.

^{32}P-5'-C G C G A T C G G T A G C A C A A T G G C - 3 '
3'-G C G C T A G C C A T C G T G T T A C C G-5'

The restriction site in the upper strand for HinP1 to act upon is not present and therefore no labeled 12-mer would be seen on the gel. The results from the Figure 15 show that in the case of all the mismatched duplexes, the wrong base at the 3' end of the primer is removed before extension has taken place since no significant level of labeled product with a chain length of 12 nucleotides was seen (lanes 1-3, 5-7,9-11). With the correctly matched duplex (G-C) as the substrate a distinct 12 mer spot is seen in the autoradiogram. This suggests that in case of the correctly matched duplex significant extension has occurred when neuronal extracts were supplemented with Pol p. Pure Pol β in the absence of any neuronal extract

gave a 12 mer in the case of correctly matched duplex (G-C, lane 16) and very little extension is seen in the case of mismatched duplexes, G-A, G-G and G-T (lanes 13-15).

This result confirms that a mismatched primer cannot be extended by pol β with facility. The over all results of the three step reaction show that during the first step of the 3-step reaction the mismatches at the 3' end are removed by the proof reading activity present in the neuronal extracts thus allowing pol β to carry out the extension activity/repair activity during the second step even at adult and old ages (Figures 14). The results also indicate that the independent 3'-5' exonuclease present in neuronal extracts is necessary for subsequent extension of the mismatched primer in an optimal way by pol p.

Since the above resulted indicated the presence of a 3'-5' exonuclease activity in the neuronal extracts of all the ages, a systematic study was undertaken to know more about the exonuclease activity. The 14 mer is labeled on the 5'-side and annealed to two 21-mers,one correctly matched (G-C) and the other mismatched(G-T).The resulting duplexes were incubated for different time periods with 'young' neuronal extracts both in the absence and presence of dNTPs. The results are shown in Figure 16. In the absence of dNTPs, the 14-mer is excised apparently in a step wise manner from 3'end both in correctly matched duplex (G-C) and incorrectly matched duplex (G-T). No extension of the primer is seen. However, when dNTPs are present in the reaction mixture, along with the exonuclease activity some feeble extension of 14-mer is also seen which is more visible in the case of properly matched duplex (G-C, lanes 9-12).This result is taken to indicate that both the 3'-5' exonuclease and primer extension activity are simultaneously occurring when dNTPs are present while only exonuclease activity is seen in the absence of dNTPs.

In the next set of experiments, the exonuclease activity was assessed in neuronal extracts prepared from 'young' , 'adult' and 'old' rat brains. The reaction was carried out for a fixed time of 10min and the results are shown in Figure 17.Excision of 14-mer is seen at all ages and with both the duplexes(G-C and G-T).From the pattern of spots, it was clear that excision is occurring from the 3'-end and also in a step wise manner. It was however not clear, under the conditions, whether there is any difference in the activity with age of the extract or with the type of the duplex. These results were noticed a number of times. While it was clear that excision activity could be seen quantification of the activity became difficult because of number of spots below the 14-mer and variation and large variations from experiment to experiment. Therefore, these experiments were repeated with the difference that the 14-mer is labeled on the 3'side instead of 5'side.

The time course of 3'-5' exonuclease activity in 'young' neuronal extracts is presented in Figure 18. While in the upper portion a typical autoradiogram is shown, the average intensity of the 3'-labeled 14-mer (the substrate) band along with SD bars is shown in the lower portion of the figure. As can be seen the 14-mer is labeled on the 3'-side by the addition of just one or two radioactive nucleotide under controlled conditions of terminal transferase reaction. The radioactivity in the substrate has disappeared gradually with time of incubation.

At 20 min of incubation time about 80% of the label has disappeared indicating the removal of radioactive nucleotide on the 3' end in a time dependent fashion. No radioactive spots at shorter lengths are seen. The excision activity could be seen both with correctly matched duplex (G-C) as well as with incorrectly paired duplex (G-T). The presence of dNTPs in the reaction mixture does not seem to make any marked difference at 20 min of

incubation although the initial rates of base removal seem to be higher when dNTPs are present, particularly in the case of mismatched G-T duplex.

Next, the change if any, in this 3'-5' exonuclease activity was examined (Figure 19) Firstly, there is excision of the 3' base by 'young' extracts with both correctly matched and mismatched duplexes (G-C and G-T) and also in the presence and absence of dNTPs (see lanes 1,4,7 and 10 and compare their densitometric values with the no enzyme controls, lanes 13 and 14). However, significant excision activity could be seen in 'adult' and 'old' extracts only in the case of G-T mismatched duplex and that too only when dNTPs were present in the reaction mixture (lanes 10-12). This activity towards mismatched base decreases with age although considerable activity could still be noticed in 'old' extracts (lane 12).

Neuronal excision activity towards single strand oligos (14-mer and 21-mer) was also tested. Initially experiments were conducted with 5'-labeled oligos using the neuronal extracts prepared from the three age groups of rats. The results are shown in Figure 20. Both 14-mer and 21-mer are excised by neuronal extracts from 'young', 'adult' and ' old' animals. The 'young' extracts seem to be highly active towards 14-mer and the activity decreased with age. On the other hand, the 21-mer is excised by all the extracts with apparently similar efficiency.

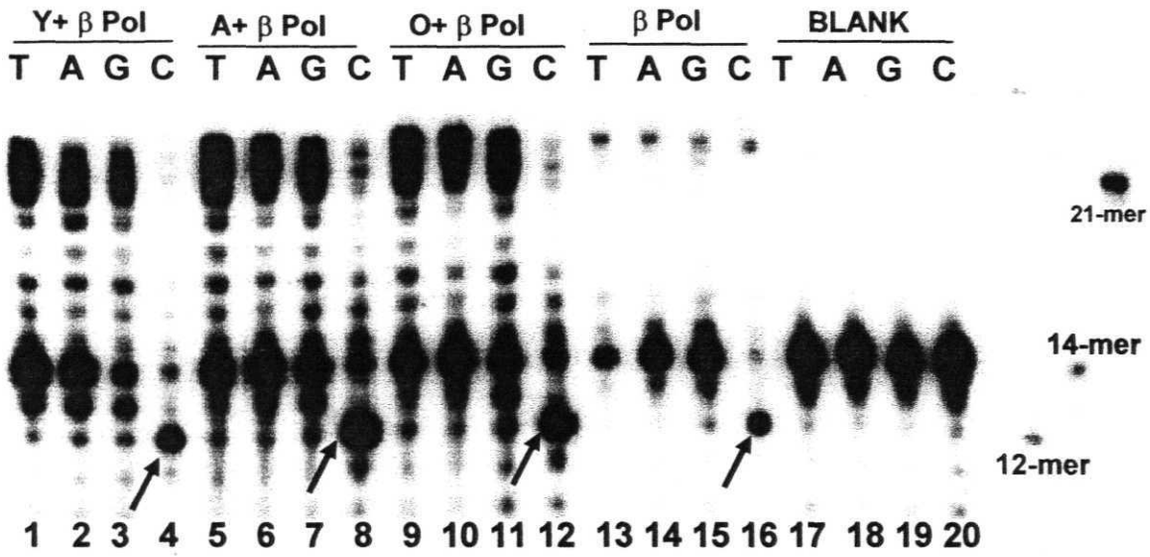
When the 14-mer and 21-mer were labeled on the 3' end and presented to neuronal extracts excision activity could be followed in a more clear cut manner. The time course of such a reaction is shown in Figure 21. It is seen that extracts of all the ages show increasing activity with time. If one compares the activity at 10min incubation, it can be assumed that

'young' extracts show higher activity (compare the spots in lane 4,8, and 12). However, 'adult' and 'old' extracts still retain considerable activity. Figure 22 shows the results with respect to the 21-mer labeled on the 3'side. 3'-5' exonuclease activity could be seen in the neuronal extracts of all the three ages and no striking changes in the activity could be visualized with respect to age. If anything the 'old' extracts showed slightly more activity.

Figure 15

Three-step reaction to examine whether the incorrect base at the 3'end of the primer is removed by the 3'-5-exonuclease activity of the neuronal extracts before the extension of the primer is affected by pol p.

In the third step, the products of the second step are subjected to HinP 1 restriction digestion. Significant amount of a labeled 12-mer (indicated by arrows) is seen on the autoradiogram only when the extension was carried out without the removal of the base at 3'end of the primer. (4,8,12). Lanes 13-16, only pol β without any neuronal extract while lanes 17-20, 'no enzyme' controls. Mobilities of standard 14-mer and 21-mer are shown. For other details regarding the strategy of this 3-step reaction and notations, chapter 2 and Figs. 13 and 14. The autoradiogram is a typical one from 2 different experiments.



5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

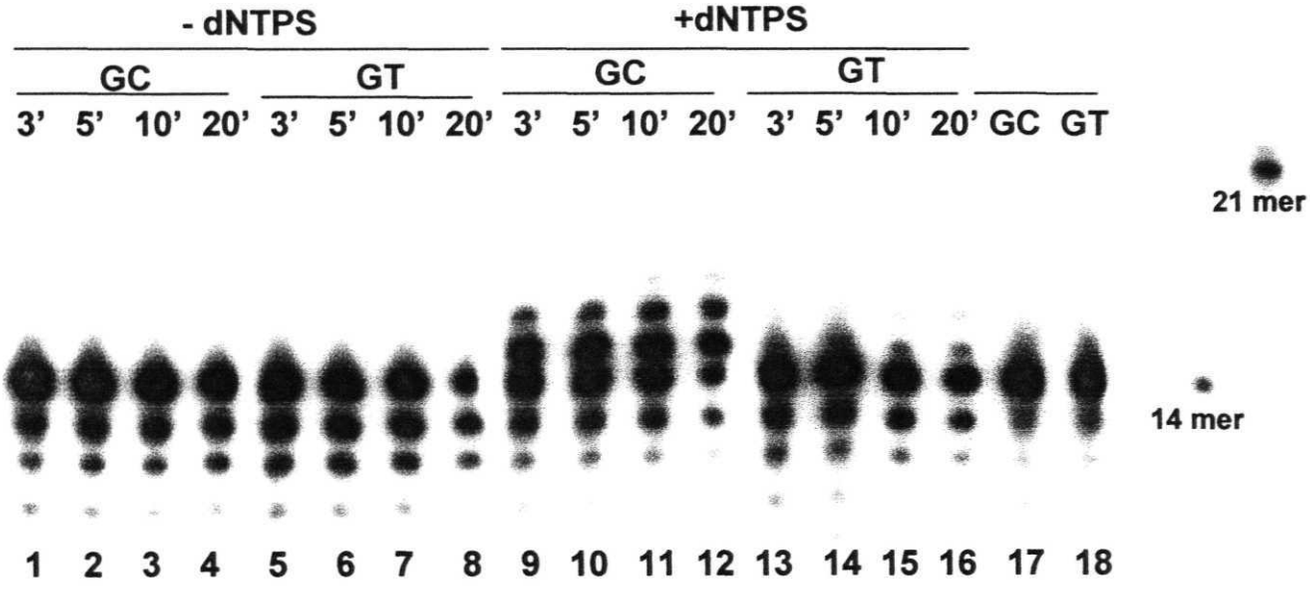
5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g A g t t a c c g - 5' (21 mer -A- oligo 4)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g G g t t a c c g - 5' (21 mer -G- oligo 5)

Figure 16

Time-course of degradation of 5'-labeled 14-mer annealed to two 21-mers *to* give either a correctly matched duplex (G-C) or mismatched duplex (G-T). Only neuronal extracts from 'young' rats were used in this experiment. Time periods of incubation are shown on **top** of each lane. Incubations were carried out without (lanes 1-8) and with 20 μM dNTPs (lanes 9 and 16). Lanes 17 and 18 are controls without neuronal extracts. Standard 14-mer **and** 21-mer are also shown. **Other details of the incubation are given in chapter 2.**



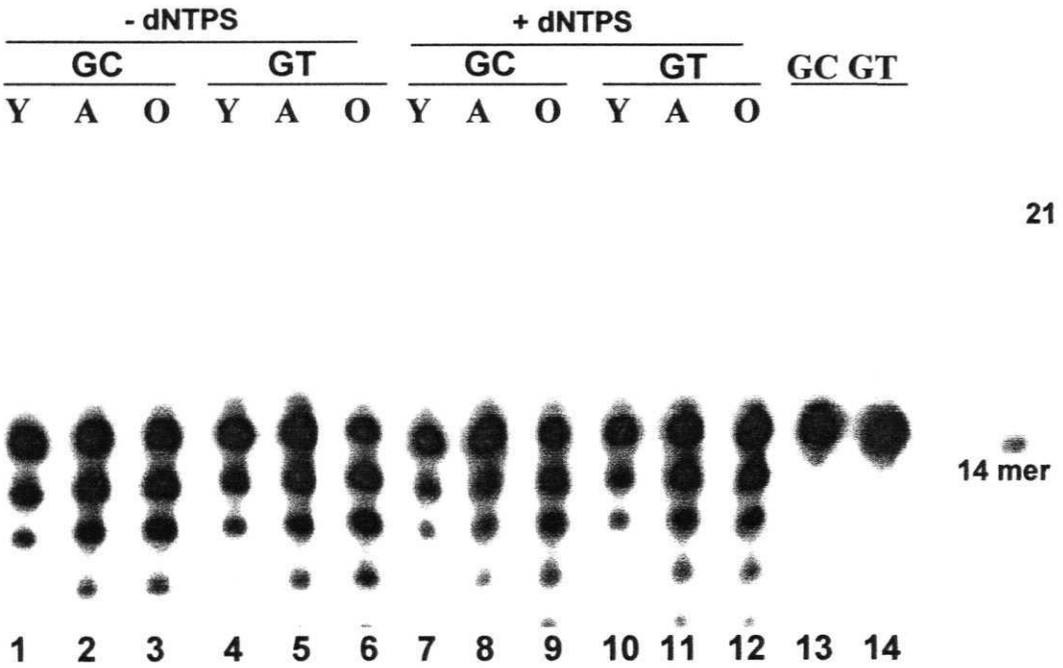
5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

Figure 17

Excision of 5'-labeled 14-mer hybridized to 21-mers by 'young'-Y, 'adult'-A and **'old'-O, neuronal extracts.**

Two duplexes, one correctly matched (G-C) and the other with a mismatch (G-T) were used. Incubation time 10 min. Reaction carried out both without (lanes 1-6) and with 20 μM dNTPs (lanes 7-12). The mobility of labeled standard 14-mer and 21-mer are also shown. A typical autoradiogram from two different experiments is shown.



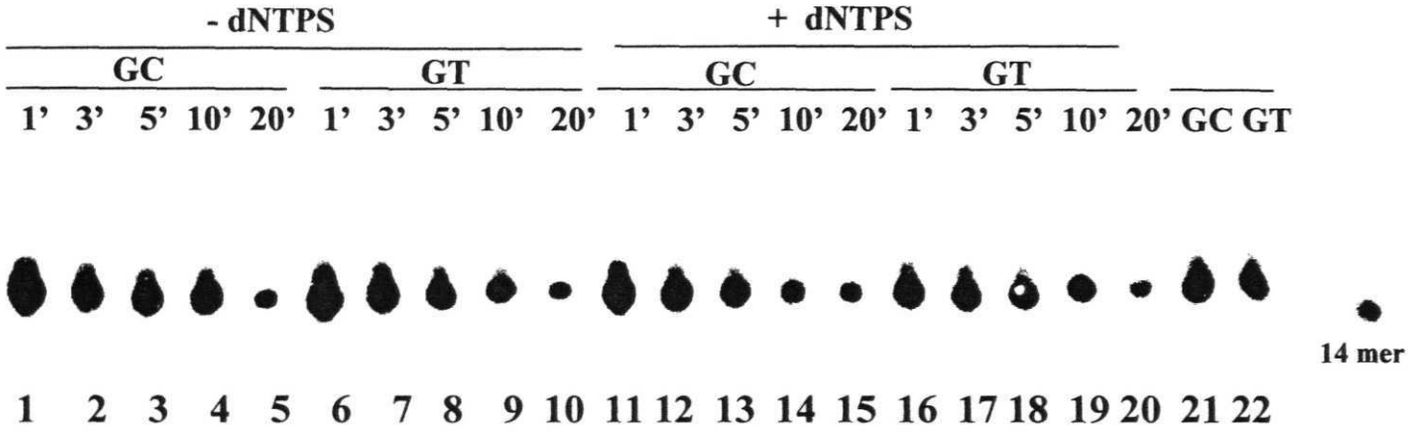
5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g C g t t a c c g - 5' (21 mer -C- oligo 2)

5' - c g c g a t c g g t a g c G -3' (14 mer- oligo 1)
3' - g c g c t a g c c a t c g T g t t a c c g - 5' (21 mer -T- oligo 3)

Figure 18

Time-course of excision of 3'-labeled 14-mer annealed to two 21-mers to give **either a correctly matched duplex (G-C) or mismatched duplex (G-T).**

Neuronal extracts from 'young' rats were only used in this experiment. Time periods of incubation are shown. Incubations were carried out without (lanes 1-10) and with 20 uM dNTPs (lanes 11-20). Lanes 21 and 22 are controls without neuronal extracts. Standard 14-mer and 21-mer are also shown. For other details of the incubation, see chapter 2. The excision activity is followed by the decrease in radioactivity in the substrate. Below the autoradiogram is given a bar diagram showing the average intensity with SD of the spots from 3 different experiments.


