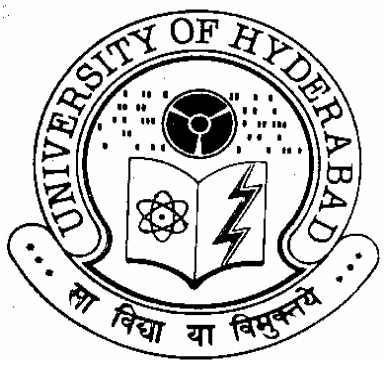
Characterization of Topoisomerase II function in neuronal development, DNA repair and HIV-1 induced inflammation

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

M. RAJAKUMAR



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DECLARATION

I hereby declare that the work presented in my thesis is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of Prof. Anand K. Kondapi. I further declare that this has not been submitted before for the award of degree or diploma from any Institute or University.

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CERTIFICATE

This is to certify that thesis entitled "**Characterization of Topoisomerase II function in neuronal development, DNA repair and HIV-1 induced inflammation**" submitted to the University of Hyderabad by **Mr. M. Rajakumar** for the degree of Doctor of Philosophy, is based on the studies carried out by him under my supervision. This work has not been submitted before for the award of degree or diploma from any University or Institution.

Prof. Anand K. Kondapi
Supervisor

Head, Department of Biochemistry

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Date:

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ABBREVIATIONS

µg	: Microgram
µl	: Microlitre
µM	: Micromolar
mM	: Millimolar
pM	: Picomolar
fM	: Femtomolar
ADP	: Adenosine di phosphate
AIDS	: Acquired Immunodeficiency syndrome
ALP	: Alkaline Phosphatase
ATP	: Adenosine 5' triphosphate
AZT	: 3'-azido-3'-deoxythymidine
BCIP	: 5- bromo – 4 – chloro – 3 indoyl phosphate
Bis-acrylamide	: *N,N'*- methylene –bis –acrylamide
bp	: Base pairs
BSA	: Bovine serum albumin
$CaCl_2$	: Calcium chloride
CCR5	: Cystein-cystein linked chemokine receptor 5
cDNA	: Complementary DNA
CO_2	: Carbon dioxide
cPPT	: Central polypurine tract
CTS	: Central termination signal
CXCR4	: Cystein-x-cystein linked chemokine receptor 4
DMEM	: Dulbecco's modified Eagle medium
DMSO	: Di methyl sulphoxide
DNA	: Deoxy ribonucleic acid
DNase I	: Deoxy ribonuclease I
dNTPs	: Deoxynucleotidetriphosphates
dsDNA	: Double-stranded RNA
DTT	: Dithiothreitol
EDTA	: *N-N*- Ethylene di amine tetra acetic acid
ELISA	: Enzyme linked immunosorbent assay

Env	: Viral envelope
EtBr	: Ethidium bromide
FBS	: Fetal Bovine Serum
FCS	: Fetal calf serum
gag	: Group-specific antigen
gp	: Glycoprotein
HCl	: Hydrochloric acid
HIV	: Human Immunodeficiency Virus
HLA	: Human leukocyte antigen
HPO_4	: Hydrogen phosphate
HSV	: Herpes simplex virus
IgG	: Immunoglobulin G
IL	: Interleukin
IN	: Integrase
Kb	: Kilo basepairs
KCl	: Potassium chloride
kDa	: Kilo daltons
LB	: Luria- Bertani
LTR	: Long Terminal Repeat
MA	: Matrix protein
MAb	: Monoclonal antibody
Met	: Methionine
mg	: milligrams
$MgCl_2$	: Magnesium chloride
min	: Minutes
ml	: Millilitre
$MnCl_2$	: Manganese chloride
mRNA	: Messenger RNA
MTT	: 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2HPO_4	: Disodium hydrogen phosphate
NaCl	: Sodium chloride
NBT	: Nitro blue tetrazolium
NC	: Nitrocellulose membrane

NC	: Nucleocapsid protein
NDGA	: Nordihydroguaiaretic acid
ng	: Nanogram
p.i.	: post infection
PAGE	: Poly acrylamide gel electrophoresis
PBCV	: Paramecium bursaria chlorella virus
PBMC	: Peripheral Blood Mononuclear Cell
PBS	: Phosphate buffer saline
Pbs	: primer binding site
PCR	: Polymerase chain reaction.
PEG	: Poly ethylene glycol
PHA	: Phytohemagglutinin
pI	: Isoelectric point
PIC	: preintegration complex
PMSF	: Phenylmethylsulphonyl fluoride
pol	: RNA polymerase
PPT	: polypurine tract
PR	: protease
PVDF	: Polyvinylidene Fluoride
RNA	: Ribonucleic acid
RNAi	: RNA mediated interference
RNase	: Ribonuclease
rNTPs	: Ribonucleotide tri-phosphate
rpm	: Revolutions per minute
RPMI	: Roswell Park Memorial Institute medium
RRE	: Rev response elements
RT	: Reverse transcriptase
SDS	: Sodium dodecyl sulphate
siRNA	: Small interference RNA
SIV	: Simian immunodeficiency virus
SU	: Surface glycoprotein
SV40	: Simian virus 40
TAR	: Transactivation-response elements
Tat	: Trans-activator of transcription

TE buffer	: Tris-ETDA buffer
TEMED	: *N',N',N',N'*- tetra methyl ethylene diamine
TFIID	: Transcription factor IID
TM	: Transmembrane protein
TRIS	: Tris (hydroxy methyl) amino methane
tRNA	: Transfer RNA
U3 region	: Unique sequence at the 3′ terminus
U5 region	: Unique sequence at the 5′ terminus
UV	: Ultraviolet

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CHAPTER 1

INTRODUCTION

Topoisomerases

Virtually every facet of nucleic acid physiology is influenced by the topological state of DNA (Wang, 1985). Topoisomerase catalyses 3 types of reactions viz., relaxation of supercoiled DNA, inter-conversions between single stranded DNA rings and linking of single stranded rings of complimentary sequences. Clearly, the cell's ability to regulate the topological state of DNA is imperative for its viability.

Two classes of ubiquitous enzymes, the type I and type II Topoisomerases, modulate DNA topology.

a) **Topoisomerase I** can relieve torsional constraints in DNA by passing a single strand of DNA through a transient nick made in the complementary strand (Wang, 1985; Osheroff, 1989). Although type I enzyme is not required for cell survival (Uemura and Yanagida, 1984), it plays important roles in DNA replication (Goto and Wang, 1985) and transcription (Muller *et al.,* Gilmour *et al.,* 1986; Garg *et al.,* 1987)

b) **Topoisomerase II** can relieve both torsional and interlocking constraints in double stranded nucleic acids by passing an intact helix through a transient double stranded break made in a second helix (Wang, 1985; Osheroff, 1989). Topoisomerase II is essential to the Eukaryotic cell (Uemura and Yanagida, 1984; Holm *et al.,* 1985). It is required for chromosome segregation (Uemura and Yanagida, 1984; Holm *et al.,* 1985) and maintenance of chromosome structure (Earnshaw *et al.,* 1985) and plays role in DNA replication and recombination (Wang, 1985). DNA Gyrase is a prokaryotic Topoisomerase II and its catalytic activity is same as that of eukaryotic enzyme.

Human Topoisomerase II:

Topoisomerase II (TopoII) is uniformly distributed along the chromosomes (Heck *et al.,* 1988; Hsiang *et al.,* 1988) and is uniquely required for segregation of completely replicated daughter molecules during mitosis (Yang *et al.,* 1987; Snapka, 1988; Uemura *et al.,* 1987; Ritcher, 1988) and meosis (Holm *et al.,* 1985; Uemura and Yanagida, 1986).

In mammals TopoII is found to be present as 170 kDa, α and 180 kDa, β isoforms (Woessner *et al.,* 1991, Coutts *et al.,* 1993). Both the isoforms show structural

similarity but are genetically, immunologically and biochemically distinct. They show distinct cellular localization and cell cycle expression profiles. TopoIIα activity is shown to be highest during the G_2/ M phase of the cell cycle (Heck *et al.,* 1988). Where as TopoIIβ is constant throughout the cell cycle (Woessner *et al.,* 1991). TopoIIα is distributed in the nucleoplasm (Woessner *et al.,* 1990) in contrast to TopoIIβ, which is localized in the nucleolus during interphase, and in the cytoplasm during mitosis (Negri *et al.,* 1992). Both the isoforms show different patterns of tissue distribution. TopoIIα is shown to be higher in testes, spleen, bone marrow and liver.

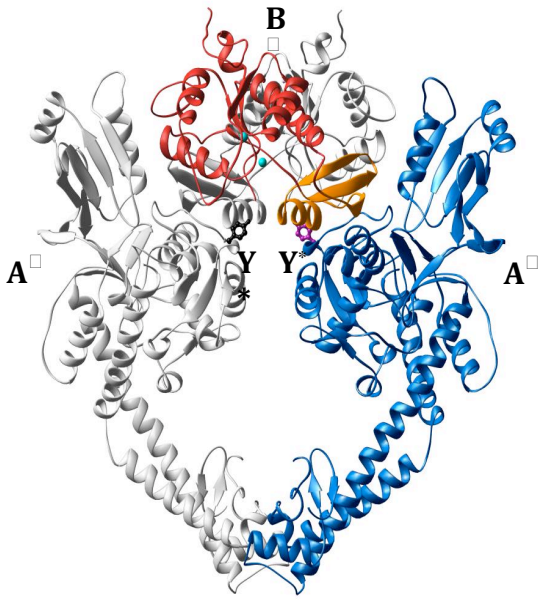
Both the isoforms of TopoII show similar catalytic activities. But the exact roles shared by these enzymes are unknown. TopoIIβ protein differs from TopoIIα in many important aspects. The genes coding for the TopoIIα and β proteins map to chromosomes 17q21-22 and 3p24 and are clearly distinct. TopoIIβ protein is less sensitive to inhibition by intercalating agents and epipodophillotoxins than the α protein.