- dNTPS
+ dNTPS
GC
GT
GC
GT
1' 3' 5' 10' 20' 1' 3' 5' 10' 20' 1' 3' 5' 10' 20' 1' 3' 5' 10' 20' GC GT
14 mer
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

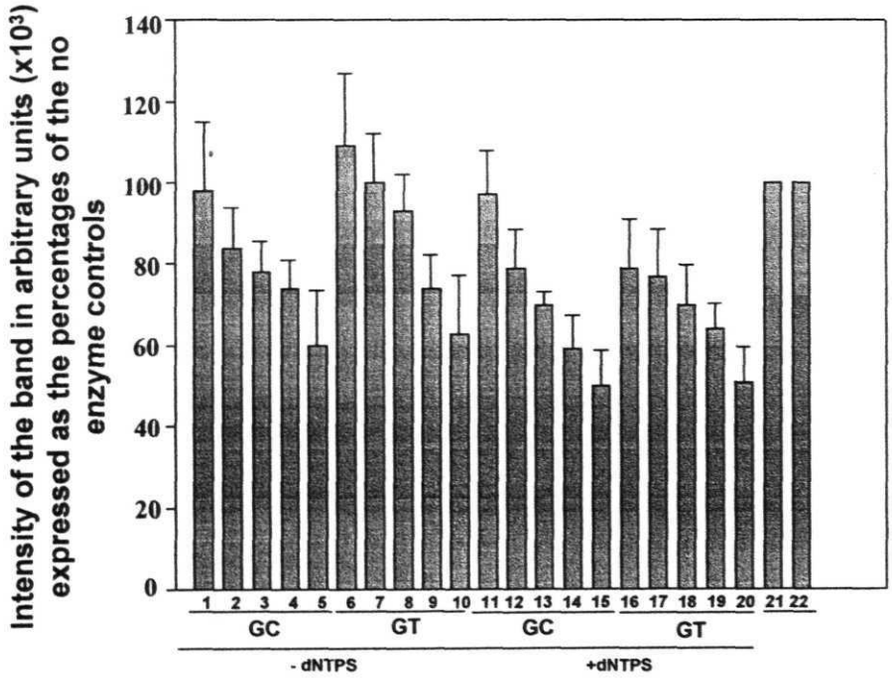
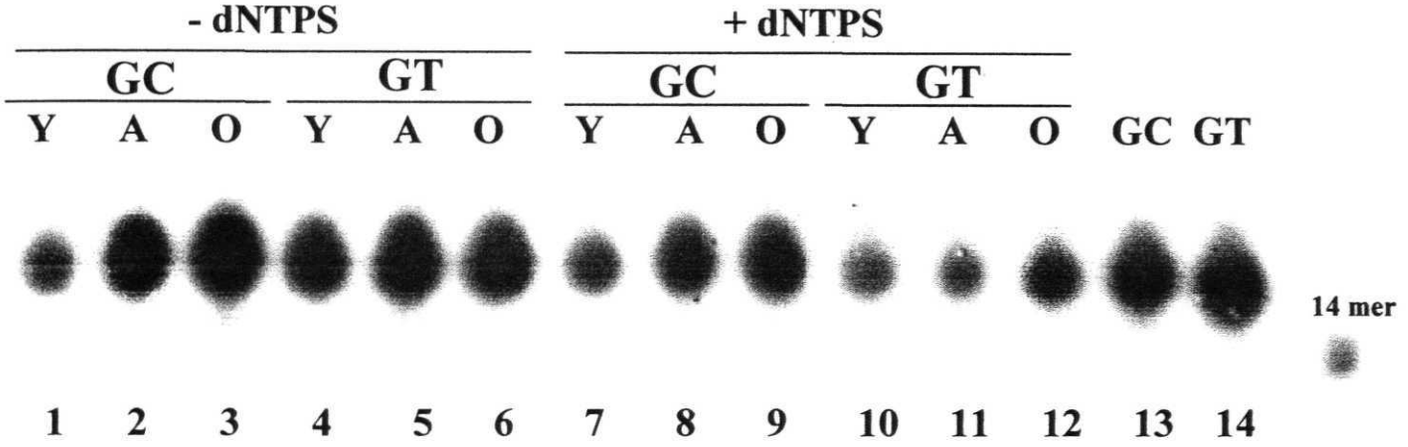

Intensity of the band in arbitrary units (x10³) expressed as the percentages of the no enzyme controls
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
GC
GT
GC
GT
- dNTPS
+dNTPS

Figure 19

Excision of 3'-labeled 14-mer hybridized to 21-mers, by 'young'-Y, 'adult'-A and 'old'-O, neuronal extracts.

Two duplexes, one correctly matched (G-C) and the other with a mismatch (G-T) were used. Incubation time 10 min. Reaction carried out both without (lanes 1-6) and with 20 μM dNTPs (lanes 7-12). Lanes 13 and 14 are no enzyme controls. The mobility of labeled standard 14-mer is also shown. Activity is followed from the decrease of label in 14-mer spots. A bar diagram is given to show the average intensity with SD of the spots from 4 different experiments.


- dNTPS
+ dNTPS
GC
GT
GC
GT
Y A O Y A O Y A O Y A O GC GT
14 mer
1 2 3 4 5 6 7 8 9 10 11 12 13 14

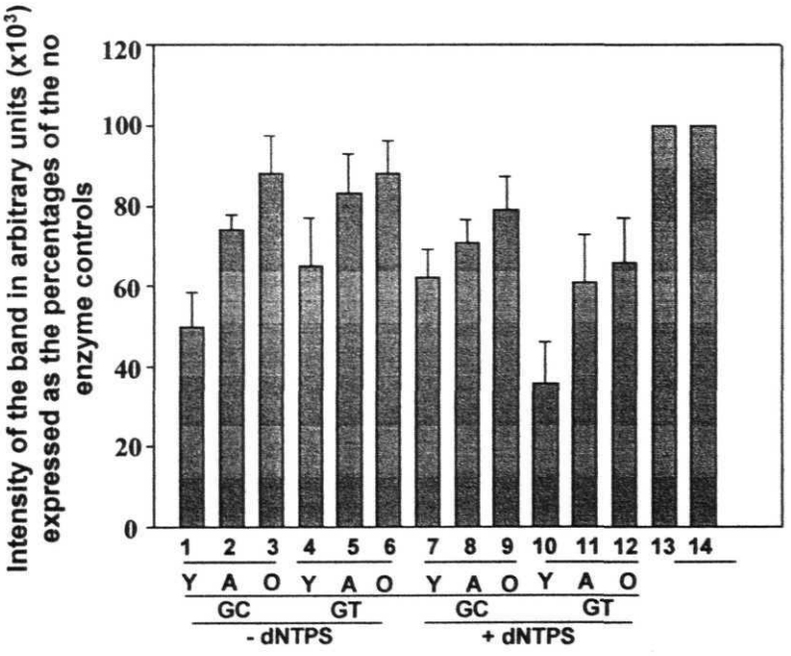

Intensity of the band in arbitrary units (x10³) expressed as the percentages of the no enzyme controls
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Y A O Y A O Y A O Y A O
GC GT GC GT
- dNTPS + dNTPS

Figure 20

Exonuclease activity of 'young'-Y, 'adult'-A and 'old'-O neuronal extracts towards single strand 14-mer (lanes **1-3)** and **21 mer (lanes 4-6) labeled on 5'side.**

Lanes 7 and 8 are no enzyme control. Standard 14 and 21- mer labeled on 5' side are also shown.

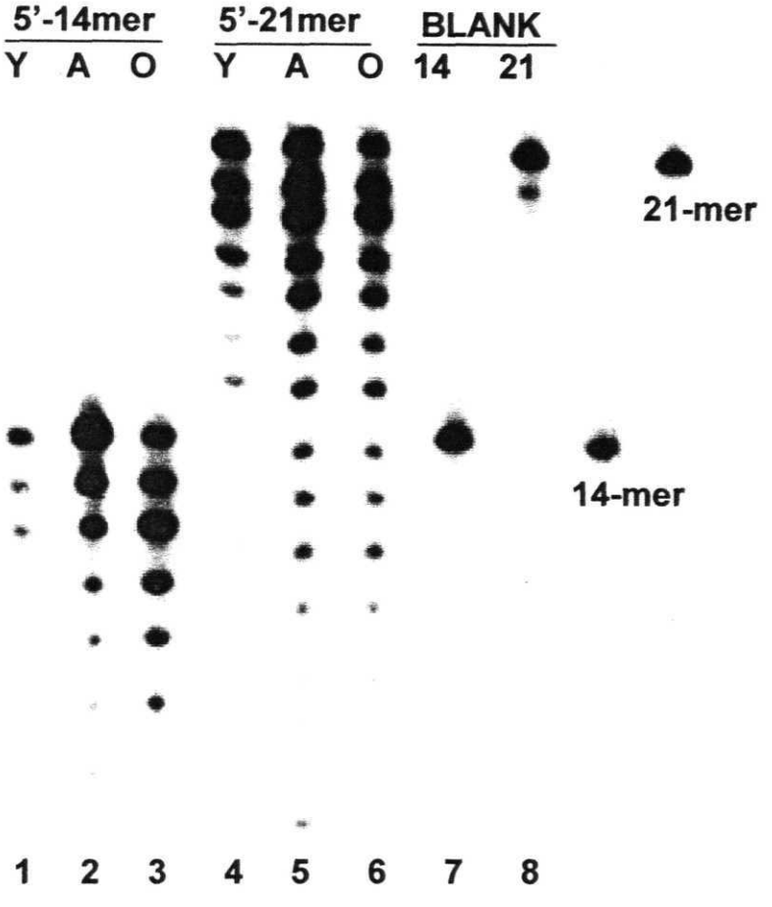

5'-14mer
Y A O
5'-21mer
Y A O
BLANK
14 21
21-mer
14-mer
1 2 3 4 5 6 7 8

Figure 21

Time course of the exonuclease activity of 'young'-Y, 'adult'-A and 'old'-O neuronal extracts towards single strand 14-mer labeled on 3'side.

The time of incubation is shown on the top of the spots. Lane 13 is no enzyme control. Standard 14 and 21- mer labeled on 5' side are also shown. Activity is followed from the disappearance of the label in substrate with time. A typical autoradiogram from two different experiments is shown.

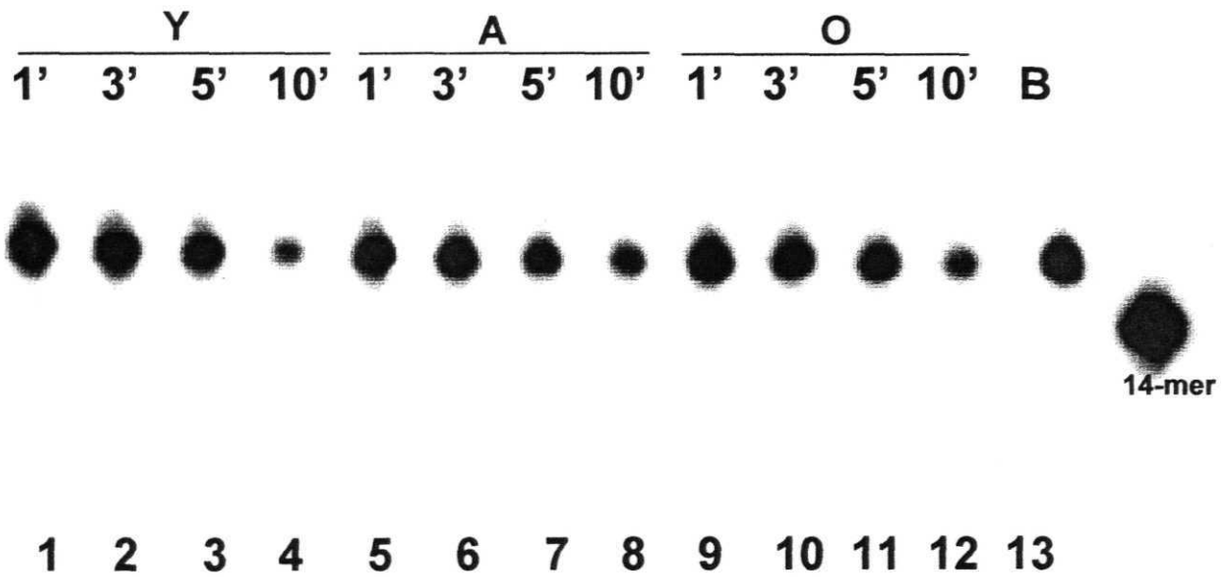
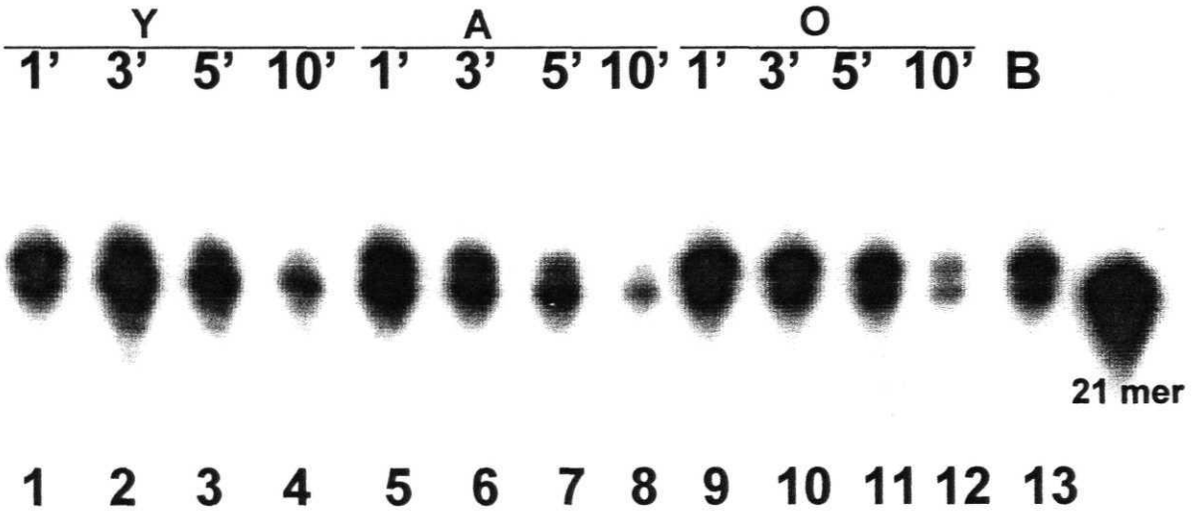

Y
A
O
1’ 3’ 5’ 10’ 1’ 3’ 5’ 10’ 1’ 3’ 5’ 10’ B
14-mer
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 22

Time course of the exonuclease activity of 'young'-Y, 'adult'-A and 'old'-O neuronai extracts towards single strand 21-mer labeled **on 3'side.**

The time of incubation is shown on the top of the spots. Lane 13 is no enzyme control. Standard 14 and 21- mer labeled on 5' side are also shown. Activity is followed from the disappearance of the label in substrate with time. A typical autoradiogram from two different experiments is shown


Y
A
O
1' 3' 5' 10' 1' 3' 5' 10' 1' 3' 5' 10' B
21 mer
1 2 3 4 5 6 7 8 9 10 11 12 13

The above studies provide some information about the nature of 3'-5' exonuclease activity in rat neurons. The exonuclease activity decreases with age of the animal although the activity still remains in the old age significantly. Both single and double stranded DNA is excised from 3' side in a sequential manner (Figures 17-22). However when a duplex template primer is presented as substrate in the presence of dNTPs, some preferential excision of the mismatched base at the 3'-end of the primer is seen (Fig 18 and 19). In the past few years, an increasing number of mammalian genes that encode for 3'-5' exonucleases not associated with a DNA polymerases have been identified in eukaryotic cells (Hubscher, 2002). However, Information regarding the exodeoxyribonuclease activity in brain is scanty. An exonuclease with single stranded DNA specificity has been isolated by Ivanov et al., (1983). A role for this enzyme, in conjunction with β-pol, in repairing depurinated DNA has been envisaged. This exonuclease however, acts in the direction of 5'-3'. A mismatch nicking (G-T and G-U) and an autonomous 3'-5' exonuclease activity in brain cells is reported only in the recent past (Brooks, 1996; Shevelev et al., 1996). However the reported activities exhibit considerable specificity towards G-T and G-U mismatches whereas in the present study no such absolute specificity is observed. In an interesting observation, (Chou and Cheng, 2002) found that human apurinic/apyrimidinic endonuclease (APE1) exhibits DNA exonuclease activity on mismatched deoxyribonucleotides at the 3' termini of nicked or gapped DNA. Since APE1, like pol p, is an important component of BER machinery and BER is the main repair pathway in brain tissue (Rao, 2002), it is possible that APE1 may be providing the proof reading activity as when necessary.

Be as it may, the present study shows the presence of 3'-5' exonuclease proofreading activity in rat neuronal extracts. This 3'-5' exonuclease activity facilitates the Pol β aided primer extension repair activity by removing the mismatched base present at the 3' end of the primer. It is not clear why the 3'-5' exonuclease activity in neurons acts on single strand DNA also. It is also not clear whether the same enzyme is acting on both recessed duplex and single strand DNA. Excision of single strand DNA may be necessary as a scavenging operation and also perhaps to generate dNTP pool in the cell for subsequent synthetic/repair activities. Although the exonuclease activity is found to decrease with age (Figure 20) the activity remaining in 'old' neurons appears to be adequate and is not a limiting factor for DNA repair activity since addition of pol p after the removal of the mismatch by endogenous proof reading activity, has markedly restored the primer extension activity (Figure 14). It thus appears that the most important limiting factor for carrying out DNA repair in brain seems to be pol β. Interestingly, pol p constitutes most of the DNA-polymerase activity in brain (Subrahmanyam and Rao 1988, Waser et al., 1979).

The importance of pol β in the general maintenance of genomic stability in brain is becoming pointedly apparent. For example, (Sugo et ah, 2000) have recently shown that mice deficient in pol p suffer from neonatal lethality and abnormal neurogenesis. Pol p also appears to be repairing the DNA-damage following cerebral injury due to a variety of reasons like hypoxia, ischemia etc., (Liu et al., 1996, Englander et al., 1999, Lin et al., 2000) and the fidelity with which such repair is carried out may determine the chances of recovery. Moreover, there are a number of genetic disorders that show elevated genomic damage and neurodegeneration with coupled symptoms of premature aging in some cases

(reviewed in Rao, 1997 and Brooks, 2002). It therefore, appears that the present results pointing out that pol β may be the most limiting component of BER machinery in aging rat brain neurons, should be of considerable importance.

CHAPTER 5

Reduced DNA gap repair in aging rat neuronal extracts and its restoration *in vitro* by DNA Polymerase β

Reduced DNA gap repair in aging rat neuronal extracts and its restoration *in vitro* by DNA Polymerase β

Introduction

Mammalian genomic DNA suffers damage from a variety of physical and chemical agents leading to the base loss or alterations. In view of the generally protected situation of the brain, the damage to the genomic DNA of the brain is mainly due to small changes in the bases of the DNA. Apurinic or apyrimidinic (AP) sites arise in DNA as a result of spontaneous hydrolysis of the N-glycosidic bond or the removal of the altered bases by DNA glycolyases (Lindahl, 1993). Approximately 10,000 AP sites are formed in each mammalian cell per day under normal physiological conditions (Lindahl and Nyberg, 1972). This should be higher in brain due to its high metabolic activity. The AP site should be promptly removed since it is a noncoding lesion which can lead to the misincorporation during replication and transcription (Friedberg, 1995).

DNA synthesis catalyzed by DNA polymerases is a cyclic process consisting of recruitment and creation of a 3'-OH primer terminus (Kornberg, 1974). The polymerase regains its initial configuration with respect to the invariant structural elements of the primer template. This is achieved by two ways. 1) processive mechanism where after the addition of the given dNMP residue, the enzyme translocates on the primer template and consequently capable of adding another dNMP, thus it is able to replicate long stretch of the same template. 2) Non processive or distributive in which the enzyme regains its configuration through dissociation and reassociation process after each catalytic step.

The major repair pathway protecting the cells against single base damages is thought to be the base excision repair (BER). The other error free repair pathway such as nucleotide excision repair can also be involved. Repair of AP sites is initiated by AP endonuclease which binds AP site and hydrolyzes the phosphodiester bond 5' to the abasic site, generating 5'terminal sugar phosphate (Lindahl, 1990; Lindahl and Wood, 1999). The 5'terminal sugar phosphate (dRp) is removed by AP lyase activity associated with DNA polymerase β (Matsumoto and Kim, 1995; Sobol et al., 2000). Pol p removes the dRp and simultaneously adds one nucleotide to the 3' ends of the nick. The most preferred substrate for Pol p seems to be double stranded DNA with a single nucleotide gap in one of the strands which is the *in vivo* situation following the removal of a damaged base by 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 atleast two sub pathways that are differentiated by repair patches and the enzymes involved. One sub pathway is Short patch BER or Single nucleotide repair and the other long patch repair pathway involving replacement of upto lOnucleotides (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 Lindhal, 1997). In cases where the terminal sugar phosphate after the 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) the repair synthesis would nevertheless continue but in a strand displacement manner. This long patch synthesis is catalyzed by either pol p or pol 5/s with associated proof reading activity.

This pathway is stimulated by Proliferating Cell Nuclear Antigen (PCNA) and requires a "flap" structure specific enonuclease-1 (FEN1) (Harrinton and Lieber, 1994) activity to cut the flap like structure produced by the strand displacement type synthesis by Pol (3 (Wu et al., 1996; Klungland and Lindahl, 1997). The role of PCNA seems to be stimulation of FEN1 activity and the repair size is about seven nucleotides (Frosina et al., 1996)

A slightly different long patch BER pathway in which Pol 8 or Pol s is involved instead of Pol p. Pol p null embryonic fibroblast cells were proficient in repairing oxidative damage although they were defective in uracil initiated repair (Sobol et al., 1996) and that the neutralizing antibody to Pol P, which inhibited repair synthesis catalyzed by pure Pol p 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 polymerase p the nick is sealed by DNA ligase. In case of the short patch repair pathway DNA ligase III along with its partner XRCC1 seals the nick (Cappelli et al., 1997). Where as DNA ligase I/III joins the nick in case of long patch repair pathway (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 short gap repair, the present study has been extended to examine Pol β aided BER with a model oligoduplex substrate containing a gap of 1 or 4 nucleotides. The gapped DNA substrates are formed as described in materials and methods (chapter 2). Using these gapped oligoduplexes the ability of the neuronal extracts to fill the gap is tested . The gap filling ability is also tested with respect to the age of the animals.

Methods

The sequences of the oligonucleotides used for generating the gapped oligoduplex are described in Chapter 2 and also shown in figures. Briefly, gapped oligoduplexes are formed by annealing a 14 mer (5'-kinased with ^{32}P-γ-ATP and T4 polynucleotide kinase) and unlabeled 14 or 17mer to a 32 mer. When the two 14 mers are annealed to the 32 mer, a 4 nucleotide gapped substrate is formed and when a 5' end labeled 14 mer and 17 mer were annealed to the 32 mer, a single nucleotide gap is formed. The annealing of these oligoduplexes were done as described in materials and methods (Chapter 2).

Gap repair assays

Gap repair assays with neuronal extracts was carried as follows. The final reaction volume of 30μl contained 20mM HEPES pH 7.5, 1mM $MgCl_2$, 0.1 mM DTT, 0.1mg/ml Bovine serum albumin, 2% glycerol and 20 μM of all the four dNTPS. 400 femtomole of the gapped oligo duplex containing 1 gap and 4 gap were incubated with 5μg of neuronal extract protein at 37^{0}C for 20minutes. The reaction was stopped by heating at 70°C for 10minutes. When the experiment is carried out with supplementation of pol β, 2.5 units of pure rat liver recombinant enzyme was added in the reaction along with neuronal extracts.

2 units of the T4 DNA ligase and/or 1 mM ATP were added to the reaction mixture as and when mentioned. Purification of the repaired products was done on sephadex G-50 spin column and the products were separated by sequencing gel electrophoresis.

Results and discussion

Figure 23 shows the gap filling activity in the presence of the 'young', 'adult' and 'old' neuronal extracts. Gap filling activity is very low in 'adult' and 'old' neuronal extracts (lanes 3-6). On the other hand, excision of labeled 14-mer to shorter lengths is seen. However, 'young' neuronal extracts showed the gap filling activity both with 1 gap and 4 gap substrates (lanes 1 and 2). But the extended 14-mer is not apparently joined to the down stream 17-mer and hence no labeled 32 –mer product is seen. Also, the number of nucleotides added to 14-mer appear to be more than the gap revealing a tendency of continued strand displacement synthesis. When the neuronal extracts are supplemented with pure recombinant rat liver DNA pol p (2.5units), a major spot corresponding to the length of 32-mer is seen. However, while the predominant spot is 32-mer, several spots/bands below 32-mer are also noticed . This ladder like bands are taken to indicate that pol β is no doubt filling up the gap but also adding nucleotides beyond the gap in a strand displacement manner reading the lower strand as template. This general pattern is the same at all the ages(lanes 7-12). In those tubes where only pol p was present (lanes 13 and 14) the pattern of extension is not very much different essentially suggesting that this is the property of the exogenously added pol β .

The pattern of results seen in Fig 23 brought up many questions. Firstly why even a short gap of 1 or 4 nucleotides is not filled and ligated by neuronal extracts even when supplemented with pure pol p. This is borne out by the fact that several bands/spots are seen below the 32-mer suggesting that pol p added nucleotides all the way to extend the 14-mer to 32-mer. It is also possible that both processive and distributive addition of nucleotides was taking place since considerable radioactivity is found in 32-mer spot apart from the

several bands below. It is pertinent to mention that Singhal and Wilson (1993) and Prasad et al., (1994) have shown that pol β can fill up a gap of upto 6 nucleotides in a processive manner and for that to happen the down stream primer must have a phosphate group on the 5' end. It is also reported that the most favored substrate for pol (3 is 1 nucleotide gap. In view of this existing information, the results seen in Fig 23 are not altogether unexpected in that the down stream primer in the model oligo substrate used did not have a phosphate group on 5' side. As a result of this, ligation after the addition of nucleotide(s) might not have taken place and the enzyme continued to add nucleotides, albeit slowly, up to the length of the template. It is not known whether the neuronal extracts used in this study possess the necessary activities to 5' phosphorylate the downstream primer and ligate the strands.

T4 DNA ligase is a bacteriophage ligases and is ATP-dependent (Armstrong et al., 1983, Nilsson and Magnusson 1982, Rossi, 1997). The ATP dependent DNA ligases catalyze the joining of single strand breaks (nicks) in the phosphodiester back bone of the double stranded DNA in a three step mechanism (Timson et al., 2000). The first step is the formation of a covalent enzyme-AMP complex and the ATP is cleaved to pyrophosphate and AMP, with the AMP being covalently joined to highly conserved lysine residue in the active site of the ligase. The activated AMP residue is then transferred to the 5'Phosphate of the nick, before nick is sealed by phosphodiester bond formation and AMP is eliminated.

In order to check these possibilities, the reaction was carried out in the presence of 1 mM ATP either alone or together with T4 DNA ligase. The results are shown in Fig 24. It may be seen that there is some improvement with ATP but still there are faint spots seen below the 32-mer spot. Addition of T4 DNA ligase did not show any benefit over and

above that already conferred by ATP. In any case it can be taken that ATP supplementation may be causing the phosphorylation of the down stream primer thus helping ligation of the added nucleotides to the down stream primer at least to some extent.

Therefore, the above experiments were repeated where in the oligo duplex with 1 and 4 gaps has a down stream primer which has been already phosphorylated on the 5' side. The results of these experiments are shown in Figs 25 and 26.

To begin with, neuronal extracts themselves, without any supplementation, have shown some improvement in repairing the gaps. In particular, 'young' neuronal extracts (lane 1 and 2, Fig 25) have shown detectable extension of the 14-mer to 32-mer. Very careful observation also reveals the even in the case of 'adult and 'old' neuronal extracts, very faint spots corresponding to 32-mer could be discerned. This means, there is significant gap repair activity present in 'young' neurons while in 'adult' and 'old' neurons, this activity is drastically reduced (lanes, 3-6). It is also apparent that for this gap repair activity to take place , presence of 5' phosphate on the down stream primer is very helpful. However, it is also clear that the gap repair is not solely processive and addition of nucleotides in a distributive and strand displacement manner is also taking place. Once the neuronal extracts were supplemented with pol p, there is a considerable increase in the 32-mer product at all the ages. This result is taken once again to pointing out that aging neurons are deficient of pol p. It is also seen that while pol β is improving the gap repair and formation of 32-mer, it is essentially through a slow strand displacement addition of nucleotides. As a positive control, cell free extracts from testis are also used in the experiment. In testis, as can be seen the gap repair is affected largely by a processive insertion of nucleotide to fill the gap followed by ligation.

When the neuronal extracts were supplemented with either ATP alone or together with T4 DNA ligase apart from pol (3, no marked difference was seen in the pattern of results (Fig 26). Thus the results are essentially similar to those seen when the extracts were supplemented with pol p alone (compare the lanes,7-12 of Fig 25 with all the lanes of Fig 26).

Pol p is considered as a repair polymerase. Several lines of evidence suggest a major role for this enzyme in gap filling repair and that too in the repair of short gaps . Even in the case of long patch BER, Podlutsky et al., (2001) have shown that in human cell extracts pol β is the major DNA polymerase incorporating the first nucleotide during the repair of reduced AP sites, thus initiating the long patch BER synthesis. With all these observations regarding the unique role of pol β in single or short gap DNA repair, the possible role for this small DNA polymerase in filling up long patches of gaps in a double strand DNA is largely ignored. The ability of pol β to extend a primer with long stretches of nucleotides, albeit slowly, using the other strand as template is also ignored.

For example, almost 30 years ago Wang et al., (1974) showed that Pol β is intrinsically capable of synthesizing 10-20 nucleotides at each nick by limited strand displacement manner. Using duplex primer template that contained limited numbers of nicks or gaps of defined average sizes, the same laboratory (Wang and Korn, 1980) again showed that KB and human liver DNA polymerase β performs limited synthetic reaction and incorporation of about 15 nucleotides at each nick where as polymerase a is unreactive towards gapped substrates. It was shown that Pol β prefers small gaps approximately 10 nucleotides long. Recently Wilson and Singhal (1993), using different levels of pol β on different gapped

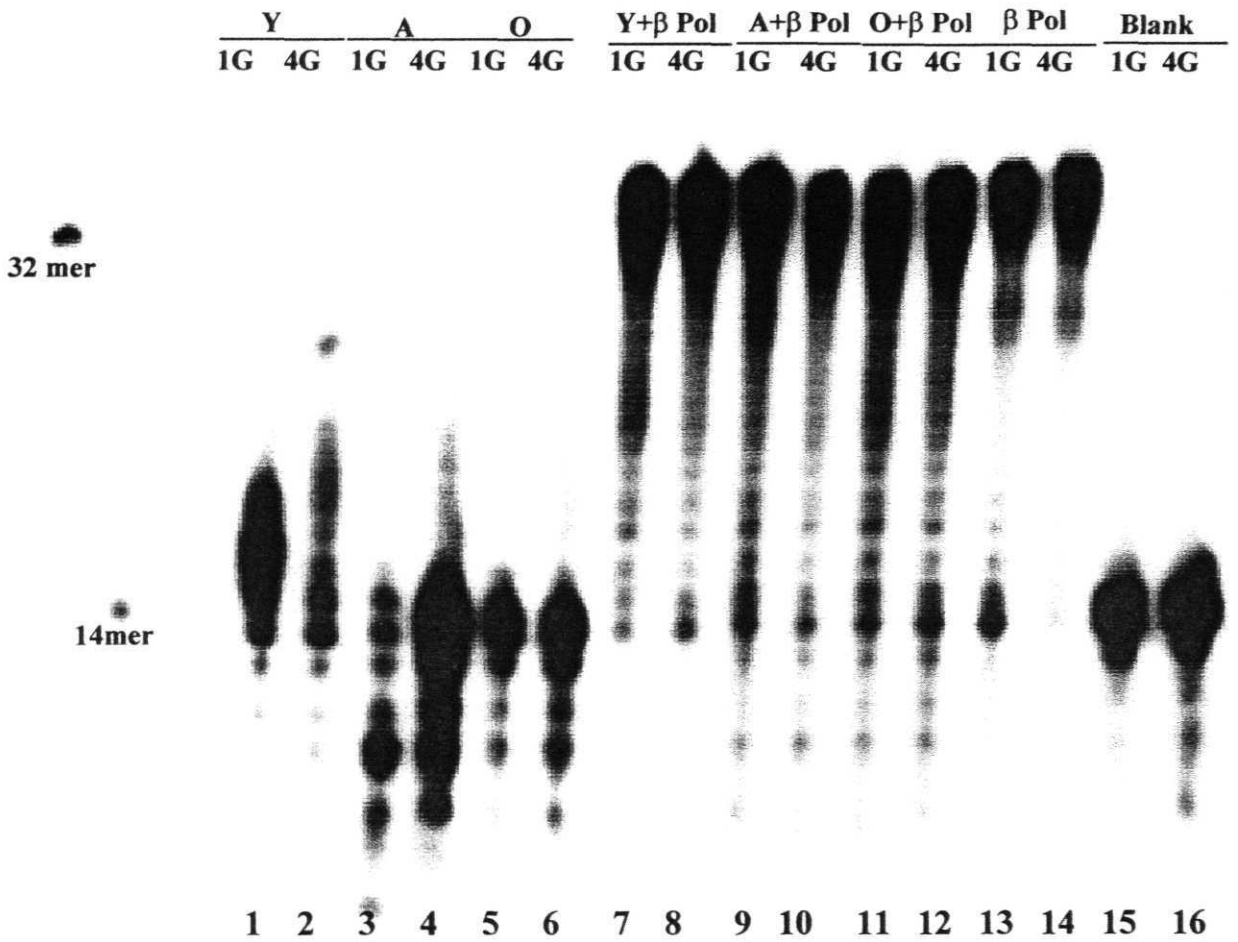
substrates showed that reactions with large amount of the enzyme, showed limited strand displacement synthesis. They have also shown that the extent of strand displacement by pol β is dependent on the time of incubation and the enzyme concentration. Limited strand displacement was observed even at low enzyme levels with longer incubation periods where as higher enzyme levels resulted in more extensive strand displacement. The results presented in this as well as in Chapter 4 indeed substantiate these earlier observations and demonstrate that pol p can incorporate a considerable number of nucleotides both to an open ended primer as well as to that in a gap. The addition, of course, seem to be a slow and distributive one displacing the down stream primer. It is possible that higher amounts of enzyme have been used in the present studies.

Be that as it may, the above results show that with gapped DNA as the substrate, the gap filling activity in neurons decreases with age of the animal and the supplementation of the reaction mixture with pure recombinant pol β restores this activity predominantly by a slow distributive strand displacement type of addition of nucleotides to the full length of that of template (in the present case, 32-mer). Save the exact mechanism of gap filling activity , the ability of pol p, the most predominant DNA polymerase present in brain, to restore the lost activity in aging neurons should be of considerable importance. This may raise possibilities for a potential with therapeutic consequences.

Figure 23

Gap filling activity in the **presence of the 'Young', 'adult' and 'old' neuronai extracts and supplemented with recombinant pure rat liver DNA polymerase β or β alone with no 5'- phosphate on the downstream primer.**

A typical autoradiogram is shown .Lanes 1-6 neuronai extracts from young brain (Y, 5 days postnatal), adult brain (A, 6 months) old brain (O,> 2 years) with out added pol β. Lanes 7-12(Y+P pol, A+P pol, O+ β pol) with added P-pol. Lanes 13 and 14 are with **p** pol alone. Lanes 15 and 16 are without any neuronai extracts (Enzyme blanks). The mobility of labeled standard 14-mer and 32-mer are also shown. Lanes 1,3,5,7,9,11,13,15 are with 1gap(lG). Lanes 2,4,6,8,10,14,16 are with 4 gap (4G) oligo duplex.