Structure of Topoisomerase II

The crystal structure of TopoII was worked out in detail by Berger *et al.,* in 1996. The study shows that TopoII in its active form is a heart shaped homodimer with a large central hole. The monomer is a flat crescent shaped fragment, which can be divided into three discrete domains. The first is the binding domain in the N-terminal region ($B^{/}$ region). It has a consensus sequence for ATP binding and has the capacity to hydrolyze ATP. This domain dimerizes with the other monomer upon binding of ATP, and imposes a conformational change all over the enzyme, required for catalytic activity.

The second is the DNA binding domain or the DNA breakage/reunion domain, present in the $A^{/}$ region. The active site tyrosine's, which associate with the broken ends of DNA during the catalytic cycle, are present in this domain. The third is the primary dimer interface in the C-terminal region, which forms the dimer interface of the enzyme by associating with the other monomer. Apart from forming the dimer interface, this region is also implicated in regulation of enzyme activity and nuclear localization.

Figure 1.1



From Berger *et al.,* 1996

Catalytic Activity of Topoisomerase II:

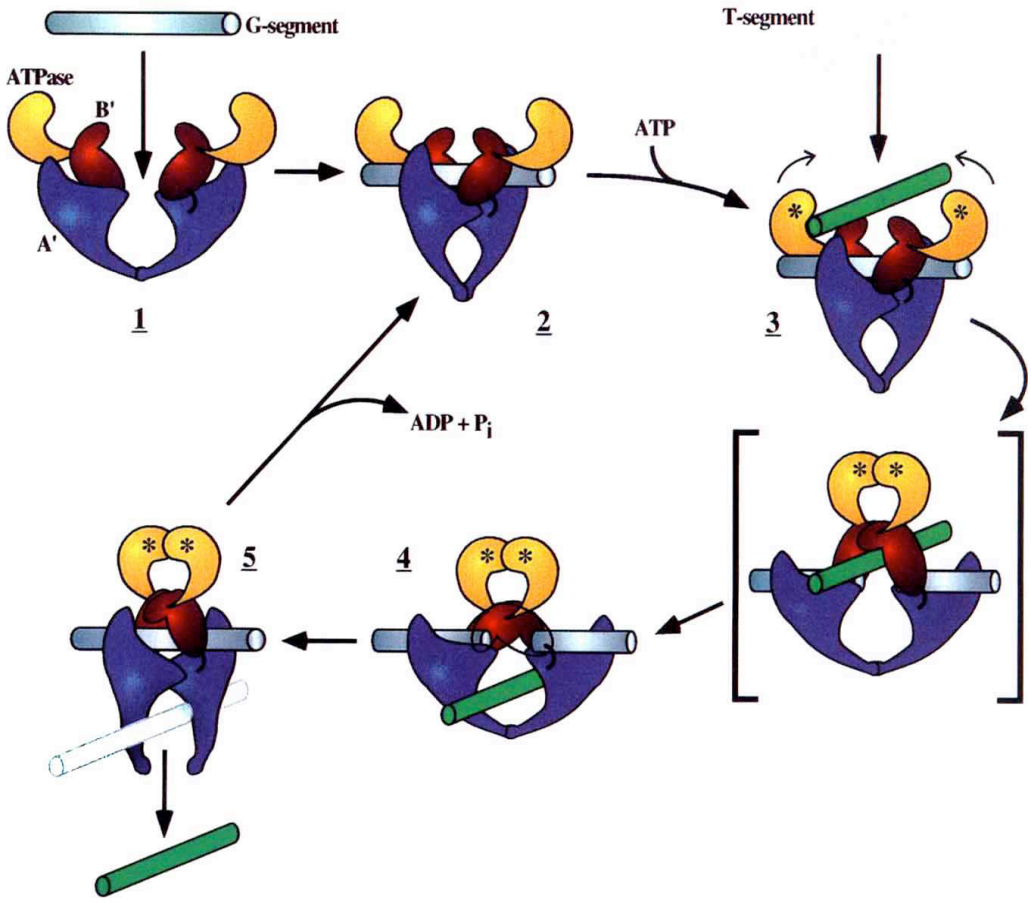
In its catalytic cycle (Berger *et al.,* 1996), the topoisomerase II dimer first binds to a duplex DNA segment termed as the 'G' (gated) segment and undergoes a conformational change. It then binds to ATP through its ATP binding domain and also binds to a second DNA segment called the 'T' (transported) segment. This binding causes a series of conformational changes in the enzyme, which causes the A′ regions to be pulled apart from each other, leading to cleavage of the G-segment in both the strands, four base pairs apart. The active site tyrosine in the DNA binding domains then form covalent bonds.

The molecular model for the catalytic reaction of TopoII

The ATPase domain, B′ and A′ subfragments are coloured yellow, red and blue respectively. The G-segment DNA (containing the DNA gate) is grey, and the transported T-segment is green with the nicked DNA strands through a trans-esterification reaction between the phenolic hydroxyl groups of the tyrosine and the 5′-phosphoryl ends of the nicked DNA. Concomitantly, the ATP domains dimerize and the T-segment is transported through the gate formed by the nicked DNA into the central hole.

Following this transport, the G-segment is rejoined by a second trans-esterification reaction and the T-segment is transported out of the enzyme through the opening formed in the dimer interface. The monomers immediately dimerize at the interface and the ATP is hydrolyzed and released. This regenerates the starting state and the enzyme is ready to begin a fresh catalytic cycle. The DNA cleavage/religation reactions do not require energy from a high-energy co-factor (like ATP) because the phosphate bond energy is conserved in the two successive trans-esterification reactions (Roca, 1995). The ATP binding and hydrolysis is only involved in introducing conformational changes in the enzyme for carrying out its catalytic functions and not for DNA nicking and resealing.

Figure 1.2



From Berger *et al.*, 1996

Functions of Topoisomerase II

TopoII enzyme has an ability to promote topological interconversions of DNA. It plays an important role in various cellular processes such as chromosome segregation, chromosome condensation, replication, transcription, maintaining the genomic integrity and recombination.

Cell cycle regulation of Topoisomerase II

The cellular TopoII levels are high in rapidly proliferating cells but they decrease, when cell growth is arrested or when cells are induced to differentiate. TopoIIα phosphorylation is higher in mitotic cells than in G1 cells. Topoisomerase II α expression and phosphorylation is maximal during the G2/M phase of cell cycle. Topoisomerase II β is uniformly expressed through out the cell cycle. TopoII enzyme is over expressed in cancer cells (Hsiang *et al.,* 1988; Tricoli *et al.,* 1985; Bodley *et al.,* 1987). TopoII from Chlorella virus PBCV-1 has an exceptionally high DNA cleavage activity (Fortune *et al.,* 2001).

The two Topoisomerase II isoforms are functionally distinct. TopoIIα function is critical for cell proliferation, but the functional aspects of Topoisomerase II β remain unknown. Significant activity of this enzyme was found in isolated nuclei from post mitotic neuronal cells. Topoisomerase II β is also shown to express in other non-proliferative and fully differentiated tissues like neurons (Capranico *et al.,* 1992 and Kondapi *et al.,* 2004) cell cycle analysis of this enzyme during cell cycle did not show any significant alteration. Topoisomerase II β is not essential for cell proliferation and survival in vitro since in some cell types this enzyme is not expressed at all. These observations suggest that Topoisomerase II β is not required for maintenance of general cellular activities but involved in more specific processes in growth, development and cellular repair processes induced by different stress responses in vivo.

Neuronal development

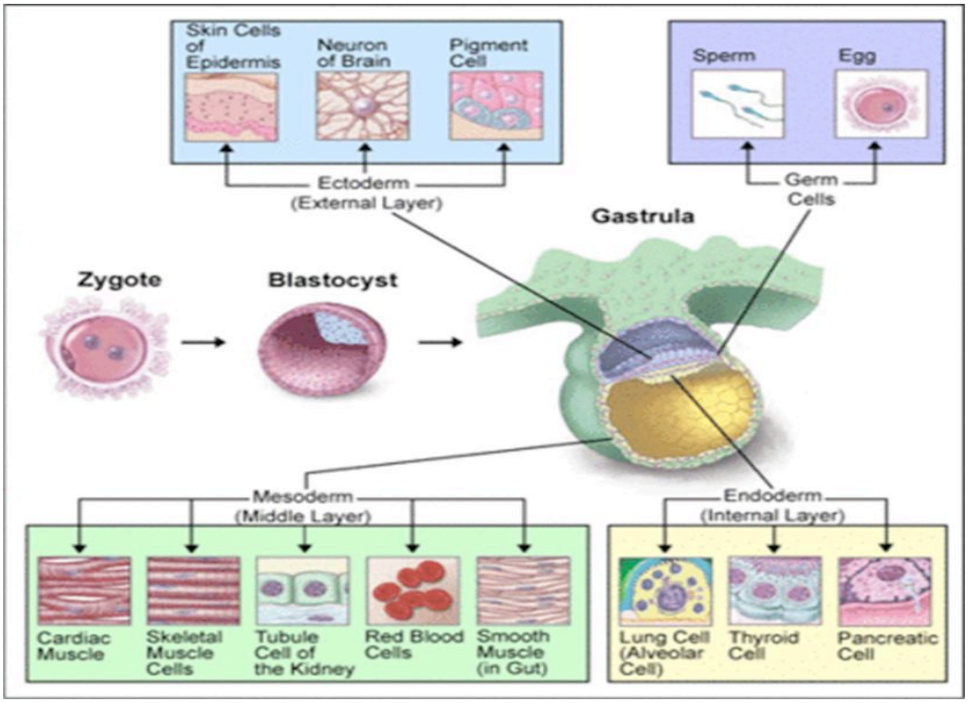
The study of **neural development** draws on both neuroscience and developmental biology to describe the cellular and molecular mechanisms by which complex nervous systems emerge during embryonic development and throughout life.

Some landmarks of embryonic neural development include the birth and differentiation of neurons from stem cell precursors, the migration of immature neurons from their birthplaces in the embryo to their final positions, outgrowth of axons from neurons and guidance of the motile growth cone through the embryo towards postsynaptic partners, the generation of synapses between these axons and their postsynaptic partners, and finally the lifelong changes in synapses which are thought to underlie learning and memory.