1 Gap Oligo duplex:

(14 mer) ^{32}P 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' (17 mer)
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' (32 mer)

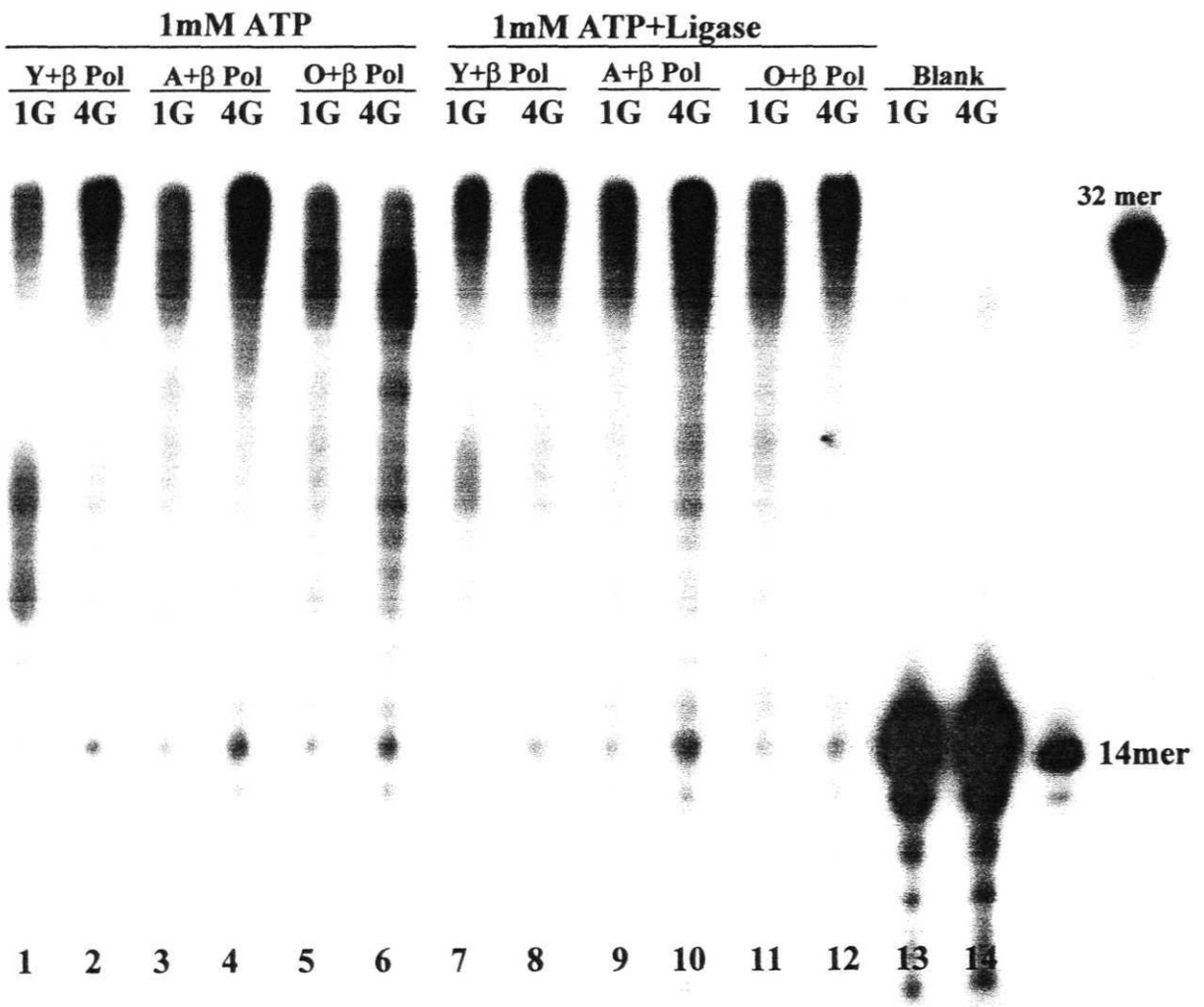
4 Gap Oligo duplex:

(14 mer) ^{32}P 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' (14 mer)
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' (32 mer)

Figure 24

Gap filling activity in the presence of 1mM ATP alone or together with T4 DNA ligase with 'Young', 'adult' and 'old' neuronal extracts and supplemented with recombinant pure rat liver DNA polymerase (3 with no 5'-phosphate on the downstream primer.

A typical autoradiogram is shown. Lanes 1-6 neuronal extracts from young brain (Y, 5 days postnatal), adult brain (A, 6 months) old brain (O, > 2 years) with out added pol β and lmM ATP. Lanes 7-12(Y+β pol, A+p pol, O+ p pol) with T4 DNA ligase and lmM ATP. Lanes 13 and 14 are without any neuronal extracts (Enzyme blanks). The mobility of labeled standard 14-mer and 32-mer are also shown. Other notations are similar to the Figure 23



1 Gap Oligo duplex:

(14 mer) ^{32}P 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' (17 mer)
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' (32 mer)

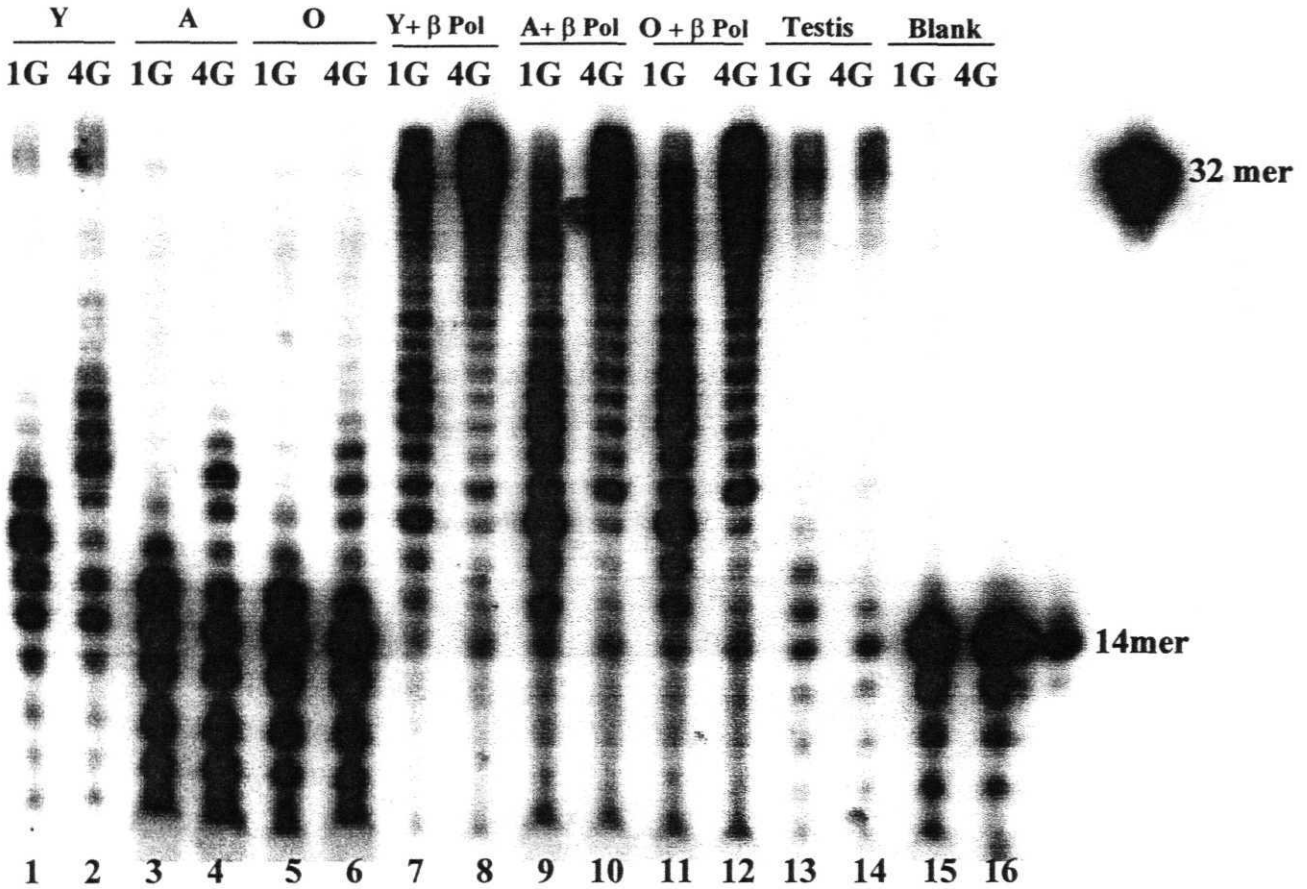
4 Gap Oligo duplex:

(14 mer) ^{32}P 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' (14 mer)
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' (32 mer)

Figure 25

Gap filling activity in the presence of the 'Young', 'adult' and 'old' neuronal extracts and supplemented with recombinant pure rat liver DNA polymerase β or β alone with 5'-PO_4 on the downstream primer.

A typical autoradiogram is shown. Lanes 1-6 neuronal extracts from young brain (Y, 5 days postnatal), adult brain (A, 6 months) old brain (O, > 2 years) with out added pol β. Lanes 7-12(Y+p pol, A+P pol, 0+ β pol). Lanes 13 and 14 are with testis extracts alone. Lanes 15 and 16 are without any neuronal extracts (Enzyme blanks). The mobility of labeled standard 14-mer and 32-mer are also shown.



1 Gap Oligo duplex:

(14 mer) ^{32}P 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' (17 mer)
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' (32 mer)

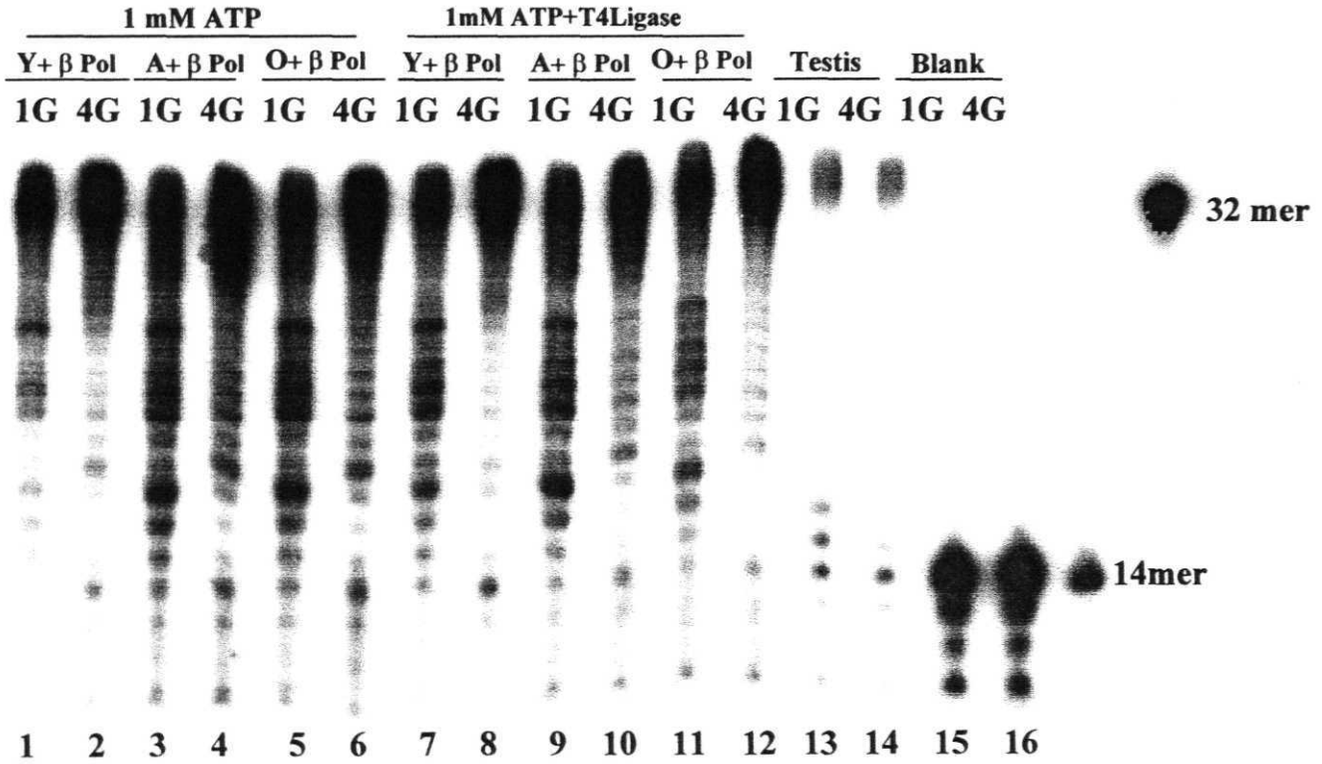
4 Gap Oligo duplex:

(14 mer) ^{32}P 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' (14 mer)
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' (32 mer)

Figure 26

Gap filling activity in the presence of lmM ATP alone or together with T4 DNA ligase with 'Young', 'adult' and **'old' neuronal extracts and supplemented with recombinant pure rat liver DNA polymerase β with 5'-phosphate on the downstream primer.**

A typical autoradiogram is shown. Lanes 1-6 (Y+p pol, A+p pol, 0+ β pol) neuronal extracts from young brain (Y, 5 days postnatal), adult brain (A, 6 months) old brain (O, > 2 years) with out added pol p and lmM ATP. Lanes 7-12(Y+p pol, A+p pol, O+ β pol) with T4 DNA ligase and lmM ATP. Lanes 13 and 14 are with testis extracts alone. Lanes 15 and 16 are without any neuronal extracts (Enzyme blanks). The mobility of labeled standard 14-mer and 32-mer are also shown. Other notations are similar to the Figure 23



1 Gap Oligo duplex:

(14 mer) ^{32}P 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' (17 mer)
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' (32 mer)

4 Gap Oligo duplex:

(14 mer) ^{32}P 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' (14 mer)
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' (32 mer)

CHAPTER 6

GENERAL DISCUSSION

SUMMARY

REFERENCES

General discussion

The process of aging is one of the most intriguing problems of today's world. Aging, although a basic and general phenomenon is a complex interplay of genetic and epigenetic factors resulting in the functional deterioration. Aging changes are manifest at all levels of organization -molecular to organismic level. It is generally accepted that changes occurring after attaining reproductive maturity comprise the phenomenon of aging or senescence. However, fundamental molecular mechanisms involved in aging remains controversial and largely unproven and the major reason for this is the obvious complexity of the problem. The nature of biochemical and molecular mechanisms underlying the aging process had been the subject of considerable speculation. Several theories have been proposed based on genetic and stochastic events as the triggering events that eventually lead to senescence. DNA damage and repair theory of Hart and Setlow (1974) has evoked tremendous attention among the scientists all over the world.

According to this concept, repair of the damaged DNA is a primordial molecular process, fundamental to the maintenance of life and genetic diversity on earth. 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. The contribution of DNA damage and repair in processes impinging on Man's mortality, such as aging and carcinogenesis, have become of particular interest.

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 the DNA are the result of continuous exposure of living organisms to DNA damaging agents like radiations, 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 damages accumulate, mRNA synthesis declines, and protein synthesis is reduced with age (Price, 1971; Chetsanga et al., 1977; Mori and Goto, 1982; ZS-Nagy and Semsei, 1984; Sajdel-Sulkowska and Marolta, 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 cells 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.

Among the four different pathways involved in repairing the DNA damage, Base excision repair pathway (BER) is the most important and relevant DNA repair pathway as far as the brain is concerned.

Five major DNA polymerases have been known for some time, the a, p, *y,* 5 and 8. In addition, a number of DNA polymerases are being discovered in recent past and the total number of DNA polymerases has now reached to 17 (Hubshcer et al., 2002). A characteristic feature of these newly identified polymerases seems to be their ability to affect traslesional DNA synthesis. It appears that cell can utilize these polymerases under the conditions of desperation both for synthesis and repair of DNA. As far as the five major DNA polymerases are concerned, DNA polymerase a is classically regarded as a major enzyme associated with cell replication. Polymerase p is essentially a repair enzyme at least for some forms of damage and polymerase y is involved in mitochondrial replication. Polymerase 8, associated with its intrinsic 3'-5' exonuclease activity and high processivity in the presence of PCNA has been assigned a role in DNA replication, more precisely in leading strand DNA synthesis at the replication fork. DNA polymerase s which is PCNA independent also associated with 3'-5' exonuclease is considered to be involved in DNA repair. (Budd, 1997; Burgers, 1998; Hubshcer et al., 2002). In a post mitotic cell like neuron, replication is absent and it has been shown over the years that whatever the DNA-polymerase activity observed in this organ was found to be exclusively DNA polymerasep. However, the possibility still exists that brain cells may possess other DNA Polymerases

in very minor proportions that may escape detection due to the type of template primers and the sensitivity of the methodology used. Moreover, each cell type of brain may harbor different proportions of the polymerases. Indeed the results presented in Chapter 3 do show that both neurons and astrocytes possess small proportions of other polymerases, the pol a and pol 5/E. This is the first report to suggest the presence of pol 5/E activity in isolated cell fractions of developing as well as aging brain. It is seen that one notable difference between neurons and atrocities is the relatively higher levels of pol a and lower levels of pol 6/s in astroglia than in neurons. In both the cell types and at all the three ages studied, pol β appeared to be the predominant polymerase which is in line with many earlier reports. Once again, the results demonstrate that pol β activity decreases with age. Next predominant activity to pol β is that of pol 6/E in neurons whereas pol a is the next predominant polymerase in astroglial cells. This could be due to the completely non replicative nature of neurons and some residual replicative activity in astrocytes. Some amount of pol 5/e may become handy for a neuron when it has to perform long patch BER in a contingent situation that demands long patch repair mode of BER.

The percentage calculations for each type of nuclear DNA Polymerase is based on the extent of inhibition exerted by various known inhibitors for these polymerases and with two different substrates, the activated DNA and Poly (dA). oligo (dT) $_{12\text{-}18}$. It may be argued that this is an oversimplification of a complex situation and the values for each polymerase presented in Chapter 3 may not represent absolute values but only an approximation. Indeed that is all that is being claimed from that data. The percentages of different polymerases arrived at are, of course, to an approximation. The value of that data does not lie in denoting the exact level of a given DNA polymerase but in suggesting that some level of the

given polymerase does exist in the brain cells. Complete characterization and assessment of these polymerases and perhaps of those that are being discovered now, may constitute the heart of a future study from some laboratory.

It is easy to predict that base excision repair pathway would be the main guardian to ensure genomic stability in a highly active organ like brain. When compared to the different polymerases found in the nuclei of mammalian cells the predominant enzyme that takes part in the BER is DNA polymerase β (Wood RD, 1997; Wilson III DM et al., 1997; Fortini et al., 1998). It thus appears that genomic maintenance in brain cells is largely taken care by Pol β dependent BER pathway. In some situations where long patch BER is required, the neurons may still able to affect it since it is noticed that neurons do possess some levels of pol δ/ε.

The present study constitutes part of the overall efforts that have been going on in this lab to examine the link between DNA-repair potential and aging phenomenon with special reference to Brain. BER pathway enjoys special relevance in brain. Pol β has a crucial role in BER. It is already established that pol β activity decreases in brain with age. Can we put these facts together and conclude that pol p plays a crucial role in the process of aging? May be a more precise assay procedure for measuring the functionally relevant activity of pol β is needed before conclusions are drawn in this aspect. This study is an attempt towards that end.

An overview of the results presented in Chapter 4 and 5 reveal a few interesting facts. Firstly, pol β is able to extend a primer in a linear synthetic oligo template primer. In fact this is perhaps nothing new in that 'activated DNA' is routinely used as a substrate for

measuring the DNA synthetic activity of pol p. Many years ago, Wang and Korn (1982) have shown that pol p is capable of adding a long stretch of nucleotides to a primer although slowly. Even in recent years pol β is observed to add nucleotides to a primer to fill up the gap to join the down stream primer or some times even without a down stream primer although with varying speeds and under different experimental conditions like time of incubation and pol β concentration (Singhal and Wilson, 1993; and Chagovetz et al, 1997) It therefore appears that attributing a narrow function of filling up a single nucleotide gap only to pol β may be far from appropriate. From the present studies it does appear that pol P can extend primers with or without downstream primer, with or without a phosphate group on 5' side, with varying efficiencies and in a distributive and strand displacement manner. The results of Chapter 4 also show that while extending a primer helical distortion (mismatched bases) at the extending end is not tolerated and results in decreased efficiency. It appears that an independent 3'-5' exonuclease activity helps circumventing this problem.

Perhaps the most important outcome of the results presented in this thesis is the demonstration that pol β dependent primer extension activity is drastically decreased in the neurons of aging rat brain and that this lost activity can be restored *in vitro* by simply adding pure recombinant rat liver pol p to the reaction mixture under appropriate conditions.

Similarly the results with what is generally considered to be the most physiologically relevant substrate for pol p, a duplex with a short gap in one of the strands (Chapter 5), are of some consequence. Whether these observations can be extended to the overall BER pathway is essentially a matter of speculation at this time. However, it may be logical to assume that once pol β dependent activity is low in aging brain, the same may be

applicable to over all BER pathway since pol p is a main player in BER pathway (Cabelof 2002, Intano et al., 2003). Nevertheless it is possible that any of the other factors involved in BER pathway may be more affected during aging than pol β. This possibility , however, appears to be less likely since , as is shown in this study, addition of pol p alone is able to bring back the activity once the mismatch removal is affected. Moreover, Intano et al., (2003) have very recently reported an 85% decrease in BER activity in aging mouse brain nuclear extracts and there was a decreased abundance of pol p, but not of other BER proteins, in old mice. On the other hand they could not restore the BER activity by the addition of pure pol p to the reaction mixture. The reasons for this variation from the present results are not known at this time. A close comparison of the conditions used in the two labs may yield some clues.

The importance of pol β in the general maintenance of genomic stability in brain is becoming pointedly apparent in recent years. For example, (Sugo et al., 2000) have shown that mice deficient in pol β suffer from neonatal lethality and abnormal neurogenesis. Pol p also appears to be repairing the DNA-damage following cerebral injury due to a variety of reasons like hypoxia, ischemia etc., (Liu et al., 1996; Englander et al., 1999, Lin et al., 2000) and the fidelity with which such repair is carried out may determine the chances of recovery. Pol β is also found to confer protection against the cytotoxicity of oxidative DNA damage (Horton et al., 2002) Moreover, there are a number of genetic disorders that show elevated genomic damage and neurodegeneration with coupled symptoms of premature aging in some cases (reviewed in Brooks, 2002 and Rao, 1997). The involvement of pol β in the maintenance of genomic stability is also becoming apparent from the results being

reported from a different direction. For example, induction of oxidative stress was found to increase the levels of pol p in mouse monocytes and fibroblasts. It was also found that it is the pol p dependent BER which was conferring protection against DNA damage (Chen et al.,1998) In other words, there is an up regulation of pol β dependent BER when cells are subjected to DNA damage.

Interestingly, the up-regulation of pol β is also connected to the genomic instability. Thus Canitrot et al.,(1998; 1999) have proposed that over expression of pol β could be a genomic instability enhancer process and indeed it has been found that pol β is up-regulated in some types of adenocarcinomas and cell lines (Srivastava et al, 1999). A role for deregulated pol β in inducing chromosome instability and tumorigenesis has also been envisaged by Bergoglio and co workers (2002). Further, regulated over expression of pol β is found to mediate early onset of cataract in mice (Sobol et al., 2003) these diverse observations surface the possibility that pol β may have slightly different roles in different tissues and a balanced level of this enzyme may be required for its proper function.

Be as it may, the present studies revealing that pol β may be one of the, if not the sole, most limiting factors for carrying out BER in aging neurons and that this deficiency can be rectified, *in vitro,* by the addition of pure pol p, should be of considerable importance. A thorough study of BER in normal and pathological/experimental brain and examination of ways and means to bring back the lost DNA repair activity may yield results with far reaching consequences.

Summary and Conclusions

1. The hypothesis that "decreased DNA repair capacity in the brain is atleast one of the major biochemical markers that is associated with advancing age and deterioration of brain function" has been tested in this investigation.
2. DNA Polymerase activities were studied in extracts of isolated neuronal and astroglial cells fractions from the rat cerebral cortex at three different ages of 'young' (4 days postnatal),'Adult' (6months), 'Old' (> 2 years).
3. DNA polymerase activity undergoes a significant decrease with the advancement of age with 'activated DNA' as the substrate in spite of the fact that considerable variation in this activity was noticed from one animal to the other.
4. DNA polymerase activity towards synthetic oligos as substrate in either type of cells is far less when compared to 'activated DNA'. No age dependent changes were observed when synthetic substrates Poly (dA). oligo $(dT)_{12-18}$ and Poly(dA.dT) were used.
5. Relative abundance of DNA polymerases using differential sensitivities of these enzymes towards inhibitors like BuPdGTP, BuAdATP, ddTTP, monoclonal antibody of human a polymerase, was assessed. The results pointed that Pol β is the predominant polymerase at all the post-natal ages, where as some amounts of Pols a,5,e are also present . Pol 5/e activity was closely behind the Pol β activity in neurons whereas Pol a is the second predominant polymerase in astroglia.
6. Base excision repair (BER) being the main DNA-repair mechanism in brain cells and Pol β being a main player of that pathway, a more *in vivo* relevant functional assay for Pol p activity was standardized.

7. With a synthetic staggered oligoduplex DNA substrate the primer extension activity was low in ‘ Young' neuronal extracts and almost undetectable in adult and old brain neuronal extracts.

8. Restoration of the lost activity by supplementing the neuronal extracts with pure DNA polymerases(calf thymus a polymerase, E.coli DNA polymerase I and rat liver DNA polymerase P) was examined *in vitro.* Only polymerase β gave consistent and encouraging results. Extension of the primer was slow and distributive in nature and primers with a mismatched base at 3’-end were extended much less efficiently than the correctly paired primer.

9. Processivity and efficiency of pol β aided primer extension (including those with a mismatch) can be enhanced by preincubating the synthetic oligoduplexes with only neuronal extracts and supplementing pol β along with dNTPS and Mn^{++} in the second step. These results pointed that pol β can restore the DNA-repair / synthetic activity of the neuronal cells irrespective of the age of the animal.

10. Pol β aided extension of a primer with a 3' mismatch will occur optimally only after the removal of the mismatch. This was established by a 3 step reaction where the products of the second step were restrict digested with HinP 1 and looked for the predicted labeled 12-mer. These experiments also suggested the existence of 3’-5’exonuclease activity in neuronal extracts.

11. Since pol β doesn't possess the proof reading ability, studies were extended to establish the actual existence of an independent 3’-5’exonuclease activity in neuronal extracts. The results indicated that indeed neuronal extracts do possess such activity. Duplex DNA Model duplex oligo is excised from the 3’-end in a sequential and time

dependent manner with no particular specificity towards a mismatched base. However, at the initial stages of the reaction, a mismatched base is removed faster

12. With age there is a decrease in 3'-5' exonuclease activity but still maintained at significant levels even in old neuronal extracts. It is concluded that this 3'-5' exonuclease activity is facilitating the extension by pol β of a primer with a mismatch at the 3'-end.

13. It was observed that neuronal extracts were also able to excise bases from 3'-end of single stranded oligos. Excision activity towards single strand oligos (14-mer and 21-mer) showed that extracts of all the ages show increasing activity with time and 'young' extracts show higher activity where as 'adult' and 'old' extracts still retain considerable activity towards primer (14 mer). No striking changes in the activity could be seen with respect to age towards 21-mer. The physiological relevance of this activity is not clear at this time.

14. Since the most preferred substrate for pol (3 is reported to be a duplex DNA with a one nucleotide gap, the ability of neuronal extracts to fill up 1 and 4 nucleotide gaps in oligo duplex was studied.

15. The results reveal that the gap filling activity decreases with age. 'Adult' and 'old' neurons could hardly exhibit any gap filling activity. Supplementation of the extracts with pure pol p restored the gap filling activity largely through a slow distributive strand displacement addition of nucleotides to the up stream primer all the way to the length of the template (32 nucleotides). This conclusion was reached because of the several radioactive spots representing various lengths between 14 and 32 nucleotides, seen on

the autoradiogram. Additional supplementation of the extracts with ATP alone or together with T4 DNA ligase did not change the situation significantly.

16. When the above experiments were repeated with the down stream primer having a phosphate group on 5'-side, still the improvement seen due to the addition of pol β was no different from what was seen in the previous set of experiments (when there was no phosphate group on the down stream primer). It was concluded that that gap filling is achieved by added pol (3 both by a processive mechanism where the filled gap is immediately ligated as well , perhaps more predominantly, by extending the up stream primer in a slow distributive manner until the template length is achieved.
17. Be as it may, the present studies show that pol β is the most predominant DNA polymerase present in brain and plays a major role in repair and maintenance of DNA in brain. It restores the lost primer extension as well as gap filling activities in aging neurons. These results surface the exciting possibility of restoring the BER activity in aging neurons to normal level, which could have far-reaching consequences.

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Interactive report

Loss of base excision repair in aging rat neurons and its restoration by DNA polymerase β[1]

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Abstract

Synthetic staggered oligodeoxynucleotide duplexes are formed by annealing a 5′-^{32}P-labeled 14-mer with four different 21-mers. These duplexes have either a correct or mismatched base pair at 3'-end of the primer. With these model template primers the ability of neuronal extracts, obtained from rats of different ages, to extend the primer to the predicted length was tested. While the neuronal extracts of all ages were able to degrade the 14-mer to shorter lengths, extension of the primers in general and in particular, the mismatched, is achieved only feebly by the young and adult neuronal extracts and undetectable with old neuronal extracts. The possibility of restoring the lost activity by supplementing the neuronal extracts with pure DNA polymerases was examined. Of the three polymerases tested (calf thymus a polymerase, *E. coli* DNA polymerase I and rat liver DNA polymerase β) only polymerase β gave consistent and encouraging results although the extension was slow and distributive in nature and mismatched primers were extended much less efficiently than the correctly paired primer. However, significantly improved extension, including those of mismatched primers, was achieved by prior removal of mismatched bases in a preincubation with just the neuronal extracts (3'-5'exonuclease activity) followed by extension by the added polymerase β and dNTPs in the presence of Mn^{2+} instead of the usual Mg^{2+}. These results are taken to indicate that the activity of polymerase (3 in brain cells is compromised with age and that this deficit can be corrected in vitro by the addition of pure recombinant rat liver polymerase (3 under appropriate conditions.

Theme: Development and regeneration

Topic: Aging process

Keywords: Neuron; Aging; DNA polymerase-β; Primer extension; Mismatch base excision

1. Introduction

We have been testing and finding support for the hypothesis that decreased DNA repair capacity in brain is at least one of the major biochemical markers that is associated with advancing age and deterioration of brain function [22,15,14,25,13] Further substantiation of this hypothesis has come from the observation that the activity of DNA polymerase β (β-pol), generally considered to be a repair enzyme, markedly decreases with age in brain. There was also the indication that this was probably due to accumulation of inactive molecules of (3-pol with age [16].

In a non-dividing cell like a neuron, the types of DNA damage that are encountered include spontaneous or induced hydrolytic depurination of DNA, deamination of cytosine and 5-methylcytosine bases resulting in mismatches, alkylated bases and products of oxidative damage caused by oxygen free radicals. Base excision repair (BER) is the DNA repair pathway that is activated to correct these alterations and there is evidence now to suggest that (3-pol plays an important role in BER in mammalian cells [30,31]. Furthermore, in adult brain there is very little DNA polymerase activity and most of it is (3-pol [28,21]. Thus, (3-pol is likely to have a prominent role in maintaining the structural integrity of DNA in brain cells.

We have designed a study to examine the age-related capacity of rat neurons to extend a primer, with or without a correctly matched base pair at its 3′-end, in a synthetic

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oligodeoxynucleotide duplex. It is shown here that neuronal extracts can efficiently remove bases from 3′-end of the primer but could elongate the primer only with negligible efficiency particularly at the advanced ages of the animal. The primer with mismatched base pair is extended much more poorly than the correctly matched primer. The results also show that this deficit to extend the primer can largely be corrected by supplementing the neuronal extracts, with pure recombinant rat liver β-pol under defined conditions.

2. Materials and methods

Cohorts of Wistar strain rats in-bred over generations and maintained in our animal house were used. The three age groups studied were 5 days postnatal, 6 months and 21 days and 28 months. We have designated these three age groups as 'Young', 'Adult' and 'Old' respectively. Animals of both sexes were used. Adequate measures were taken to minimize pain or discomfort for the animals.

Radiolabeled nucleotides (α-^{32}P-dCTP; (γ-^{32}P-ATP) were purchased from BARC (Bombay). dNTPs, RIA grade bovine serum albumin, Sephadex-G-50, Sigmacote were obtained from Sigma (St. Louis, MO, USA). *E. coli* DNA polymerase I (Pol I), T4 polynucleotide kinase, were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents used were of analytical grade.

The sequences of the synthetic deoxyoligonucleotides used in this study are shown below. These oligos were supplied (HPLC purified) by Rama Biotechnologies (Hyderabad, India) and also by Bangalore Genei (Bangalore, India). Out of the four 21-mers, when hybridized with 14-mer, one has the correct complimentary base (cytosine) at 14th position from 3′-end (that is opposite to the 3′-end of the 14-mer primer) whereas the other three have at that position the three mismatched bases as follows

1. 5'-cgcgatcggtagc G-3' (oligo 1-14 mer)
2. 3'-gcgctagccatcgCgttaccg-5' (oligo 2-21 mer-C)
3. 3'-gcgctagccatcgTgttaccg-5' (oligo 3-21 mer-T)
4. 3'-gcgctagccatcgGgttaccg-5'(oligo 4-21 mer-G)
5. 3'-gcgctagccatcgAgttaccg-5' (oligo 5-21 mer-A)

Prior to hybridization with the four different 21-mers, the 14-mer (oligo-1) was 5'-kinased using equimolar 7-^{32}P-ATP (specific activity, 5000 Ci/mmol) and T4-polynucleotide kinase (2.5 units/pmol of substrate). The reaction was carried out for 40 min. at 37°C in a total volume of 40 μl containing the buffer supplied by manufacturer to a final 1× concentration (70 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$ and 5 mM dithiothreitol). The 5′-^{32}P-kinased 14-mer was then hybridized (10 min at 70°C and then gradual cooling to room temperature) with each of the four different 21-mers (oligos 2-5) in equimolar concentrations in the presence of 50 mM NaCl and 5 mM $MgCl_2$. Thus one of the duplexes so formed would have the correct base against the 3'-guanine of 14-mer while the other three would have mismatches of thymine or adenine or guanine. These duplexes are referred to as G-C, G–T, G-A and G-G, respectively.

2.1. Preparation of neuronal extracts and DNA polymerase assay with 'activated' DNA as substrate

Neuronal cell enriched fractions from rat brain cerebral cortex of different ages were prepared essentially as standardised in this laboratory [24]. The final preparation of the neuronal cells was suspended in extraction medium consisting of 20 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 0.1 mM PMSF (just before use), 5 mM β-mercaptoethanol, 1 mM $MgCl_2$, 1 μg/ml leupeptin and 1 |xg/ml pepstatin A (both just before use) and 0.5 M KC1. The suspension was sonicated for 5 s, three times with the setting at 5 in a Branson sonifier. The suspension was centrifuged at 100 000 *g* for 1 h in a Beckman ultracentrifuge and the clear supernatant was used as the source for both 3′–5′ exonuclease and DNA polymerase. For measuring the DNA polymerase activity, the reaction mixture (20 μl) contained 5 μg of 'activated' calf thymus DNA, 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 dATP, dCTP and TTP and 5 μM dCTP and appropriate amount of neuronal extract usually ranging from 5 to 10 μg of protein. In addition, 1 jxCi of α-^{32}P-dCTP (specific activity, 4000 Ci/mmol) was included as radioactive nucleotide. Incubation was carried out for 20 min at 37°C. The reaction was stopped with 2 ml of stop solution (1 M perchloric acid and 0.01 M sodium pyrophosphate) and 200 μg of denatured calf thymus DNA was added as carrier. The tubes were kept in ice for 10 min and centrifuged at 3000 *g* for 10 min in a refrigerated centrifuge. The supernatant was aspirated out carefully and the precipitate was dissolved in 0.5 ml of 0.2 M NaOH. A 2-ml volume of stop solution was added again and centrifuged after 10 min. The alkali solubilization step and addition of stop solution were repeated. The whole solution, along with the precipitate was transferred to 2.5-cm glass fiber filters (Schleicher & Schuell) and the filters were washed three times with ice-cold stop solution and twice with 95% ethanol. The washed filters were directly taken into counting vials and dried by keeping in oven at 40°C for 20 min. Then, toluene-based scintillation fluid having Triton-X-100 was added and the radioactivity was counted in a liquid scintillation counter.

2.2. Assay of oligo duplex template-primer dependent polymerase activity

DNA polymerase activity in neuronal extracts with the

hybridized oligo duplex as template primer was measured essentially as standard polymerase assay described above except that instead of activated DNA, 400 nmol of oligoduplex template primers were used as substrate.

2.3. Assay of mismatch removal and primer extension (exo-extension) activity of neuronal extracts

The 5'- P labeled 14-mer is hybridized with 21-mers as outlined above. Four hundred fmol of the hybridized oligo duplex either with correctly matched base pair (G-C) or with mismatched base pair (G-A, *G-G* and G-T) at the 3′-end of the primer, was incubated for 20 min at 37°C with appropriate volume of neuronal extract in a total reaction mixtures of 30 μl having 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. The reaction was stopped by heating at 70°C for 10 min. The volume was then made up to 100 μl with water. The whole sample was loaded on to a Sephadex-G-50 spin column (previously washed twice with 100 |xl of water). The collected sample was dried and resuspended in 5 |xl of loading buffer and denatured just before performing the electrophoresis. The chain length of the labeled products (was assessed by electrophoresis on 20% poly acry lamide. Electrophoresis was run at 3000 V in 90 mM Tris-borate teDTA buffer, pH 8.3, for 5 h. Oligos of different chain lengths were identified by autoradiography [10]. Under these conditions, oligos could be separated on a single base difference.

In the two-step reaction, the first step consisted of incubating the oligo template primer with neuronal extract (reaction mixture volume, 10 μl) in the absence of added dNTPs for 10 min so as to allow the 3′–5′ exonuclease activity to proceed without much interference from the extension activity. The reaction was then stopped by heating at 70°C for 10 min. and in the second step the total volume of the reaction mixture was made up to 30 μl with dNTPs (20 μM final concentration) and either pure calf thymus α-polymerase (0.25 units) or *E. coli* pol I (2 units) or rat liver β-pol (2.5 units) and the buffer. The extension reaction was carried out for another 20 min at 37°C. The chain length of the radioactive products was assessed through electrophoresis followed by autoradiography as mentioned above.