Neurulation

Neurulation is the formation of the neural tube from the ectoderm of the embryo. It follows gastrulation in all vertebrates. During gastrulation cells migrate to the interior of embryo, forming three germ layers the endoderm (the deepest layer), mesoderm and ectoderm (the surface layer) from which all tissues and organs will arise. In a simplified way, it can be said that the ectoderm gives rise to skin and nervous system, the endoderm to the guts and the mesoderm to the rest of the organs.

Figure 1.3



Development of different organs from blastula stage of embryo

After gastrulation the notochord a flexible, rod-shaped body that runs along the back of the embryo has been formed from the mesoderm. The notochord sends

signals to the overlying ectoderm, inducing it to become neuroectoderm. This results in a strip of neuronal stem cells that runs along the back of the fetus. This strip is called the neural plate, and is the origin of the entire nervous system.

The neural groove forms during the third week of gestation as the plate folds outwards. Beginning in the future neck region, the neural folds of this groove close to create the neural tube. The anterior (front) part of the neural tube is called the basal plate; the posterior (rear) part is called the agar plate. The hollow interior is called the neural canal. By the end of the fourth week of gestation, the open ends of the neural tube (the neuropores) close off (Fitzgeral *et al*., 2007)

Neuronal migration

Neuronal migration is the method by which neurons travel from their origin or birth place to their final position in the brain. There are several ways they can do this, e.g. by radial migration or tangential migration.

Radial migration

Neuronal precursor cells proliferate in the ventricular zone of the developing neocortex. The first postmitotic cells to migrate form the preplate, which are destined to become Cajal-Retzius cells and subplate neurons. These cells do so by somal translocation. Neurons migrating with this mode of locomotion are bipolar and attachs the leading edge of the process to the pia. The soma is then transported to the pial surface by nucleokinesis, a process by which a microtubule "cage" around the nucleus elongates and contracts in association with the centrosome to guide the nucleus to its final destination (Samuels et al 2004). Radial fibres (also known as radial glia) can translocate to the cortical plate and differentiate either into astrocytes or neurons. Somal translocation can occur at any time during development (Nadarajah *et al.,* 2001).

Tangential migration

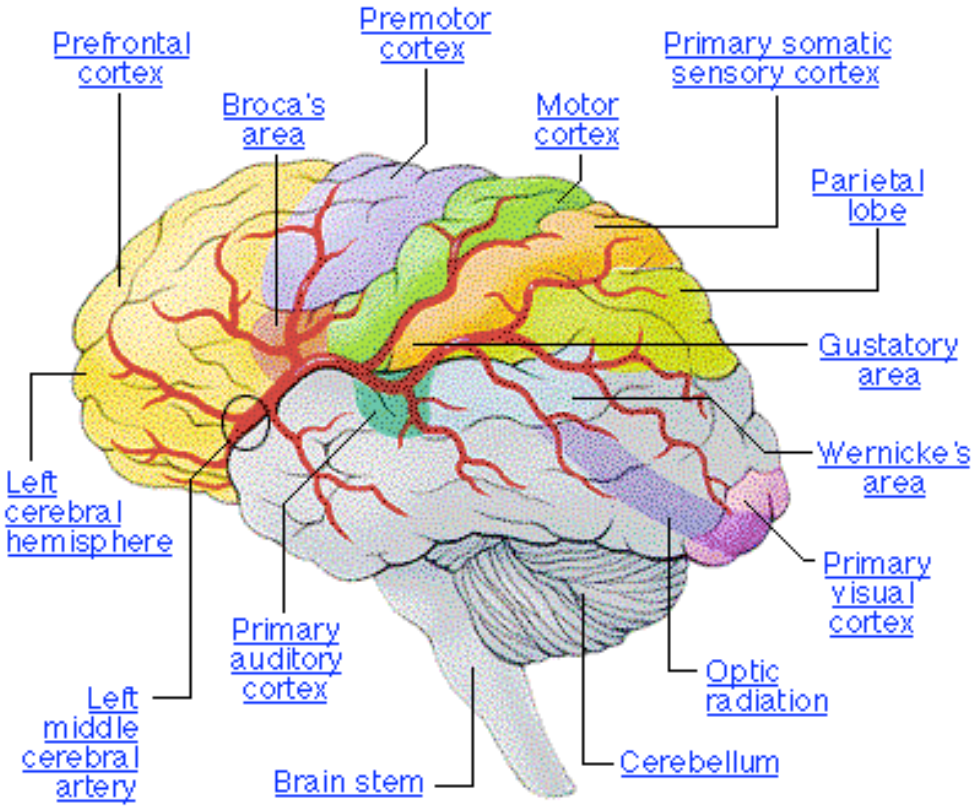
Most interneurons migrate tangentially through multiple modes of migration to reach their appropriate location in the cortex. An example of tangential migration is the movement of Cajal-Retzius cells from the ganglionic eminence to the cerebral cortex. There is also a method of neuronal migration called multipolar migration (Tabata *et al.,* 2003 and Nadarajah *et al.,* 2003). This is seen in multipolar cells, which re abundantly present in the cortical intermediate zone. They do not resemble

the cells migrating by locomotion or somal translocation. Instead these multipolar cells express neuronal markers and extend multiple thin processes in various directions independently of the radial glial fibers.

Early brain development

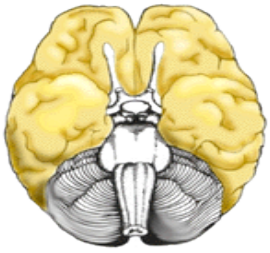
The anterior segment of the neural tube forms the three main parts of the brain: the forebrain, midbrain, and the hindbrain. Formation of these structures begins with a swelling of the neural tube in a pattern specified by Hox genes. Ion pumps are used to increase the fluid pressure within the tube and create a bulge. A blockage between the brain and the spinal cord prevents the fluid accumulation from leaking out. These brain regions further divide into subregions. The hindbrain divides into different segments called rhombomeres. Neural crest cells form ganglia above each rhombomere. The neural tube becomes the germinal neuroepithelium and serves as a source of new neurons during brain development. The brain develops from the inside-out.

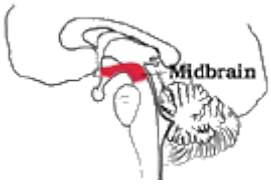
Figure 1.4



Different parts of a Brain

Table 1: Function of cerebral cortex and cerebellum and associated disorders

Brain Structure	Function	Associated Signs and Symptoms
Cerebral Cortex  **Ventral View (From bottom)**	The outermost layer of the cerebral hemisphere, which is composed of gray matter. Cortices are asymmetrical. Both hemispheres are able to analyze sensory data, perform memory functions, learn new information, form thoughts and make decisions.	
Left Hemisphere	Sequential Analysis: systematic, logical interpretation of information. Interpretation and production of symbolic information: language, mathematics, abstraction and reasoning. Memory stored in a language format.	
Right Hemisphere	Holistic Functioning: processing multi-sensory input simultaneously to provide "holistic" picture of one's environment. Visual spatial skills. Holistic functions such as	

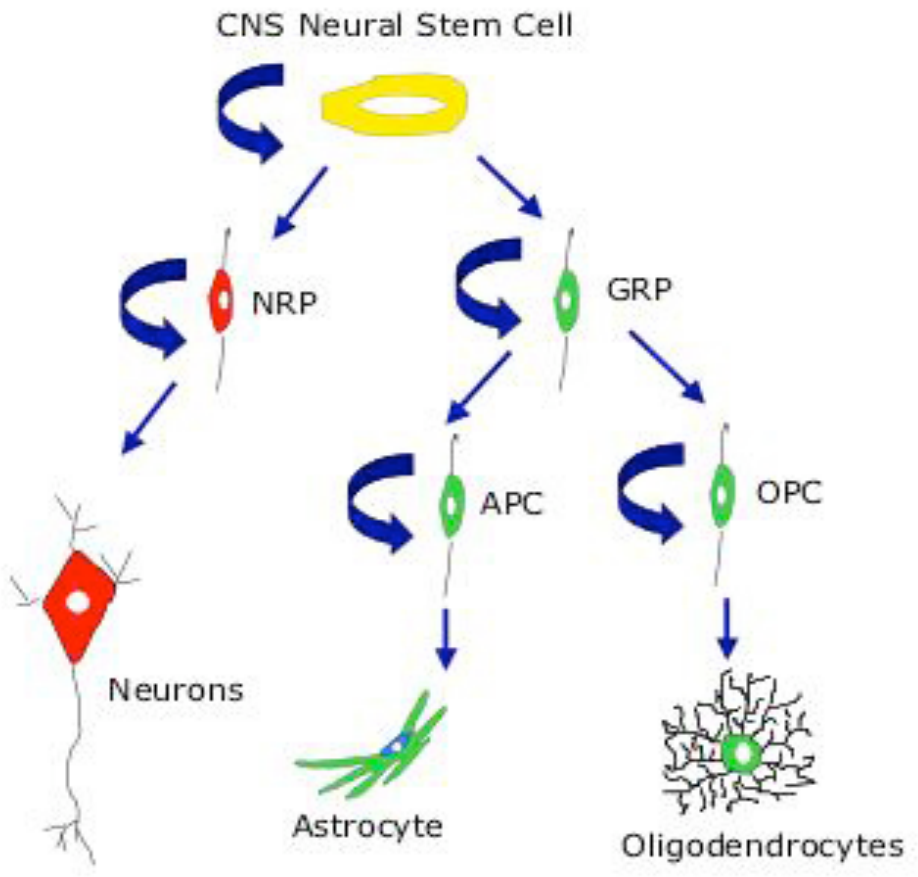
	dancing and gymnastics are coordinated by the right hemisphere. Memory is stored in auditory, visual and spatial modalities.	
Cerebellum 	Coordination and control of voluntary movement.	• Tremors. • Nystagmus (Involuntary movement of the eye). • Ataxia, lack of coordination.
• **Midbrain** 	Nerve pathway of cerebral hemispheres. Auditory and Visual reflex centers. Cranial Nerves: · CN III - Oculomotor (Related to eye movement), [motor]. · CN IV - Trochlear (Superior oblique muscle of the eye which rotates the eye down and out), [motor].	• Weber's: CN III palsy and ptosis (drooping) ipsalateral (same side of body). • LOC (Loss of consciousness): Varies • Movement: Abnormal extensor (muscle that extends a part). • Respiratory: Hyperventilating. • CN (Cranial Nerve) Deficits: CN III, CN IV.

Cells of the brain

The brain and spinal cord are made up of many cells, including neurons and glial cells. Neurons are cells that send and receive electro-chemical signals to and from the brain and nervous system. There are about 100 billion neurons in the brain. There are many more glial cells; they provide support functions for the neurons, and are far more numerous than neurons.

There are many types of neurons. They vary in size from 4 microns (.004 mm) to 100 microns (.1 mm) in diameter. Their length varies from a fraction of an inch to several feet.

Figure 1.5



Neuronal Development from Stem Cell

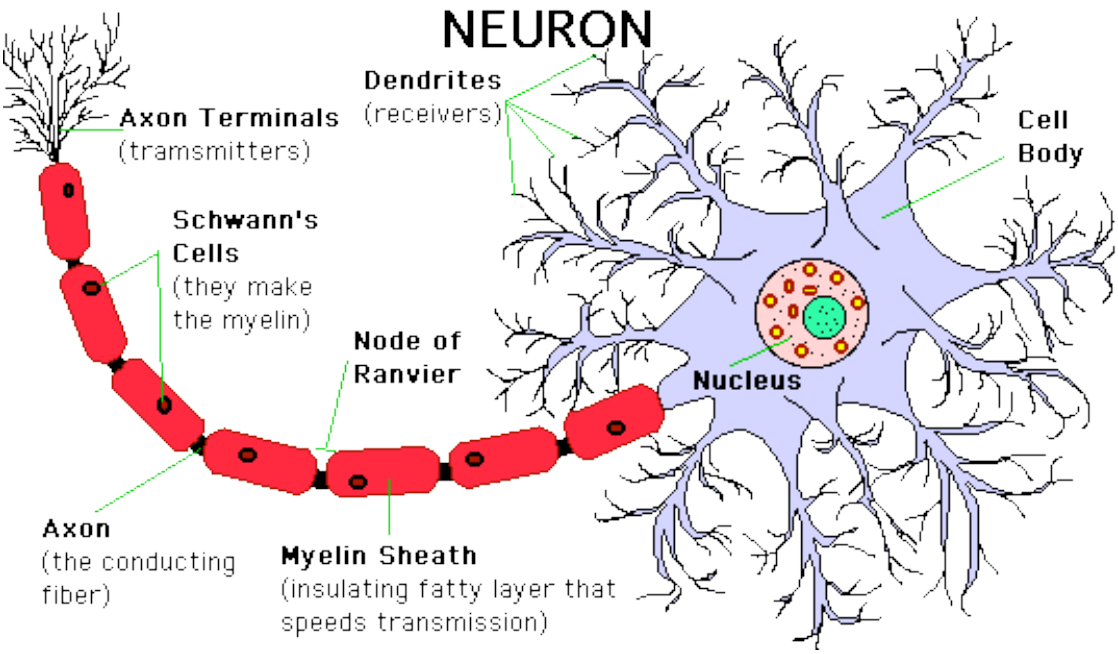
Neurons

Neurons are nerve cells that transmit nerve signals to and from the brain at up to 200 mph. The neuron consists of a cell body (or soma) with branching dendrites (signal receivers) and a projection called an axon, which conduct the nerve signal. At the other end of the axon, the axon terminals transmit the electro-chemical signal across a synapse (the gap between the axon terminal and the receiving cell). The German scientist Heinrich Wilhelm Gottfried von Waldeyer-Hartz coined the term "neuron" in 1891 (he also coined the term "chromosome").

The axon is a long extension of a nerve cell that takes information away from the cell body. Bundles of axons are known as nerves or, within the CNS (central nervous system), as nerve tracts or pathways. Dendrites bring information to the cell body.

Myelin coats and insulates the axon (except for periodic breaks called nodes of Ranvier), increasing transmission speed along the axon. Myelin is manufactured by Schwann's cells, and consists of 70-80% lipids (fat) and 20-30% protein. The cell body (soma) contains the neuron's nucleus (with DNA and typical nuclear organelles). Dendrites branch from the cell body and receive messages. A typical neuron has about 1,000 to 10,000 synapses (that is, it communicates with 1,000-10,000 other neurons, muscle cells, glands, etc.).

Figure 1.6



Typical structure of the neuron

Different types of neurons

There are different types of neurons. They all carry electro-chemical nerve signals, but differ in structure (the number of processes, or axons, emanating from the cell body) and are found in different parts of the body.

- Sensory neurons or Bipolar neurons carry messages from the body's sense receptors (eyes, ears, etc.) to the CNS. These neurons have two processes. Sensory neuron accounts for 0.9% of all neurons. (Examples are retinal cells and olfactory epithelium cells)
- Motoneurons or Multipolar neurons carry signals from the CNS muscles and glands. These neurons have many processes originating from the cell body. Motoneurons account for 9% of all neurons. (Examples are spinal motor neurons, pyramidal neurons and Purkinje cells.)
- Interneurons or Pseudopolare (Spelling) cells form all the neural wiring within the CNS. These have two axons (instead of an axon and a dendrite). One axon communicates with the spinal cord; one with either the skin or muscle. These neurons have two processes. (Examples are dorsal root ganglia cells.)

Glial cells

Glial cells make up 90 percent of the brain's cells. Glial cells are nerve cells that don't carry nerve impulses. The various glial (meaning "glue") cells perform many important functions, including: digestion of parts of dead neurons, manufacturing myelin for neurons, providing physical and nutritional support for neurons, and more. Types of glial cells include Schwann's Cells, Satellite Cells, Microglia, Oligodendroglia, and Astroglia.

TopoII role in neuronal development

Previous studies indicate that TopoIIα involving in DNA replication and segregation required for cell proliferation. Studies on TopoIIβ shows that TopoIIβ expression high in terminally differentiated cells especially in neurons (Kondapi *et al.,* 2004). Studies of whole-body TopoIIβ knockout mice have demonstrated a prenatal death phenotype. It has been demonstrated that motor neuron axons fail to

innervate the diaphragm muscles and the sensory neuron axons fail to enter the spinal cord, suggesting a role of TopoIIβ in axon growth and/or guidance (Yang *et al.,* 2000). Experiments on brain-specific TopoIIβ knockout mice have also revealed a major defect in corticogenesis during brain development (Lyu and Wang, 2003). Investigations of developing rat cerebellum have demonstrated that post-mitotic granule cells in the external germinal layer show an abrupt switch-over of expression of TopoII isoforms from TopoIIα to TopoIIβ, again suggesting an association between TopoIIβ and neuronal differentiation (Kondapi *et al.,* 2004; Tsutsui *et al.,* 2001). Recent reports shows that TopoIIβ is required for neurite outgrowth and growth cone formation in cultured cerebral cortical neurons, cerebellar granule neurons, and dorsal root ganglion as well as PC12 cells. In addition, the expression of a number of neuronal genes requires TopoIIβ (Kamal *et al.,* 2007). The brain during ageing exposed to various environmental factors such as damaging agents and infectious agents, such an event would induce stress in the brain.

Neurons are unique cell types in an organism. The differentiated neurons are more vulnerable to different type stress responses and these leads to various neurodegenerative disorders. Various exogenous and endogenous agents cause stress in the brain and to neuronal cells.

The present study analyzes the role of TopoIIα and β in stress caused due to

a) DNA damage due to ROS intermediates
b) HIV-1 infection mediated inflammatory response

DNA damage in brain:

Genomic integrity is very essential for the survival of any organism and damage to it may be dangerous to the survival of the organism. Any deleterious mutation in the coding region of an important enzyme or protein of an open reading frame of a gene results in the abnormal expression of the gene. DNA is subject to both endogenous and exogenous events. Many of the DNA modifications have been shown to be mutagenic in vitro and in vivo (Loeb *et al.,* 1989). The endogenous DNA damaging agents are free radicals, reactive oxygen species (ROS) and alkylation of DNA, free radicals are generated during several metabolic processes in the cell and also ionizing radiation and they react with many cellular components

especially DNA. Free radicals produce a variety of oxidative DNA damage including single strand breaks (SSB) and double strand breaks (DSB) AP sites and cross links (Rao *et al.,* 1996). ROS can be cause of spontaneous DNA damage (Lee and Ceramini, 1987) and alkylation of DNA by S-adenosyl-L-methionine the normal intracellular methyl group donor (Barrows *et al.,* 1982; Rydberg *et al.,* 1982). This leads to the formation of N7 methyl guanine, N3b methyl adenine, N3 methyl Thymine and small amounts of O6-methyl guanine. The methylation of adenine and guanine bases cause destabilization of the N-glucosylic bond, resulting in an increased spontaneous cleavage and the formation of AP sites (Lindahl and Nyberg, 1972).

The major source of exogenous DNA damage is UV, X-ray, gamma rays and mutagens and carcinogens present in human diet such as polycyclic aromatic hydrocarbons (PAH), aflotoxin B1 and nitrosamine (Ames *et al.,* 1983). UV forms cyclobutane pyrimidine dimers (Setlow, 1982, Niggli and Rothlisherher, 1988). UV irradiation also induces DNA-protien cross links and single strand breaks (Peak *et al.,* 1985 and Lai *et al.,* 1966). Ionizing radiation causes single and double strand breaks and cross-links (Scholes, 1983; Hutchinson, 1985). PAH, aflotoxin B1 and nitrosamines react with DNA, inducing several types of damages including single and double strand breaks and bulky adducts.