5. Results

3.1. DNA polymerase activity in rat neuronal extracts at young, adult and old ages

Table 1 shows the overall DNA Polymerase activity in neuronal extracts with 'activated' calf thymus DNA as the substrate and the effect of age of the animal on such activity. As can be seen, there was considerable variation in this activity from one neuronal extract to the other. However the pattern of changes in the activity with the age is clearly visible. There is a drastic reduction in the polymerase activity by the time the animals become 6 months old (88% decrease). Between 6 and 28 months of postnatal ages, there was a small increase but this difference was not statistically significant. These results once again confirm our earlier observations about the age dependent reduction of DNA polymerase activity in brain [21,22].

Table 1
DNA polymerase activity in rat neuronal cells of different ages with 'activated' calf thymus DNA as template primer[a]

Substrate	Age		
	Young	Adult	Old
'Activated' calf thymus DNA	5389.8±4336	626.6±638	865.9±942

[a] Isolation of neuronal cells from young (5 days postnatal), adult (6 months and 21 days) and old (28 months) rat brains. Preparation of neuronal extracts and DNA polymerase assay procedure are as described in Materials and methods. The values are averages±S.D. from nine experiments from five independent extracts and represent picomoles of dCMP incorporated into acid insoluble fraction/mg protein/h. While the difference between adult and old values is not significant, the difference between young and old or adult is highly significant ($P<0.007$).

3.2. 3′-5′ Exonuclease and primer extension activities (exo-extension activities) of neuronal extracts with synthetic oligo template primer as substrate

Fig. 1 shows the ability of neuronal extracts to degrade and /or extend the 14-mer primer to the template length in the absence or presence of 20 μM dNTPs. As can be seen, neuronal extracts of any age were able to excise the primer to shorter lengths with good efficiency. On the other hand, significant extension of the primer could be seen only with young extracts (lanes 5-8) and there was age dependent decrease in this activity (compare the activity in lanes 5—8 to 13-16). In fact, in old neuronal extracts (lanes 21-24), the activity was hardly detectable. It can also be noted that maximum extension activity is seen in the case of correctly matched primer (G-C) with the activity in the case of mismatches being diminished. Among the mismatches, the G-G appears to be least extended. Omission of dNTPs from the incubation resulted in negligible activity of extension with no apparent effect on the excision activity.

When the neuronal extracts were incubated under the same conditions with unlabelled oligo duplexes but with one of the four dNTPs being labeled (α-^{32}P-dCTP), detectable template primer dependent incorporation of the label into acid insoluble fraction was noticed both in the case of correctly matched G-C pair and the three mismatches (Table 2). The activity was lower in the case of mismatches, and decreased with age signifying the limitation of this activity with advancement of age. These results

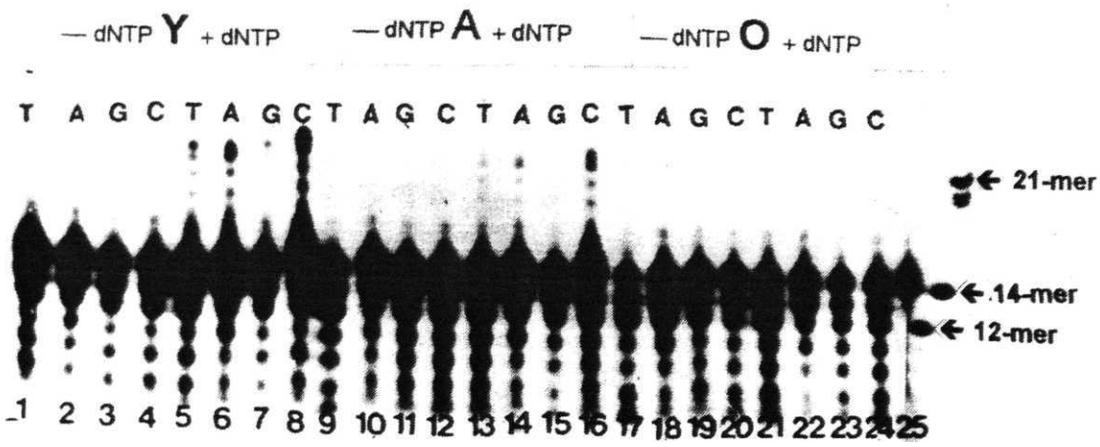

Fig. 1. Exo-extension activities of rat brain neuronal extracts with correctly matched and mismatched oligo duplex template primers. Details regarding the incubation conditions and identification of the products through sequencing gel electrophoresis are given in Materials and methods. The reaction was carried out both in the presence (20 μM) and absence of dNTPs. Lanes 1-8 contain neuronal extracts from young brain (Y, 5 days postnatal), lanes 9-16 contain neuronal extracts from adult brain (A, 6 months and 21 days old) and lanes 17-24 contain neuronal extracts from old brain (O, 28 months old). At each age, the first four tubes are without dNTPs while the next four are with dNTPs. Lane 25 is without any neuronal extract (enzyme blank). The mobilities of standard 21-, 14- and 12-mer are shown. The base in 21-mer corresponding to that at the 3'-end of the primer is shown on the top. In all the tubes protein in neuronal extracts was normalised to a fixed amount by adjusting the volume (usually 5 or 10 μg). Similarly equal amounts of products were loaded for the sequencing gel electrophoresis.

are in line with those in autoradiography experiments (Fig. 1).

3,3. Effect of exogenous calf thymus α-polymerase, E. coli pol I and rat liver β-pol on the primer extension activity of neuronal extracts

In order to examine the possibility of restoring the age dependent loss of primer extension activity, the above experiments were repeated with the same neuronal extracts but supplemented with either calf thymus a-polymerase or *E. coli* Pol I or rat liver (3 -pol.

Pure calf thymus a-polymerase, either alone or in association with neuronal extracts could extend the 14-mer only in the case of correctly paired duplex (G-C). In the case of mismatched primers, the activity was almost negligible confirming the known inability of DNA polymerase a to extend mismatched primers [11] probably because of the absence of proof-reading 3′–5′ exonuclease activity in the enzyme and also perhaps, in the present case, the inappropriateness of oligoduplex substrate. The results with *E. coli* pol I either alone or when added to neuronal extracts were inconsistent and erratic and no set pattern could be discerned (data not shown). Therefore further experiments were conducted only with (3-pol.

Addition of pure rat liver (3-pol to neuronal extracts (Fig. 2) restored the extension activity significantly and with a pattern. Further what ever the extension activity found was consistent in different experiments with a number of characteristic features. Firstly, β-pol either itself (lanes 13-16) or in the presence of neuronal extracts could not extend the mismatched primers efficiently whereas the correctly matched primer (G-C) was extended with good facility. Among the mismatches, G-T appears to be the most preferred template-primer to act upon. Secondly, the addition of nucleotides to the primer is a slow and distributive process. A number of products of intermediate length can be seen. Thirdly, β-pol is able to extend the primer strand much more efficiently in the presence of Young neuronal extracts (lanes 1-4). It is interesting that the extension has occurred to completion (to a length of 21 nucleotides), at least to some extent even in the case of mismatched primers. The same enzyme, however, did not show the same efficiency in the company of adult (lanes 5-8) and old (lanes 9-12) neuronal extracts in that the extensions occurred only up to 18 or 19 nucleotides lengths, that is, addition of only four or five nucleotides Thus the facility with which (3-pol can extend the primer (particularly the mismatched ones) to the predicted tem-

Table 2
DNA polymerase activity in rat neuronal extracts of different ages with the four synthetic oligo duplex molecules as template primers[a]

Base pair at primer 3′-end	Age		
	Young	Adult	Old
G-T	37.0±13.1	24.2±6.8*	22.5±17.7*
G-A	27.3±11.3	21.5±8.6*	9.3±6.5**,**
G-G	25.2±14.6	18.4±8.0	6.8±5.5**,**
G-C	51.3±34.3	33.6±15.6*	26.0±9.1**,**

[a] Experimental conditions and method of assay are described in Materials and methods. The results are the average ±S.D. of 12 independent experiments. The activities are expressed as femtomoles of radioactive deoxynucleotide incorporated into acid insoluble fraction. These activities are over and above of those in appropriate controls like substrate and enzyme blanks. The volume of neuronal extracts were adjusted so that in all cases the protein was 10 μg. *, Values are significantly lower than those of the corresponding young group and **, those of the corresponding adult group, at a P value <0.01.

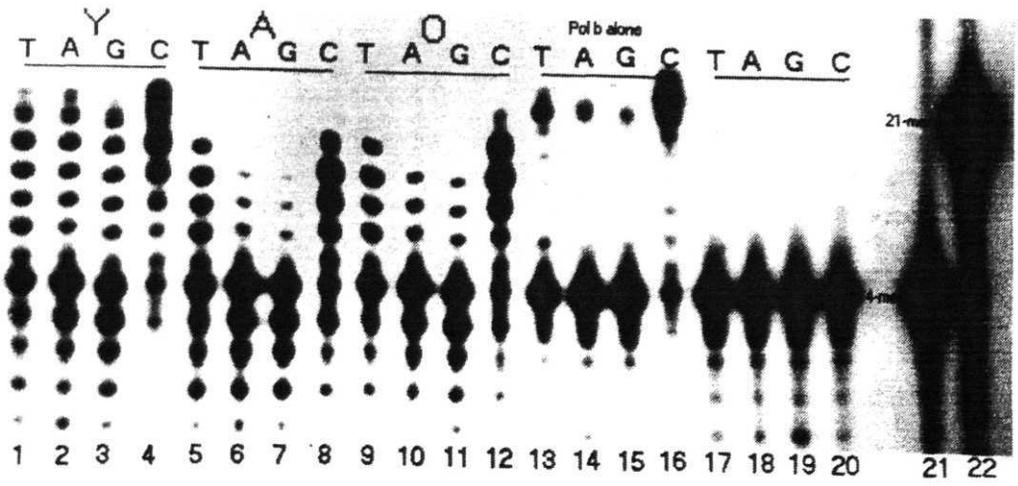

Fig. 2. Exo-extension activity in rat brain neuronal extracts of different ages when supplemented with rat liver β-pol with the four different oligo duplex template primers (lanes 1-4, Y, young; lanes 5–8, A, adult and lanes 9-12, O, old). 2.5 units of the purified β-pol and 10 μg of neuronal extract protein were used in all cases. One unit of polymerase activity is equivalent to 1 n mole of total nucleotides incorporated into acid insoluble fraction in 1 h. at 37°C. Lanes 13-16, β-pol alone without neuronal extracts and lanes 17-20,no enzyme controls. For other details see text and Fig. 1.

plate length seems to depend upon some factor the levels of which are different in young and adult/old extracts. One such factor could be the 3'-5'exonuclease activity degrading the primer to shorter lengths and in the process removing the mismatch. The final result seen on autoradiogram may be the outcome of the balance between two opposing reactions i.e. the one trying to degrade the primer and the other trying to extend the primer. Furthermore, it is also possible that the young extracts may have a factor which contributes to the processivity of the β-pol catalysis.

To gain information in this direction, a two-step reaction was designed. Firstly, the synthetic oligo template primers (with the ^{32}P-label on 5′-end of the primer) were incubated with neuronal extracts for 10 min in the absence both dNTPs and β-pol. In the second step the products of the first step were incubated for another 20 min. with β-pol and 20 |xM dNTPs under standard polymerase assay conditions. The results shown in Fig. 3 were obtained. The usage of rat liver polymerase in the second extension step resulted in further enhancement in the extension activity as compared to the single step results shown in Fig. 2. Preincubation of the oligo substrate with neuronal extracts seems to have brought significant improvement in β-pol's ability to extend the primers even with incorrect base pairing. Not only the extension activity but also the length of the products is improved. In almost all the cases (except the G-G mismatch in old extracts) extension occurred up to 20 or 21 nucleotide length, which means β-pol has added at least 7-8 nucleotides to a significant number of mismatched primer molecules. These results point out once again that β-pol is inefficient in adding nucleotides to a mismatched primer in a staggered DNA strands. But once the mismatch is removed, it is able to add nucleotides to the 3'- end of the primer. It thus appears that β-pol's

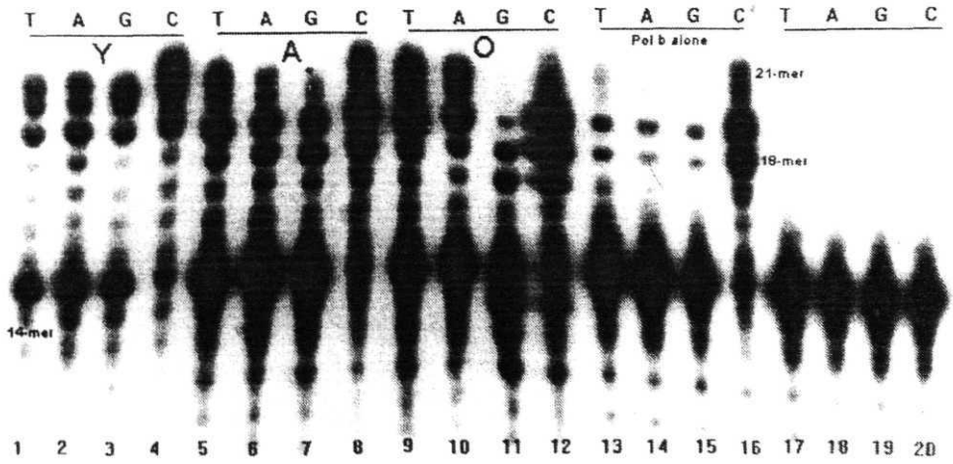

Fig. 3. Two-step reaction to assess the effect of prior incubation of the oligo duplex template primers with the neuronal extracts (Y, young, A, adult and O, old) on the primer elongation achieved by β-pol in the subsequent step. During the first step, the four oligo duplexes were incubated with neuronal extracts for 10 min at 37°C in the absence of any exogenous polymerase and dNTPs. The reaction was stopped by heating at 70°C for 10 min. In the second step 20 μM dNTPs and β-pol (2.5 units) were added and the incubation at 37°C was continued for 20 min. For rest of the details and notations please see Fig. 1 and Materials and methods.

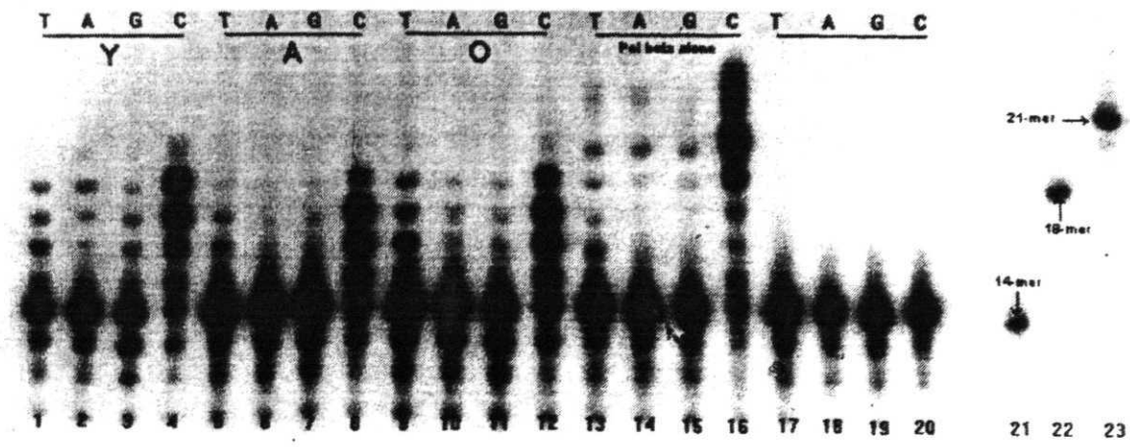

Fig. 4. Effect of 5 mM GMP on the exo-extension activity of rat brain neuronal extracts (Y, young, A, adult and O, old) supplemented with rat liver β-pol (2.5 units) or of β-pol alone with the four oligo duplexes as template primers. Notations are the same as in earlier figures. Note the inhibition of the extension activity, particularly of the mismatched primers.

catalytic activity towards a linear primer-template is affected by two factors — the removal of the mismatch at the 3'-end of the primer and secondly factors that could probably improve the processivity of the nucleotide addition.

In order to further examine the above possibilities, extension activity of neuronal extracts supplemented with β-pol is tested in presence of 5 mM GMP, a known inhibitor of 3'-exonuclease activity [1]. The results are shown in Fig. 4. The extension of mismatched primers (G-T, G-A, and G-G) particularly in terms of the number of nucleotides added was inhibited. The maximum chain length achieved was 18 with spots at lower lengths also being present. These results substantiate the contention that β-pol can successfully extend the primer to the full template length only after the removal of the mismatch. With the correctly matched primer (G-C), the extension activity, as expected, was not affected but even here the maximum chain length achieved is only 19. This may be due to high concentration of GMP present in the mixture as compared to the other deoxy nucleotides which might be affecting the processivity of the nucleotide addition.