Reactive oxygen species and DNA damage in neurons

Reactive oxygen species (ROS) are continuously formed as a consequence of normal metabolism and in response to environmental factors such as UV light, ionizing radiation, heat and pollution (Ames *et al.,* 1986 and Halliwell *et al.,* 1999). ROS encompass a variety of chemical species including superoxide anions (O_2•), hydroxyl radicals (•OH) and hydrogen peroxide (H_2O_2) (Halliwell *et al.,* 1999). Metabolic stress inevitably rises the levels of ROS in tissue. If the amount of ROS overwhelms the capacity of cells to counteract these harmful species, oxidative stress occurs and can induce various types of cell damage such as modifications of proteins, lipids and DNA leading to mitochondrial and ultimately cellular dysfunction (Young *et al.,* 2001 and Beckman *et al.,* 1998). Neurons seem to be particularly vulnerable to oxidative stress due to the substantial amount of oxygen consumption by the brain, the low glutathione content and a high proportion of polyunsaturated fatty acids in neurons (Christen *et al.,* 2000 and Rutten *et al.,*

2002). Furthermore, neuron is a post-mitotic cell, which implies that, in case they are irreversibly damaged or lost, they cannot be replaced. Accumulation of DNA damage in neurons has long been suggested to be one of the major forms of damage involved in brain aging and neurodegeneration (Bohr *et al.,* 1998 and Hamilton *et al.,* 2001). More than 100 different types of DNA lesions have been reported, including base modifications (for instance, 8-oxo- 7,8-dihydro-2`-deoxyguanosine [8-oxo-dG], thymidine glycol, and 8-hydroxycytosine), single- and double-strand breaks, and interstrand cross-links (Vijg *et al.,* 1987 and Cook *et al.,* 2003). Among these, DNA singlestrand breaks (SSBs) are the most common ones (thousands per cell per day) and are the most common lesion induced by ROS and exogenous genotoxins such as ionizing radiation and alkylating agents (Caldecott *et al.,* 2004).

DNA repair in neurons

Damaged or inappropriate bases can be repaired by several mechanisms:

- **Direct chemical reversal** of the damage
- **Excision Repair**, in which the damaged base or bases are removed and then replaced with the correct ones in a localized burst of DNA synthesis. There are three modes of excision repair, each of which employs specialized sets of enzymes.
    1. **Base Excision Repair** (**BER**)
    2. **Nucleotide Excision Repair** (**NER**)
    3. **Mismatch Repair** (**MMR**)

Repairing Strand Breaks

Reactive oxygen intermediates and ionizing radiation and certain chemicals can produce both single-strand breaks (**SSB**s) and double-strand breaks (**DSB**s) in the DNA backbone.

1. Single-Strand Breaks (SSBs)

Breaks in a single strand of the DNA molecule are repaired using the same enzyme systems that are used in Base-Excision Repair (BER).

2. Double-Strand Breaks (DSBs)

There are two mechanisms by which the cell attempts to repair a complete break in a DNA molecule:

a) **Direct joining** of the broken ends. This requires proteins that recognize and bind to the exposed ends and bring them together for ligating. They would prefer to see some complementary nucleotides but can proceed without them so this type of joining is also called **non-homologous end-joining** (**NHEJ**).

b) **Homologous Recombination.** Here the broken ends are repaired using the information on the intact

- **sister chromatid** (available in G_2 after chromosome duplication), **or** on the
- **homologous chromosome** (in G_1; that is, before each chromosome has been duplicated). This requires searching around in the nucleus for the homolog — a task sufficiently uncertain that G_1 cells usually prefer to mend their DSBs by **NHEJ**. **or** on the
- **same chromosome** if there are duplicate copies of the gene on the chromosome oriented in opposite directions (head-to-head or back-to-back).

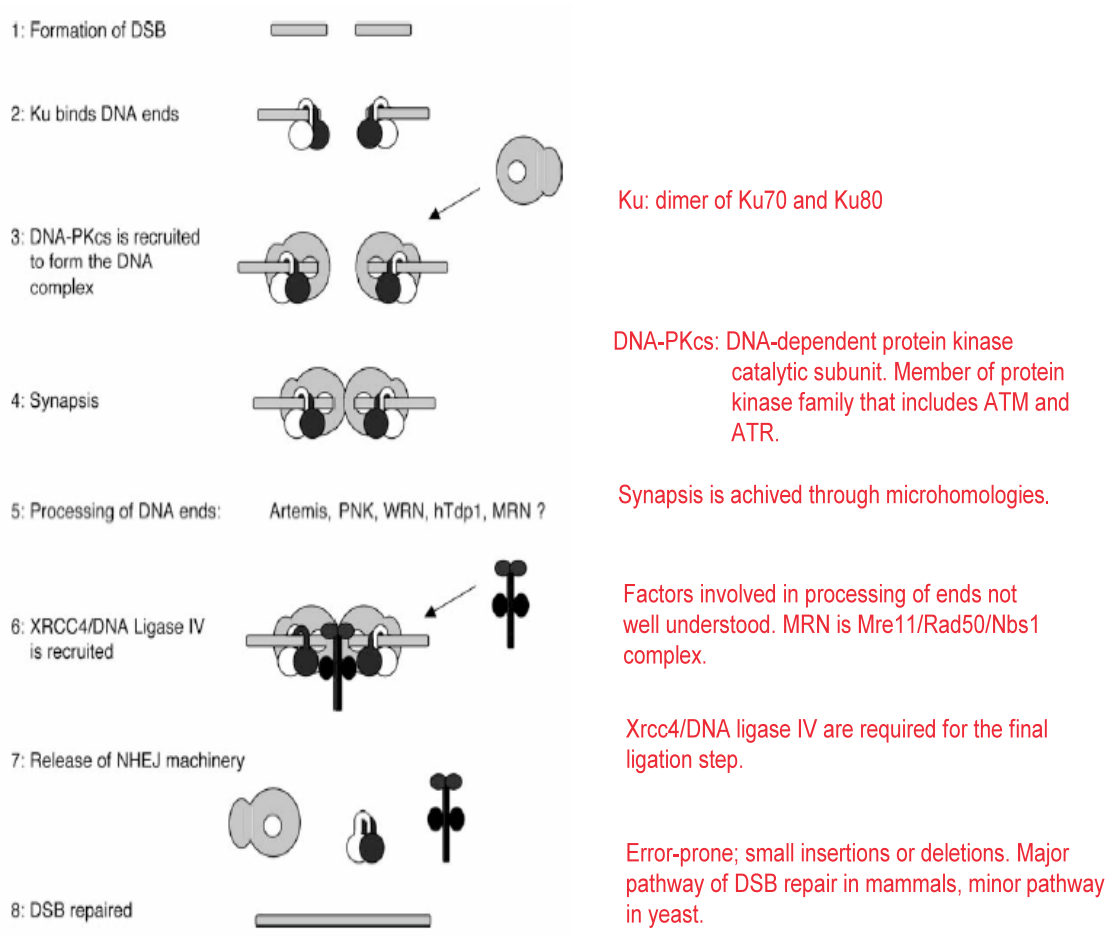
Double strand breaks (DSBs) are one of the most serious forms of DNA damage that can occur in a cell's genome. DNA replication in cells containing DSBs or following incorrect repair may result in the loss of large amounts of genetic material, aneuploid daughter cells and cell death. Much progress in our understanding of DSB repair in eukaryotes has come from studies of mutant *Saccharomyces cerevisiae* and mammalian cell lines. Two major pathways have been identified: homologous recombination (HR) is the main pathway in *S. cerevisiae* **whereas non-homologous end-joining (NHEJ) predominates in mammalian cells.**

Non-homologous end-joining pathway

Double strand breaks (DSBs) are a particularly dangerous form of DNA damage. Unrepaired DSBs can lead to induction of cell death and neurodegenerative

diseases. The consequences of errors in DSB repair can be chromosome translocation, genomic instability, and a predisposition to cancer (Khanna *et al.,* 2001 and Van gent *et al.,* 2001). Mammalian cells use predominantly non-homologous end joining (NHEJ) to repair DSBs, which can yield accurate restoration of the sequence or loss or addition of nucleotides before religation. The alternative mechanism of homologous recombination is used to a lesser extent in higher eukaryotes and is mainly proficient during late S and G2 phases of the cell cycle when a sister chromatid is available to serve as a template and thus may be less important in terminally differentiated neurons. Genetic and biochemical studies identified five major components of the NHEJ machinery. These include the large catalytic subunit of DNA-dependent protein kinase catalytic subunit (DNAPKcs; 469 kDa), Ku70 (70 kDa), Ku 80 (or Ku86; 83 kDa), DNA ligase IV (96 kDa), and XRCC4 (38 kDa) (Leiber *et al.,* 2003, Valerie *et al.,* 2003 and Van Gent *et al.,* 2001). DNA-PKcs is a member of the phosphatidylinositol-3 kinase-related serine/threonine protein kinase family that includes the cell cycle checkpoint kinases ATM and ATR. Its substrates include itself, Ku70 and Ku80, and XRCC4. Ku70 and Ku80 heterodimerize to form an open ring-like structure that can accommodate double stranded DNA. Ku70/80 binding to DSBs recruits DNA-PKcs to DNA ends resulting in kinase activation and also binding of the DNA ligase IV/XRCC4 multimer. Additional proteins, such as Artemis and the complex of Mre11/Rad50/NBS1, have been demonstrated to interact with DNA-PKcs or Ku and may play a role in processing DNA ends by contributing nuclease activity (Khanna *et al.,* 2001).

Figure 1.7



Mechanism of DSB`s repair by NHEJ pathway

TopoII in DNA repair

Previous studies indicate that TopoII may be required for multi protein repair complexes to gain access to localized damaged lesions of DNA. However, no direct evidence for the participation of TopoII in DNA repair in intact cells has been reported. In vitro data indicate that the catalytic activity of TopoII can be stimulated by abasic, oxidized and mono-alkylated DNA (Osheroff *et al.,* 2000). However, these studies were performed with short double stranded oligonucleotides, and thus it is difficult to extrapolate the significance of these findings for highly structured cellular DNA. Emmons *et al.,* (2007) indicated that TopoIIβ is required for repair of alkylating agents induced DNA aducts. Other studies also show that TopoIIβ may involving in DNA repair process.