In the next set of experiments, the effect of replacing Mn^{2+} for Mg^{2+} in the reaction mixture was tested. It was shown earlier by Wang and Korn [26] that Mn^{2+} substituted for Mg^{2+} can enhance the processivity of the β-pol activity. The reaction was carried out both in single and two steps as mentioned above. The results are shown in Figs. 5 and 6. It can be seen that in either case, there is marked improvement in the processivity of the nucleotide addition. In the single step reaction (Fig. 5), the major product seen is of 21 nucleotide length. However, as seen in earlier experiment (Fig. 2), activity towards mismatched primers is less as compared to that with correctly matched primer. But these differences have essentially disappeared once the reaction was carried out in two steps — allowing

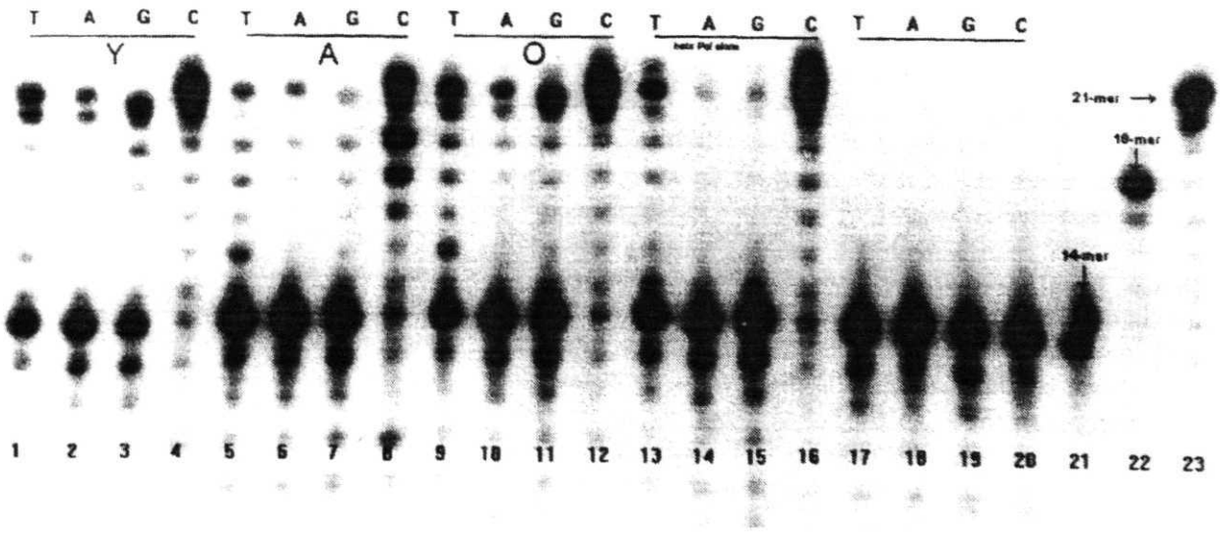

Fig. 5. Effect of 1 mM Mn^{2+} in place of Mg^{2+} in the reaction mixture on the exo-extension activity (single step) of rat brain neuronal extracts (Y, young, A, adult and O, old), supplemented with 2.5 units of rat liver β-pol or β-pol alone (no neuronal extract). Notations same as in earlier figures. Note the improved extension activity and the processivity of the extension.

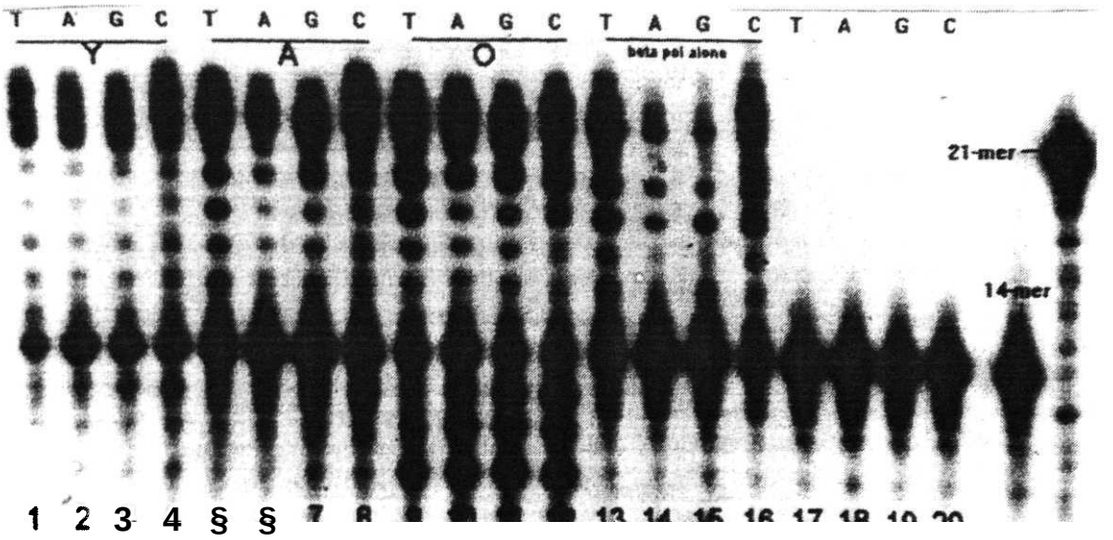

Fig. 6. Effect of 1 mM Mn in place of Mg^{+} in the second step of the two-step reaction to assess the exo-extension activity of rat brain neuronal extracts (Y, young, A, adult and O, old), supplemented with 2.5 units of rat liver 3-pol. For details regarding the two step reaction, please see Fig. 3 and Materials and methods. Notations are as in earlier figures. Note the marked improvement in the extension activity at all ages with product being the predicted 21-mer.

he exonuclease to act first and then adding the β-pol (Fig. 6). The major product in all the cases is 21-mer and in good measure. These results thus suggest that under appropriate conditions, β-pol can restore the DNA repair/synthetic activity of the neuronal cells irrespective of the age of the animal from which these extracts are made.

4. Discussion

A variety of DNA repair strategies are necessary to maintain genomic integrity in a cell. In a postmitotic cell like a neuron the structural perturbations that genomic DNA is likely to encounter are mismatched base pairs, modified bases, abasic sites and oxidative damage. It is estimated that 1-20 5-methylcytosines and 100-500 cytosine residues are spontaneously deaminated per human cell per day [6,18]. These two activities would result in G-T and G-U mismatches, respectively. Similarly spontaneous formation of abasic sites and oxidative damage in genomic DNA are well documented [7,17]. It is therefore necessary that a neuron, endowed with high transcriptional activity but with no replication, should have a strategy to repair these forms of DNA damage. BER seems to be the route through which this type of damages are generally repaired and as already mentioned β-pol appears to be the major polymerase involved in BER and it is only logical that most of the DNA polymerase present in brain cells is β-pol.

BER occurs in two different pathways, the short and long patch pathways. While in the case of short patch repair, a single nucleotide is added essentially by β-pol, the long patch repair involves β-pol or 8/e pol with the addition of six or more nucleotides [3,30]. There is also a suggestion that the long patch pathway itself can further be divided into two sub routes — one involving PCNA and (3-pol with the addition of up to six nucleotides and the other involving again PCNA but 8/e -pol with addition of more than six nucleotides [29,32]. The mechanism of repair of double strand oligonucleotide substrate harbouring a damaged or modified base, or abasic site or a mismatch by (3-pol involves the addition of a base and also to release the 5′ terminal deoxyribonucleotide phosphate from the abasic site generated by the class II apurinic endonuclease [8]. It therefore appears that (3-pol is most efficient in filling up gaps of one to six nucleotides in a duplex DNA. However, it has also been found [26] that (3-pol can also add several nucleotides to 3' end of a primer in a staggered double stranded DNA molecules.

We have therefore, chosen a simple and easy-to-work-with model for measuring the DNA polymerase activity in aging neuronal extracts. The oligo template-primer model used in the present study offers a dual advantage. Both mismatch removing activity and the subsequent or simultaneous primer extension activity could be monitored. It may not exactly represent the type of substrate that β-pol would encounter during BER but nevertheless serves as a good model to follow the proof-reading and overall polymerase (including that of (3-pol) activities.

The present study makes two facts apparent. Firstly, neuronal cells have the ability to excise a mismatch located at the 3′-end of a primer of a synthetic oligoduplex. Thus the neuronal cells possess 3′–5′-exonuclease activity and that too at all the ages studied (Fig. 1). It was not possible under the present experimental conditions to discern whether there were any changes in this activity due to age. Also not clear is the exact nature of 3′–5′-exonuclease present in neuronal extracts. The activity appears to be of a generalised nature lacking any absolute specificity since all the four primers, including the correctly matched one, are

excised to shorter lengths. A so-called 'correxonuclease' which has been isolated from human placenta, acts in both the 3′–5′ and 5′–3′ directions to release oligonucleotides [4]. DNase of Novikoff hepatoma cells also hydrolyses DNA in the 3′–5′ and 5′–3′ directions and supposed to act in synchrony with β-pol to affect DNA repair [9]. From brain tissue, an exodeoxyribonuclease with single-stranded DNA specificity has been isolated. A role for this enzyme, in conjunction with β-pol, in repairing depurinated DNA has been envisaged [5]. This exonuclease however, acts in the direction of 5′–3′.

Information about the mismatch recognising endonucleases or the 3′–5′ exonuclease activities in brain ceils is scanty. Only in the recent past while this work was in progress, some evidence for the existence of mismatch (G-T and G-U) nicking activity and of an autonomous 3'-5' exonuclease in brain has become available [19,2,23]. Two mammalian DNA polymerases the 5 and e that have been exhaustively studied were found to possess tightly associated 3′–5′ exonuclease activity and are thus capable of proof-reading while adding nucleotides at the 3′-end of the primer [27]. There is some indirect evidence for the presence of δ-polymerase, although as a minor component, in embryonic [20] and adult [12] brain. In contrast, Yang and Lu [33] failed to notice any a, 8 and ϵ-polymerase activities in mouse brain on 17th postnatal day. However, all these studies are indirect in nature and more direct and accurate measurements are needed in this respect. In any case it appears unlikely that the mismatch removal activity of neuronal extracts observed in the present study was coming from δ or e polymerase. If that were to be the case then extension of the primer also would have occurred at all ages due to these activities. Thus, the present studies provide evidence to show a proof-reading 3′–5′ exonuclease in brain of young, adult and old rats. It will be a separate study to characterise this activity in detail.

Secondly, either with activated DNA or synthetic oligoduplex as substrate, the overall DNA polymerase activity in neurons is markedly reduced with age (Tables 1 and 2) Also, with the oligo duplex substrate the primer elongation activity is very limited when there is a mispaired base at the 3′-end of the primer. This age-dependent decrease in the primer extension activity could perhaps be attributed to the decrease in the expression of β-pol gene and more importantly due to the accumulation of catalytically inactive β-pol molecules in aging rat brain as was observed earlier [16]. The present studies showing the effectiveness of β-pol addition in vitro to restore the lost primer extension activity in aging neurons can be taken to substantiate the contention that proper β-pol is a limiting factor in aging brain. The present results also indicate that even when a good and pure β-pol is added to the neuronal extracts, there are certain other factors, which seem to influence the restoration of the primer extension activity to its full extent. For example, it appears to be necessary that the mismatched base at the 3′-end of primer be removed before β-pol could efficiently lengthen the primer (Fig. 3). Thus any alteration in the proof-reading status can be expected to affect the β-pol activity towards the kind of substrate used. Furthermore, any situation which could influence the processivity of β-pol catalysis (see Figs. 5 and 6 where the usual Mg^{2+} is replaced with Mn^{2+} in the reaction mixture) would also have a bearing on the primer extension activity by β-pol; replacement of Mg^{2+} with Mn^{2+} resulted in notable improvement in the extension of the primer to its full predicted length (21 nucleotides). This accounts for the addition of eight nucleotides even if one assumes that only one base is excised by the proof-reading exonuclease. Thus, under appropriate conditions β-pol is capable of adding several nucleotides to a staggered 3'-end of a primer. It would be necessary to extend these studies with other types of substrates like nicked or gapped DNA duplexes or a DNA duplex with a mismatch somewhere in the middle.

Be that as it may, the present study shows that the β-polymerase activity, the major DNA polymerase present in brain cells decreases with age. This may well be an important factor contributing to the senescence and death of brain cells. A more intriguing finding of this study is the ability of the exogenously added β-pol to restore the lost activity even in old neuronal extracts. The technological consequences of this observation are a matter for speculation.

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DNA-polymerase a, (3, 8 and ε activities in isolated neuronal and astroglial cell fractions from developing and aging rat cerebral cortex

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Abstract

The relative proportions of DNA-polymerases a, B, 8 and ε (pols α, B, 8 and £) activities in isolated neuronal and astroglial cell fractions from developing, adult and aging rat brain cerebral cortex, were examined. This was achieved through a protocol that takes advantage of the reported differential sensitivities of different DNA-polymerases towards certain inhibitors like butylphenyl and butylanilino nucleotide analogs, 2′,3′-dideoxythymidine triphosphate (ddTTP), monoclonal antibody of human a polymerase and the use of two template primers as substrates. The results indicate that while DNA-polymerase ß (pol B) is the predominant enzyme, significant levels of DNA-polymerases a and 8/e (pols a and δ/ε) are also present in both cell types at all the post-natal ages studied. A notable difference regarding the relative abundance of DNA-polymerases other than B is the higher percentage of pol 8/e in neurons and a more sustained pol a activity through the life span in astroglia. The presence of detectable proportion of DNA-polymerases other than ß (particularly the 8/e type) may be taken to indicate their role in long patch base excision repair as well as in other modes of DNA repair.

Keywords: DNA-polymerases 8 and e; Neurons and astroglia; Aging brain

1. Introduction

The family of eukaryotic DNA-polymerases is a growing one. A number of new DNA-polymerases performing various functions to contribute to the overall DNA-replication and maintenance of its structural integrity (Hubscher et al., 2000; Livneh, 2001; Burgers et al., 2001) have been discovered in the recent years. Nevertheless, four mammalian nuclear DNA-polymerases are generally considered to be major ones in discharging the above responsibilities—DNA-polymerases a, β, 8 and e (pols a, (3, 8 and e) with, of course, their functions overlapping (Wood and Shivji, 1997). However, in a post-mitotic cell like neuron, where replicative activity is absent, whatever DNA-polymerase activity observed was found to be almost exclusively that of pol β (Waser et al., 1979). Studies from this laboratory over the years have also revealed that pol β is the most predominant DNA-polymerase in rat brain (Rao, 1997). In a careful study, using whole brain extracts and specific inhibitors towards various DNA-polymerases and a monoclonal antibody to pol a, we noticed indications that while the most predominant activity was that of pol β, some activity that could be attributable to pols a and 8/e was also present (Prapurna and Rao, 1997). However, brain consists of heterogeneous populations of cells with two major types—neurons and astroglia with different characteristics. The present study is, therefore, taken up to examine the DNA-polymerase activities in extracts of isolated neuronal and astroglial cell fractions from the rat cerebral cortex at three different ages. It is noticed that while pol β is the most predominant DNA-polymerase in these brain cells at all the post-natal ages, some activity attributable to pols a, 8 and e is also present.

Abbreviations: BER, base excision repair; BuAdATP, N^2-(*p*-*n*-butylaniIino)-2'-deoxyadenosine-5'-triphosphate; BuPdGTP, N^2-(*p*-*n*-butylphenyl)-2′-deoxyguanosine-5′-triphosphate; DdTTP, 2'.3'-dideoxythymidine-5′-triphosphate; FEN1, flap endonuclease 1; PCNA, proliferating cell nuclear antigen; pol a, DNA-polymerase or, pol B, DNA-polymerase B; pol 5, DNA-polymerase 8; pol ε, DNA-polymerase e

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2. Experimental procedures

2.1. Materials

Highly polymerized calf thymus DNA, 'activated' calf thymus DNA (may be defined as the DNA partially digested with small amounts of DNase I to induce

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nicks), bovine serum albumin (BSA), ATP and deoxynucleotide triphosphates were purchased from Sigma (St Louis, MO, USA). Poly(dA)·oligo(dT)$_{12-18}$ and poly(dA·dT) were purchased from Midland Certified Reagent Co. (Midland, TX, USA). Monoclonal antibody, SJK 132-20 against human pol a was obtained from PL-Biochemicals, WI, USA. Dimethyl sulphoxide (DMSO) was from Sisco Research Laboratory (Bombay, India). 2′,3′-Dideoxythymidine-5′-triphosphate (ddTTP) was from Boehringer Mannheim, Germany. The nucleotide analogs, N^2-(*p*-*n*-butylphenyl)-2′-deoxyguanosine-5′-triphosphate (BuPdGTP) and N^2-(*p*-*n*-butylanilino)-2′-deoxyadenosine-5′-triphosphate (BuAdATP), were generous gifts from Dr. George Wright (Department of Pharmacology, University of Massachusetts Medical School, Worcester, MA, USA). ^{3}H-TTP (specific activity 78 Ci/mmol) was purchased from New England Nuclear, USA. All other chemicals used were of analytical grade.

2.2. Animals

In-bred Wistar rats of different ages (1-4 days, 6 months and 26-28 months post-natal) were obtained from the University of Hyderabad animal facility. Rats were maintained in a pathogen free environment with a 12 h: 12 h light-dark cycle; food and water were provided ad libitum.

2.3. Preparation of cell extracts

Animals were killed by decapitation. Neurons and astroglia from rat brain cerebral cortex of different ages were prepared essentially as standardized and followed in this laboratory over the years (Usha Rani et al., 1983). The final preparation of the cells were suspended at a concentration of 10 million cells per ml in extraction medium consisting of 20 mM Tris pH 7.5, 0.1 mM dithiothreitol, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 0.1 mM PMSF (just before use), 5 mM β-mercaptoethanol, 1 mM $MgCl_2$, 1 }xg/ml leupeptin and 1 μg/ml pepstatin A (both just before use) and 0.5 M KC1 and sonicated for 5 s, three times with the setting of five in a Branson sonifier. The suspension was kept at 0–4 °C for 30 min and then centrifuged at 100,000 x *g* for 1 h in a Beckman ultracentrifuge and the clear supernatant was used as the source for DNA-polymerases.

2.4. DNA-polymerase assay

Polymerase assays were performed with either 'activated' calf thymus DNA or with poly(dA)·oligo(dT)$_{12-18}$ or poly(dA·dT) as template primer. With 'activated calf thymus DNA' the incubation mixture (total volume 50 μl) contained 40 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 1 mM β-mercaptoethanol, 4 mM ATP, 100 μM each dATP, dGTP, dTTP, 25 μM dCTP, 5 μg 'activated' DNA, 1 μ Ci α-^{32}P-dCTP (4000 Ci/mmol) and cell extract as enzyme source (10 μg protein). With poly(dA)·oligo(dT)$_{12-18}$ or poly(dA·dT) as template primer, the reaction mixture (total volume 50 μl) contains 40 mM Tris-HCl pH 7.5, 0.8 mM $MgCl_2$, 5 μg of BSA, 2% glycerol, 2 mM dithiothreitol, 50 |xM dATP (when poly(dAdT) was the substrate), 25 (xM TTP, 1 μ Ci α-^{32}P-TTP (4000 Ci/mmol) and enzyme (20 μg protein). In either case, the mixture was incubated for 20 min at 37 °C, 200 μg each of DNA and BSA were added and the reaction stopped with 1 M:10 mM perchloric acid:tetra sodium pyrophosphate, respectively. The samples were kept on ice and centrifuged at 3000 x *g* for 10 min. The supernatant was aspirated carefully and the precipitate was dissolved in 0.5 ml of 0.2 M NaOH. A 2 ml volume of stop solution was again added and centrifuged after 10 min. The alkali solubilization step and reprecipitation with stop solution was repeated. The whole solution along with the precipitate was transferred to 2.5 cm glass fiber filters (Schleicher & Schuell) and washed three times with ice-cold stop solution and twice with 95% ethanol. The washed filters were directly taken into counting vials and dried by keeping in oven at 40 °C for 20 min or keeping in a hood overnight. Then toluene-based scintillation fluid having Triton-X 100 was added and the radioactivity was counted in a Wallac 1409 counter. Activity is expressed as picomoles of radioactivity incorporated into acid insoluble fraction.