In the present study we have investigated the role of TopoII isoforms in peroxide induced DNA double strand breaks repair as oxidative intermediates are the most common agents responsible for neurodegeneration. Hydrogen peroxide is produced by a wide variety of enzymes including several oxidases in brain and induces oxidative stress. It induces DNA damage in neurons, which are continuously repaired in the healthy neurons. The neurons deficient of repair proteins could not repair those damages, concomitantly leads to cell death. Peroxide contributes to tissue injury and leads to neurodegenerative diseases including Parkinson`s disease, Alzheimer's disease and Huntington`s disease.

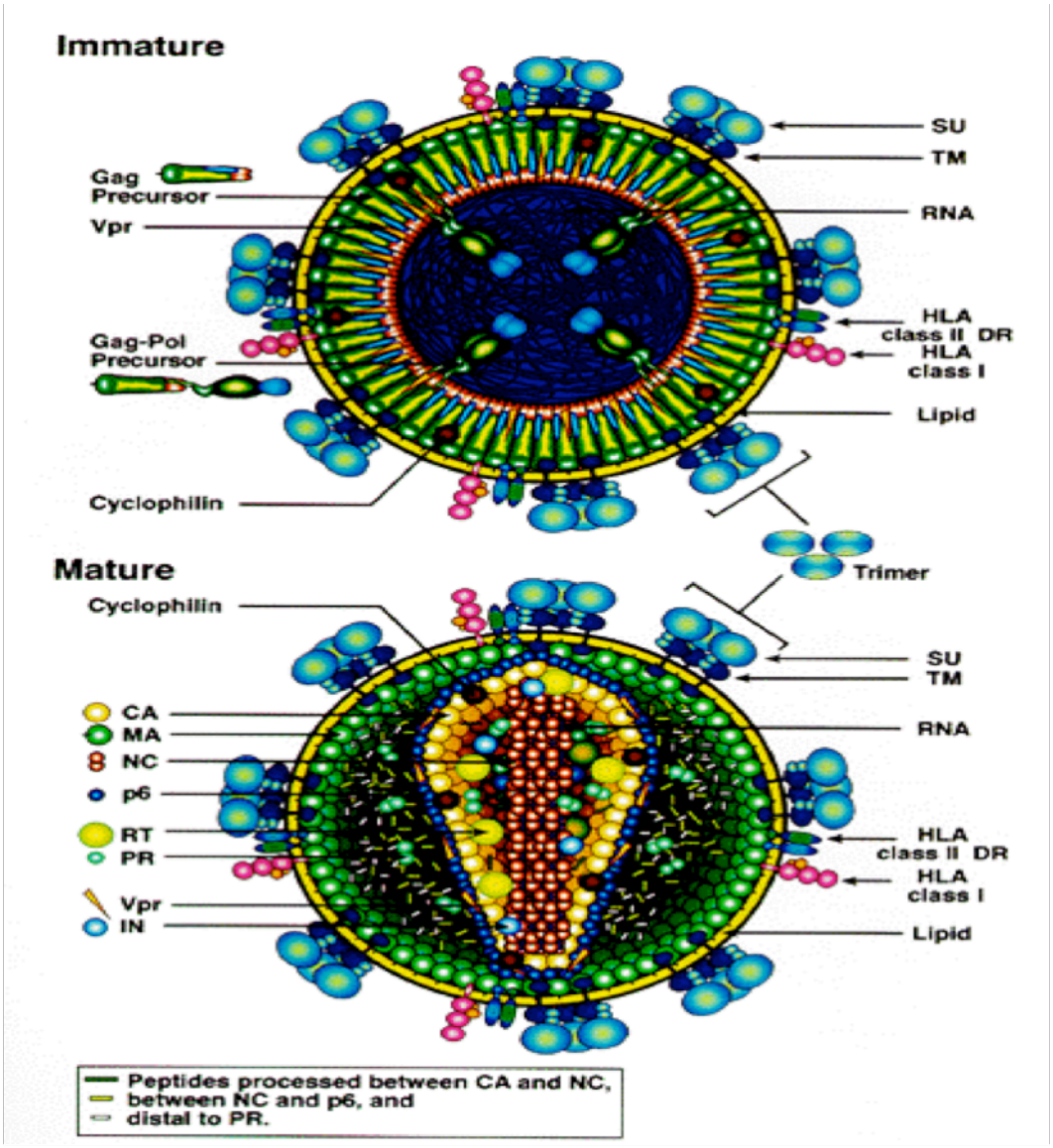
Human immunodeficiency virus

Human immunodeficiency virus (HIV) and its subtypes are retroviruses, belong to a large family of ribonucleic acid (RNA) lentiviruses that are characterized by association with diseases of immuno-suppression or central nervous system involvement and with long incubation periods following infection before manifestations of illness become apparent (Fauci, 1993; Sierra *et al.,* 2005). Lentiviruses similar to HIV, have been found in a variety of primate species and some of these are associated with a disease process called simian AIDS.

HIV and brain

Human immunodeficiency virus type-1 produces central nervous system dysfunction. NeuroAIDS is mediated by elevated levels of immunoactive substances (cytokines, prostaglandins and nitric oxide (NO)). HIV-1 is associated with neuroinflammation by stimulating release of immunoactive substances from brain cells. Therefore, HIV-1 entering the brain across the blood–brain barrier (BBB) is thought to play an important role in the incidence of the neuroAIDS syndrome. The immunoactive substances may increase the permeability of the BBB to free virus.

Figure 1.8



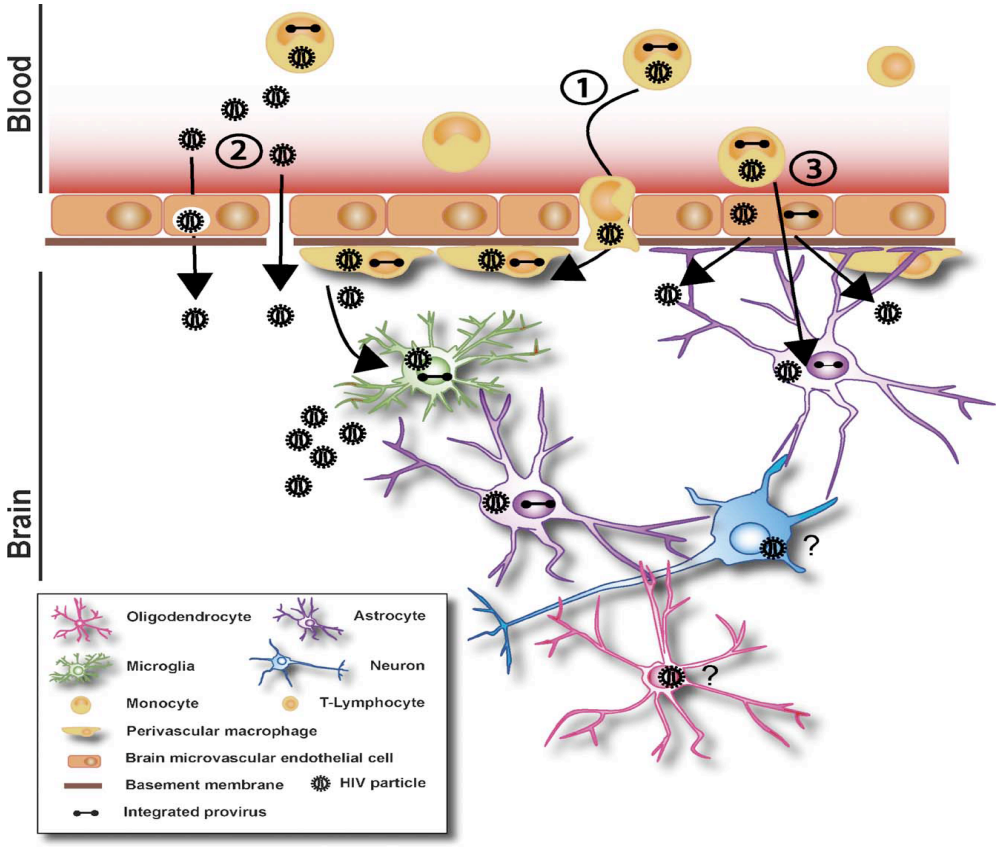
HIV-1 Models from Lou Henderson

The blood–brain barrier and invasion of the brain by HIV-1

The blood–brain barrier controls the influx of compounds from the blood in capillaries to the brain parenchyma and represents one of the cohesive barriers in the human body. It consists of a single layer of specialized endothelial cells surrounded by a basal membrane, astrocytic feet processes that ensheath the vessels and pericytes embedded in the basal membrane that stabilize the vesselwalls. Brain microvascular endothelial cells (BMVECs) are connected by tight junctions and characterized by the absence of fenestrations and sparse pinocytosis (Ballabh *et al.,* 2004). Nevertheless, HIV-1 is able to overcome this tight barrier and invade the brain.

Three pathways have been proposed for HIV-1 entry into the brain (Albright *et al.,* 2003): (a) carriage of HIV-1 into the brain by infected leukocytes ("Trojan horse" hypothesis) (b) passage of cellfree virus into the brain and (c) release of virus into the brain by infected endothelial cells.