The strategy adopted to assess the levels of different polymerases, based on their substrate preferences and differential sensitivities towards various inhibitors was to use two different concentrations of inhibitors during incubation (Prapurna and Rao, 1997). For BuPdGTP, the concentrations were 1 and 200 μM; for BuAdATP, 1 and 100 μM; and for ddTTP, 50 μM and 1 mM. These concentrations of inhibitors were arrived at after careful examination of the sensivities exhibited to various inhibitors by pols α, 8 and (3 and the $I_{0.5}$ (the amount of analog that gave 50% inhibition of the polymerase activity) values reported by Lee et al. (1985) with respect to a given template primer. The reported sensitivity of β and δ polymerases towards ddTTP were also taken into account (Dresler and Kimbro, 1987; Wahl et al., 1986). Accordingly, the polymerase activity (with activated DNA as substrate) inhibited by 1 μM BuPdGTP or BuAdATP was considered as the activity of pol a, whereas, at higher concentrations the additional reduction in activity is taken to be associated with pols 8 or e. Similarly, the reduction in activity with 50 μM ddTTP was taken to be pol (3 and further inhibition noticed at 1 mM level of ddTTP was attributed to pols 8 or ε. Pol α activity was considered to be the activity inhibited by the pol a specific monoclonal antibody, SJK 132-20. With poly(dA)·oligo(dT)$_{12-18}$ as substrate, the same procedure was followed, except that the two concentrations of BuPdGTP used were 10 and 100 μM and those for BuAdATP were 15 and 200 μM, respectively. When using inhibitors the extracts were pre-incubated with the inhibitor for 10 min on ice prior to the start of the reaction.

Statistical analysis of the data was done using Sigma plot 2000 software.

able 1
)NA-polymerase activity in rat neuronal and astroglial cells of different ges with three different template primers

ubstrate and cell action used	Age		
	Young	Adult	Old
ctivated DNA			
Neurons	2023 ± 1076	719 ± 541[a]	568 ± 412[a]
Astroglia	1471 ± 550	822 ± 512[a]	694 ± 652[a]
oly(dA)·oligo(dT)$_{12-18}$			
Neurons	18.4 3.9	17.7 ± 3.2	17.7 ± 3.6
Astroglia	37.6 ± 18.6	33.0 ± 14.9	31.2 ± 11.8
oly(dA·dT)			
Neurons	16.7 ± 3.0	18.0 ± 4.8	19.1 ± 3.6
Astroglia	35.9 ± 16.5	35.7 ± 14.9	34.5 ± 12.0

)lues are averages ± S.D. and expressed as picomoles of the radioactive eoxynucleotide incorporated into the acid insoluble fraction in 1 h/mg tetein. Eleven and nine independent experiments (with separate extracts) ere performed in the case of neurons and astroglia, respectively. While ere was considerable variation in the activities from one animal to the her, the trend of changes with age and a given substrate were quite milar.

) [a]These values are significantly different ($P < 0.001$ for neurons id 0.02 for astroglia, respectively) from the corresponding value when

Results

The DNA-polymerase activities in isolated neuronal and troglial cells from rat cerebral cortex of three different ges are shown in Table 1. The three different ages are des-)nated as young, 1-4 days; adult, 6 months; and old, more tan 24 months post-natal. Three different template primers)re used to measure the polymerase activity, the 'activated)NA', poly(dA)·oligo(dT)$_{12-18}$ and poly(dA·dT). As can) seen from Table 1, the activity, in either type of cells, ith synthetic oligos is far less as compared to that with)ctivated' DNA. Also, no age dependent changes were seen L the activity with synthetic oligos, whereas, the activity th 'activated' DNA undergoes a significant decrease by e time the animal reaches adult stages of life. There is con-derable variation in the activities from one animal to the her. However, the pattern of changes with age are quite insistent. These results with activated DNA confirm our rlier observations (Rao et al., 2000).

The extent of inhibition exerted by the various inhibitors)d are shown in Tables 2 and 3 with activated DNA as sub-rate. Table 2 shows results with neuronal extracts. The ac-)ty without any inhibitor (control) is taken as 100 and other ilues are relative to the control. The polymerase activity in)ng neurons is inhibited by 35% by 1 μM BuPdGTP. This tivity was, therefore, attributed to pol a. This assumption)eared to be reasonable, since the monoclonal antibody human α polymerase also exerted inhibition to a similar ent (as shown in the last row in Table 2). However, when e concentration of BuPdGTP was raised to 200 μM, the)vity was inhibited by 71%. The rise in inhibition by 36%

Table 2
Effect of various inhibitors on the activity of DNA-polymerases in extracts of neuronal cells isolated from rat brain of different ages using calf thymus 'activated' DNA as template primer

Inhibitors concentration	Activity (%) at different ages		
	Young	Adult	Old
Control	100	100	100
+BuPdGTP, 1 μM	65.0 ± 4.8	77.3 ± 12.2	82.2 ± 3.2
+BuPdGTP, 200 μM	28.7 ± 7.4	42.1 ± 0.6	33.5 ± 15.0
+BuAdATP, 1 μM	68.6 ± 2.7	92.3 ± 2.2	78.5 ± 12.4
+BuAdATP, 200 μM	31.1* ± 4.7	49.5 ± 7.5	27.3 ± 9.4
+ddTTP, 50 μM	50.0 ± 14.6	57.4 ± 1.6	57.6 ± 13.0
+ddTTP, 1 mM	22.3 ± 5.5	37.3 ± 6.0	31.3 ± 8.4
+SKJ 132-20 ab, 1 μ	68.4 ± 1.3	72.1 ± 6.2	77.5* ± 3.7

Activity expressed as shown in Table 1. The control value is taken as 100 and the other values in the presence of various inhibitors are expressed average ± S.D. The values are from three different experiments (separate extracts) except in two cases marked with an asterisk.

was thought to be due to the inhibition of pols 5 and/or £. A similar rationale applies to our interpretation of data with the other nucleotide analog inhibitor, BuAdATP. At 50 μM level, ddTTP inhibited 50% the polymerase activity and at the raised concentration of 1 mM, the inhibition went up to 78%. Here, the percentage of activity inhibited by 50 μM ddTTP was attributed to pol β activity and the additional inhibition at 1 mM level was attributed to pols 8 and/or ε activity.

Table 3 shows the range of inhibitions exerted by the same inhibitors with astroglial extracts. One notable difference between neurons and astroglia seems to be relatively higher levels of pol α and lower levels of pol 8/e in astroglia than in neurons. As per the rationale explained above, the relative percentages of different DNA-polymerases, out of the total polymerase activity, are calculated and presented in Table 4. The value obtained for each of the inhibitor used with respect to calculating the percentage of a given polymerase is shown and the average is taken to minimize the error. In

Table 3
Effect of various inhibitors on the activity of DNA-polymerases in extracts of astroglial cells isolated from rat brain of different ages using calf thymus 'activated' DNA as template primer

Inhibitor concentration	Activity (%) at different ages		
	Young	Adult	Old
Control	100	100	100
+BuPdGTP, 1 μM	57.7 ± 7.4	80.8 ± 7.0	58.6 ± 11.3
+BuPdGTP, 200 μM	24.5 ± 2.0	60.2 ± 4.2	44.7 ± 4.2
+BuAdATP, 1 μM	49.8 ± 1.0	70.8 ± 7.66	79.4 ± 2.9
+BuAdATP, 200 μM	19.7 ± 2.2	46.3 ± 10.5	79.4 ± 2.9
+ddTTP, 50 μM	33.6 ± 6.2	49.7 ± 6.2	47.2 ± 7.6
+ddTTP, 1 mM	21.8 ± 1.4	39.4 ± 5.8	33.5 ± 5.2
+SKJ 132-20 ab, 1 μ	50.9 ± 13.1	76.1 ± 8.5	57.6 ± 4.5

Activity expressed as shown in Table 1. The control value is taken as 100 and the other values in the presence of various inhibitors are expressed average ± S.D. The values are from three different experiments (separate extracts).

Table 4
Percentage of different DNA-polymerases present in neuronal and astroglial cell fractions isolated from rat brain of different ages using calf thymus 'activated' DNA as template primer

	Neurons									Astroglia								
	a			δ/ε			P			a			δ/ε			P		
	Young	Adult	Old	Young	Adult	Old	Young	Adult	Old	Young	Adult	Old	Young	Adult	Old	Young	Adult	Old
BuPdATP	35	23	18	36	35	49	NS	NS	NS	42	19	41	33	21	14	NS	NS	NS
BuAdGTP	31	8	22	38	42	51	NS	NS	NS	50	29	21	30	24	20	NS	NS	NS
ddTTP	NS	NS	NS	28	20	27	50	43	42	NS	NS	NS	12	10	14	66	50	53
SJK 132-20	32	28	22	NS	NS	NS	NS	NS	NS	49	24	42	NS	NS	NS	NS	NS	NS
Average	33	20	21	34	32	42	50	43	42	47	24	35	25	19	16	66	50	53

These data are recalculated, as explained in text, from the values in Tables 2 and 3. NS, not sensitive.

Table 5
Effect of various inhibitors on the activity of DNA-polymerases in extracts of neuronal cells isolated from rat brain of different ages using poly(dA)·oligo(dT)$_{12-18}$ as template primer

Inhibitor concentration	Activity (%) at different ages		
	Young	Adult	Old
Control	100	100	100
+BuPdGTP, 1 μM	61.9 ± 3.9	78.9 ± 1.6	77.5 ± 1.5
+ BuPdGTP. 200 μM	43.4 ± 2.5	68.3 ± 3.6	43.5 ± 0.9
+BuAdATP. 1 μM	64.1 ± 2.4	82.5 ± 1.8	81.7 ± 4.6
+ BuAdATP. 200 μM	48.6 ± 3.5	70.7 ± 3.3	48.9 ± 2.7
+ddTTP. 50 μM	59.5 ± 5.2	59.3 ± 3.7	73.1 ± 1.3
+ddTTP. 1 mM	35.0 ± 4.4	50.5 ± 1.4	50.1 ± 3.8
+SKJ 132-20 ab, 1 μ	56.6 ± 11.5	78.2 ± 4.6	76.4 ± 7.8

Activity expressed as in Table 1. The control value is taken as 100 and the other values in the presence of various inhibitors are expressed average ± S.D. The values are from three different experiments (separate extracts).

Table 6
Effect of various inhibitors on the activity of DNA-polymerases in extracts of astroglial cells isolated from rat brain of different ages using poly(dA)·oligo(dT)$_{12-18}$ as template primer

Inhibitor concentration	Activity (%) at different ages		
	Young	Adult	Old
Control	100	100	100
+BuPdGTP,1 μM	73.0 ± 0.4	80.2 ± 0.6	73.3 ± 1.2
+BuPdGTP, 200 μM	59.8 ± 1.3	61.3 ± 1.3	61.2 ± 5.1
+BuAdATP, 1 μM	65.1 ± 1.4	71.1 ± 2.6	78.3 ± 1.2
+BuAdATP, 200 μM	49.9 ± 0.6	58.4 ± 2.5	70.4 ± 0.4
+ddTTP. 50 μM	63.4 ± 0.8	63.9 ± 1.1	61.2 ± 2.3
+ddTTP. 1 mM	44.5 ± 1.2	50.8 ± 2.4	53.3 ± 1.0
+SKJ 132-20 ab, 1 μ	68.8 ± 8.4	76.6 ± 1.3	75.7 ± 0.2

Activity expressed as in Table 1. The control value is taken as 100 and the other values in the presence of various inhibitors are expressed average ± S.D. The values are from three different experiments (separate extracts).

both the type of cells and at all the three ages studied. pol (3 appears to be the predominant polymerase, an observation in line with many earlier reports. However, the pol 8/e activity is closely behind the pol β activity in neurons, whereas, this second position is taken by pol a in astroglia. Thus, the relative percentages of pol β in young, adult and old neurons are 50, 43 and 42, respectively. For pol α in the same order, the values are 33. 20 and 21. For pol 5/e, the values are read as 34, 32 and 42. In astroglia, the relative abundance of pol β at young, adult and old ages are 66. 50, and 53, respectively; for pol a, in the same order, 47. 24, and 35; for pol 5/e, the figures are 25, 19 and 16.

In Tables 5-7, the results of similar experiments with poly(dA)·oligo(dT)$_{12-18}$ as template primer are shown. Firstly, the basal activity itself is very low with this substrate (Table 1). The pattern of results with various inhibitors, in both neurons and astroglia, is comparable to that noticed with activated DNA as substrate. One striking difference

Table 7
Percentage of different DNA-polymerases present in neuronal and astroglial cell fractions isolated from rat brain of different ages using poly(dA)·oligo(dT)$_{12-18}$ template primer

	Neurons									Astroglia								
	a			5/e			P			a			5/e			β		
	Young	Adult	Old	Young	Adult	Old	Young	Adult	Old	Young	Adult	Old	Young	Adult	Old	Young	Adult	Old
BuPdATP	38	21	22	19	11	34	NS	NS	NS	27	20	27	13	19	12	NS	NS	NS
BuAdGTP	36	18	18	16	12	33	NS	NS	NS	35	29	22	15	13	8	NS	NS	NS
ddTTP	NS	NS	NS	24	9	23	41	41	27	NS	NS	NS	19	13	8	37	36	39
SJK 132-20	43	22	24	NS	NS	NS	NS	NS	NS	31	23	24	NS	NS	NS	NS	NS	NS
Average	39	20	21	20	11	30	41	41	27	31	24	24	16	15	9	37	36	39

These data are recalculated, as explained in text, from the values in Tables 5 and 6. NS, not sensitive.

between the two cell types is the higher percentage of pols 8/e activity in neurons even in aging brain with a more sustained pol a activity in astroglia. Also, the ddTTP inhibition is less potent in astroglia with the synthetic substrate leading to lower percentages of pols β and 8/e (compare the data in Tables 4 and 7). It thus appears that this synthetic primer may be more useful to distinguish various DNA-polymerase activities with pure proteins (Syvaoja et al., 1990) rather than with crude extracts. Nonetheless, the data do indicate the presence of activity that is attributable to pols 8/e throughout the lifespan.

4. Discussion

A major outcome of this investigation is the finding that both neuronal and astroglia] cells of rat brain cerebral cortex contain significant activity that can be considered to be due to pols α and 8/e. This is the first report to suggest the presence of pol 8/e activity in isolated cell fractions of developing as well as aging rat brain. In-line with earlier observations pol β is the most predominant polymerase in both cell types and at all the three ages studied. This activity, of course, shows a marked reduction with age (Table 1). However, the activity towards synthetic substrates (poly(dA·dT) and poly(dA)·oligo(dT)$_{12-18}$) is low and does not show significant change with the advancement of age (Table 1). It is possible that these synthetic substrates are not preferred ones for pol (3. On the other hand, it was reported earlier by Syvaoja et al. (1990) that pols 8 and e are active towards these substrates. It is, therefore, taken that this low but steady activity is due to pols δ/ε present in the cell extracts. The percentage calculations for each type of nuclear DNA-polymerase is based on the extent of inhibition exerted by various known inhibitors for these polymerases and with two different substrates, the activated DNA and poly(dA)·oligo(dT)$_{12-18}$. It may be argued that this is an oversimplification of a complex situation since the inhibitors used in this study are not absolutely specific and the calculated percent distribution of the polymerases is only approximate. In spite of this limitation, these data do give sufficient hint regarding the relative abundance of the various DNA-polymerase activities in two major cell types of rodent brain as sufficient care was taken in choosing the two different concentrations of the inhibitors.

It has been the general conclusion during the past that adult brain contains almost exclusively one DNA-polymerase and that is the β type. Traces of other polymerase activity was suspected to be the α type (Waser et al., 1979; Shrivastaw et al., 1983; Subrahmanyam and Rao, 1988; Rao, 1997). These observations appeared logical since only repair but not replication of DNA is required in a post-mitotic tissue like brain and pol (3 is considered to be a repair enzyme (Wood and Shivji, 1997; Hubscher et al., 2000). Neuronal cells, once differentiated, do not replicate, but they show high metabolic activity and the gene expression in these cells is two-three times more as compared to the cells in the rest of the body (Chaudhari and Hahn, 1983; Tobin, 1994). Also the genomic DNA in brain cells is shown to suffer various types of damage including oxidative damage and double strand breaks even in the absence of any specific environmental insult (Rao, 1997). Abasic sites and many types of base modification alterations in DNA can be repaired in brain cells through base excision repair pathway (BER) and it is well established now that pol β is the enzyme that participates in BER. In a 'short patch' BER, a single nucleotide gap is filled by pol β, whereas, in the 'long patch' BER, 2–13 nucleotides are inserted. In the later version, the participating DNA-polymerase was shown to be either pol β (upto 6 nucleotides) or pol 8/e (upto 13 nucleotides). Moreover, additional factors like proliferating cell nuclear antigen (PCNA) and flap nuclease (FEN1) are required for the long patch BER (Wilson, 1998; Prasad et al., 2000; Rao, 2002). Similarly, double strand breaks, which could be lethal if left unrepaired, are usually processed by non-homologous end joining (NHEJ) which is an error prone mode of repair and is suspected to be requiring some DNA-polymerase activity in order to process/extend the ends before joining by ligase (Pospiech et al., 2001; Ren and de Ortiz, 2002). It is not known which DNA-polymerase is involved in this kind of double strand break repair in brain. It is quite possible that pol 8/e present in neuronal and astroglial cells, as suggested by the present data, are aptly suited for this purpose.

There is now considerable evidence that even in adult brain, some progenitor stem cells are present capable of differentiating into mature neurons (Van Praag et al., 2002). It is possible that a small portion of the different polymerase activities noticed in the present study could have come from these maturing neurons. It is not. however, clear what are the in vivo stimuli that would induce this differentiation and that too in the specific area of brain used in this study-viz. the cerebral cortex. In any case the present results may largely reflect the situation in terminally differentiated neurons which are essentially incapable of dividing.

It was not possible, with protocol used in these studies, to distinguish between pols 8 and e activities. We faced severe difficulties in our attempts to purify these activities. Continued attempts using various other possibilities would constitute our future studies.

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