Figure 1.9



Entry of HIV-1 through Blood brain barrier

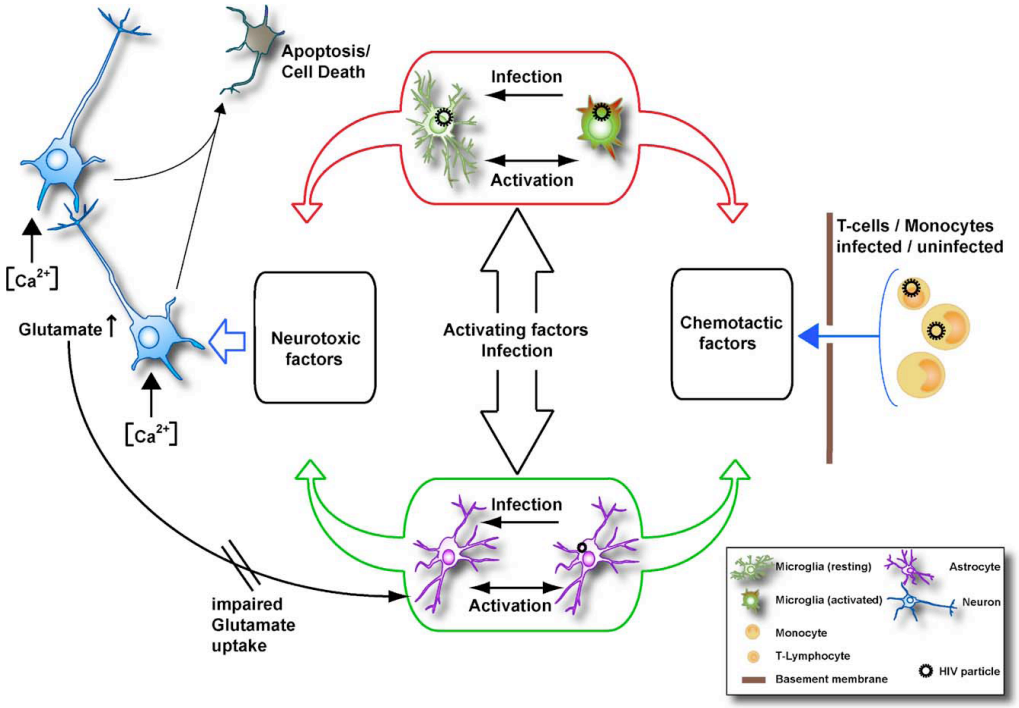
Susanne (2005)

It has been reported that 40 to 50% of acquired immunodeficiency syndrome (AIDS) patients, both adults and children, have neurologic complications, which are directly attributable to infection of the brain by the human immunodeficiency virus type 1 (HIV-1) (Bacellar *et al.,* 1994; Lipton, 1994). Brain HIV-1 infection may result in a syndrome of profound cognitive, behavioral, and motor impairment termed the AIDS dementia complex in adults (Janssen *et al.,* 1989) and HIV-1-related encephalopathy in children. The underlaying cause of HIV-1 dementia is unknown, but since productive HIV-1 infection in the central nervous system (CNS) occurs mostly in microglia, or brain macrophages, it is generally thought that these cells play a key role in the development of neurological abnormalities. HIV-1-infected neurons have been described in brains from AIDS patients (Budka, 1989; Nuovo *et al.,* 1994) and primary human neuroblasts as well as neuronal cell lines can be infected by HIV-1 *"in vitro"* (Ensoli *et al.,* 1995; Obregon *et al.,* 1999). Traditionally, HIV-1 dementia has been reported to be the consequence of neuronal damage or dysfunction resulting from the release of putative neurotoxic products by infected microglia or, alternatively, by neuronal interaction with viral proteins released or expressed by the infected cells. On the other hand, CNS disorders are more frequent and severe, particularly in infants and children, suggesting that a developing CNS is more vulnerable to HIV-1 (Belman, 1994; Epstein *et al.,*1986). Infection of T lymphocytes by HIV-1 requires the presence of the CD4 surface molecule, which serves as the virus receptor (Maddon *et al.,* 1986). However, HIV-1 infects some cells that do not express CD4, including some brain-derived glial and neuronal cells as well as retinal cells, brain capillary endothelial cells, human skin fibroblast, muscle- and bone-derived fibroblastoid cell lines, human trophoblast cells, follicular dendritic cells, fetal adrenal cells, and human liver carcinoma cells (Cheng-Mayer *et al.,* 1987; Harouse *et al.,* 1989; Li *et al.,* 1990). In T cells, HIV-1 entry also requires binding to coreceptors.

The chemokine receptor CXCR4 was the first molecule identified as a coreceptor working in conjunction with CD4 to mediate cellular entry for HIV-1 (Feng *et al.,* 1996). Since this discovery, 11 other seven-transmembrane domain molecules, many of which are chemokine receptors, have been shown to facilitate HIV-1 entry into cells. These include CCR5, CCR3, CCR2, CCR1, CCR8, CXC3CR1, STRL33 (BONZO), GPR15 (BOB), GPR1, US28, and APJ (Locati and Murphy, 1999). This

number is likely to increase. How many molecules can serve as HIV-1 coreceptors is at present unknown. In CNS cells it is possible that some HIV-1 entry cofactors may not be proteins but rather glycolipids. Thus, it has been described that a neural glycolipid, with three sugar groups in the polar head, can serve as an alternative and/or additional cofactor in CD4-dependent HIV-1 fusion (Puri *et al.,* 1999). Other studies have reported that antibodies raised against galactosylceramide (GalC) block HIV-1 infection on two CD4-negative, but, GalC-positive cell lines, SK-N-MC and U-373, derived from nervous system (Harouse *et al.,* 1991). Furthermore, the HIV-1 surface envelope glycoprotein, gp120, bound specifically and with high affinity to GalC (Bhat *et al.,* 1993). From these observations, it was concluded that GalC, or another closely related glycolipid, could serve as an alternative receptor for HIV-1 infection of some CD4-negative cells. On the other hand, nucleolin, originally described as nuclear protein, can be expressed in the cell surface and can also act as a ligand for HIV-1 (Nisole *et al.,* 1999). The role of nucleolin in HIV-1 infection is far from clear but it has been suggested to play a role in attachment of the virus to the cell.

Figure 1.10



HIV-1 related injury to the central nervous system

Susanne (2005)

The cytokines TNFα and IFNγ have been implicated in the development and progression of multiple sclerosis (MS) and AIDS associated dementia complex. TNFα is a 17 KDa peptide produced by a wide range of cells (Tracy *et al.,* 1993). TNFα plays an important role in (1) inflammation, (2) modulation of immunoresponse by affecting the expression of class I and class II MHC molecules and adhesion molecules, (3) stimulation of cytokines such as IL-1, IL-6, IL-8 and IFNc (Etty *et al.,* 1995). TNFα participates in the inflammatory reaction within the CNS. TNFα positive macrophages and astrocytes have been identified in the brain of MS patients, particularly in the plaque region (Hofman *et al.,* 1989). TNFα induction of IL-6 expression in astrocytes occurs by protein kinase C dependent pathway. Inflammatory markers shown to be enhanced in HIV-1 associated dementia, HIV-1 gp120 is shown to be involved in such response in neuroblastoma cells (Griffin *et al.,* 1994).

The objective of the present investigation is to examine if systemic inflammation can regulate TopoIIα and β in neuroblatoma and astrocytoma cell lines. The regulation of TopoIIα and β were analyzed.

Rationale:

Our earlier observation (Kondapi *et al.,* 2004) showed that

1. TopoIIβ isoform is predominant in brain especially in cerebellum (Kondapi *et al.,* 2004, Watanabe *et al.,* 1994).
2. The levels of TopoIIβ decrease during ageing (Kondapi *et al.,* 2004)

The observed decrease in TopoIIβ levels during ageing show synergetic cellular functions of TopoII isoforms. The cellular process may have programmed to different regulatory processes that are controlled by various factors expressed during development, senescence and response to stress. The thesis investigates some of the functional activities of TopoII by analysis of the expression profile of TopoII isoforms in cellular development and response to stress due to reactive oxygen intermediates generated by H_2O_2 and HIV-1 infection with an aim to elucidate the biological function of TopoII isoforms.

Objectives

The work presented in this thesis mainly deals with the following objectives.

1. Analysis of the function of Topoisomerase II α and β in neuronal development.
2. Investigation of the role of Topoisomerase II α and β in peroxide induced DNA damage and repair in neurons.
3. A study on the function of Topoisomerase II α and β, in HIV-1 induced inflammation.

CHAPTER 2

Materials & Methods

Materials

Reagents

Roswell Park Memorial Institute medium 1640 (RPMI 1640), Dulbecco's modified Eagle medium (DMEM-F12) DMEM, Eagle Essential medium with earal`s salts (EMEM), Fetal Bovine Serum (FBS), non-essential amino acids, sodium pyruvate (Invitrogen, Carlsbad, CA). Oligonucleotides, Taq DNA polymerase, dNTPs (Integrated DNA Technologies (IDT, USA). ATP, PMSF, BSA, Leupeptin, Pepstatin, Aprotinin and Protein-A agarose, Triton X 100, Ficoll, DTT, AZT, NDGA (Sigma Co., USA). PVDF, Nitrocellulose membrane (NC) (PALL Life Sciences, USA), HIV-1 p24 Antigen Capture Assay Kit (NCI-Frederick Cancer Research and Development Center), all other chemicals were from Sigma unless specified.

Animals:

Rats - Wistar

Age group:

E11 (Embryonic day 11); E18 (Embryonic day 11); Young (>10 days), 2M (2 months)

Primary neurons and astrocytes

Purkinje neurons were isolated from 18-day rat embryos cerebellum.

Cortical neurons were isolated from 18-day rat embryos cerebral cortex.

Granule neurons were isolated from 8-day old rat pups cerebellum.

Astrocytes were isolated from 6-day old rat cerebellum.

Cell lines

SK-N-SH (Neuroblastoma), 1321N1 (Astrocytoma), GO-G-CCM (Glioblastoma) cell lines were obtained from NCCS, Pune, India.

SupT1 obtained from NIH USA (Dr. J. Hoxie, NIH, USA)

Virus

HIV-$1_{93IN101}$ is biotype-NSI (X5) (Dr. R. Bollinger), isolated from a seropositive individual in India (Dr. R. Bollinger).

pNL4-3 is a HIV-1 full length chimeric form (Dr. Malcolm Mortin, NIH USA)

Antibodies

Mouse anti-human Topoisomerase IIA (Clone: 31) and Mouse anti-human Topoisomerase II β (Clone: 40) were from Pharmingen group of Becton–Dickinson biosciences.

Mouse anti-human Ku-70, Mouse anti-human XRCC2, Mouse anti-human CD4, Mouse anti-human CXCR4, Mouse anti-human CCR5, Rabbit anti-human pol-β, Rabbit anti-human WRN, Rabbit anti-human TNF α, Rabbit anti-human Lox-5 and Rabbit anti-human Cox-2 (Sigma Co., USA).

The secondary antibodies were purchased from US Biological.

Peptides

V3-peptide (TRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHNH2,(5),

MN-peptide (306-327 YNKRKRIHIQRGPGRAFYTTKNII(C), (19,20) from NIH, USA.

Methods

2.1 Preparation of tissue extracts from embryos.

Tissue extracts were prepared from cerebral cortex, cerebellum and mid brain tissue of E11 (Embryo day11), E18 (Embryo day 18) and post-natal day 1 pups by sacrificing them through decapitation and collecting the cerebellum tissue. At E11 stage there is no organ differentiation so we have taken cephalic region of the embryos as brain source. The tissue was homogenized in extraction buffer (20mM Tris HCl pH 7.5, 0.1mM β-mercaptoethanol, 1mM $MgCl_2$, 0.1mM EDTA, 5% glycerol, 0.1% triton X- 100, 0.5mM KCl, 0.5mM PMSF and 1μg/μl pepstatin and leupeptin). The homogenate was kept at 4^0C for one hour and centrifuged at 1,00,000 g for an hour in an ultracentrifuge. The supernatant containing the

cytosolic and nuclear proteins was used as a source for Topo II. The total protein was estimated by using Bradford method.

2.2 Western blot analysis

20 μg of total protein of the brain extracts prepared from, cerebellum, cerebral cortex and mid brain were separated on 7.5% sodium dodecyl sulfate (SDS) gels and then transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in TBS containing 0.05% Tween 20 for 1 h and then incubated overnight at 4^0C with corresponding protein specific primary antibodies. After washing and incubated for 1 h at 22^0C with a secondary antibody conjugated with horseradish peroxidase, the membranes were washed thrice and immunoreactive bands were visualized by chemiluminescence (Pierce Western Blot Chemiluminescence Reagent). The relative levels of protein in different lanes were compared by analyzing scanned images using the NIH IMAGE-J program.

2.3 Immunoprecipitation of Topoisomerase IIα and β from Brain extracts

100μg total protein of the brain extracts prepared from, cerebellum, cerebral cortex and mid brain of E11, E18 and post-natal day1 pups are taken in separate eppendorf tube for immunoprecipitation of Topoisomerase IIα and β. Topoisomerase IIα and β antibody (1:1000 dilution in IP buffer containing 100 mM Tris HCl pH 8, 750 mM NaCl, 2 mM EDTA, 1 mM PMSF, 0.75% Nonidet) is added to each sample. The antigen- antibody mixture is incubated at room temperature for one hour and 25 μl of 6% protein-A agarose beads are added. The beads are incubated at 4^0C for 15 minutes the beads are spun down and the supernatant is removed. The protein-A agarose beads are washed with 0.5% triton X-100. The beads were directly used for monitoring the relaxation activity of topoisomerase II captured by immunoprecipitation.

2.4 Enzymatic activity of Topoisomerase IIα and β

DNA relaxation by Topoisomerase II involves the change in the linking number of DNA by 2. During relaxation the supercoiled DNA band (form I) disappears and complete relaxed plasmid DNA (form II) appears. ~ 0.6μg of supercoiled plasmid

DNA is incubated with the immunoprecipitated Topoisomerase II captured on to the Protein-A agarose beads in relaxation buffer (50 mM Tris HCl pH 8.0,120 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 30µg/ml BSA, 1 mM ATP) for 30 min. The beads were spun down and the supernatants were collected separately. The reaction was stopped using 2µl of 10% SDS and the DNA products were resolved on 1% agarose gel stained with ethidium bromide and photographed.

2.5 Isolation and culture of cerebellar granule neurons from young (7 days) rat pups

7 days old rat pups were killed and the intact brain was taken. The cerebellum was carefully removed and cleared off for the waste tissue and blood vessels. The cerebellum was then minced using a razor blade with two passes at 90^o to each other at approximately 0.5 intervals. The minced tissue was then transferred to 10mL buffer A (10mL EBBS, 200 solution 4, 100µl 2.5mg/ml trypsin).

Tissue disaggregation

The buffer A containing the minced tissue was transferred to the shaking water bath at $37\ ^0$ C for 15 min. 10ml of buffer B (9 ml EBBS, 200 µl solution 4, 1 ml trypsin inhibitor, 200 µl deoxyribonuclease, 200µl magnesium chloride) containing the trypsin inhibitor and DNase was then added to the buffer/ tissue mixture and inversed two to three times. The solution was divided equally in two sterile screw-top 15 ml conical CF tubes and centrifuged at room temperature for 10 seconds at 180g to pellet the tissue debris.

The supernatant was discarded and the minced tissue is gently resuspended in 1.5 ml buffer C (8 ml EBBS, 200µl solution 4, 1 ml trypsin inhibitor, 600µl deoxyribonuclease, 200µl magnesium chloride) per tube. The tissue pieces were disaggregated into single cell suspension by triturating using siliconized, sterile, long form glass Pasteur pipettes. The supernatant was removed after 2 minutes, transferred to two fresh conical tubes and 1.25 ml of buffer C was added and disaggregated up to the last piece. The above step was repeated until single cell suspension is obtained. The cell supernatant was underlayed with 2 ml of buffer D (10 ml EBBS, 400mg BSA, 200µl solution 4) using a graduate plastic Pasteur

pipette and centrifuged at 180g for 5`. The supernatant was discarded and the pellet was re-suspended in 1 ml of buffer C, 50µl of cell suspension was taken and mixed this gently with an equal volume of trypan blue solution and counted using haemocytometer.

Seeding density

10^6 cells / 35mm dish (1, 2) with 3 ml of medium (MEM Eagle's modified with Earle's salts, 0.30g/ml glucose, 0.18g/ml KCl, 29mg/ml glutamine and 10%FBS).

2.6. Isolation and culture of cerebellar purkinje neurons from 18-day rat embryos

Purkinje neurons were isolated as described in Furuya *et al.,* 1998. The pregnant rat was anesthetized on gestation day-18 with ether vapor, the abdomen was sterilized with 80% ethanol solution and the peritoneal cavity was opened and the uterine horns was excised. The two uterine horns were transferred to a 35 mm cell culture dish containing PBS (70–80 ml). The embryos were removed using scissors and forceps, and transferred to a second cell culture dish (35 mm) containing dissection medium (Ca^{++} /Mg^{++} free Hank's BSS containing 10 mg/ml gentamicin). The whole brains were removed from the embryos and transferred to 35 ml ice-cold dissection medium in a Falcon tube. The ice-cold Ca^{++} Mg^{++} free Hank's BSS containing the brains was poured onto a third cell culture dish (35 mm) and cerebella from whole brains was dissected using microdissection forceps under the dissection microscope. The cerebellum was picked up using forceps without cutting into small pieces. Then, the cerebellum was transferred to ice-cold dissection medium (12 ml) in a Falcon tube. The cerebellum was washed by centrifugation at 1000 rpm for 1–2 min. The cerebellum was washed again in 10 ml of fresh dissection medium. 2 ml of 0.1% trypsin was added, diluted with dissection medium to the tube and incubated at 33–35^0 C for 10 min in a thermo-controlled water bath. After incubation, 10 ml of dissection medium was added to the tube and centrifuged at 1000 rpm for 3 min. The supernatant was removed carefully using an aspirator. 1 ml of DNase (0.05%) solution was added to the tube and the trypsin-treated cerebella was dissociated by gentle trituration with a Pasteur pipette. After the dissociation of the large aggregates, further trituration was done with a Pasteur

pipette equipped with a sterilized pipette tip. After complete dissociation of aggregates of the cerebella, 10 ml of dissection medium was added to the tube and centrifuged at 1200 rpm for 5 min. the cell pellet was suspended in 1–1.5 ml of seeding medium (DMEM/F12 containing 10% FBS) by trituration with a Pasteur pipette. To remove non-neuronal cells such as fibroblasts and glial cells, the single cell suspension was seeded onto a 10mg/ml poly-L-ornithine-coated culture dish (35mm), and incubated at 20 min in a CO_2 incubator (37^0C, 5%CO2). After incubation, the single cell suspension was recovered, counted the number of cells and diluted the single cell suspension to a density of $5x10^6$ cells/ml. The dilution of cell density below $5x10^6$ cells/ml will cause a decrease in the viability of both Purkinje cells and granule cells.

Seeding and feeding the cultures

The cell suspension was seeded onto culture dishes (40 µl/ 22.2 mm ø well or 13.5 mm ø plastic cover slip). The cultures were incubated for at least 3 h in a CO_2 incubator (37^0C, 5% CO_2). 0.4 ml of serum-free DMEM/F-12 containing N3 supplement and T3 (0.5 ng/ml) was added to each well. This results in a medium containing approximately 1% FCS. The serum-free condition was established by washing cultures in serum-free D/F and adding serum-free D/F containing N3 supplement, T3 (0.5 ng/ml) and bovine serum albumin (100 µg/ml). Half the medium was replaced with fresh serum-free DMEM/F-12 containing the supplements and AraC (4 mM at 9 and 16 DIV).

2.7 Isolation and culture of astrocytes from young rat cerebellum

The rat pups (6 days) were killed by cervical dislocation and the heads were removed then scrubbed with 70% alcohol and taken cerebellum out carefully. The white tissue and blood vessels were removed. Then tissue was placed in a 100 mm petri dish containing 5 ml of trypsin solution (EBSS/ DNase/ Trypsin). Chopped the tissue with scalpel blade and added 5 ml of trypsin solution. The suspension was transferred in to 15 ml conical tube and incubated at 37^0c with gentle agitation/ 15 minutes. An equal volume of D/L-valine DMEM was added to terminate trypsinization and spun at 250 g. The supernatant was discarded and the pellet was

triturated about 20 times in 5 ml EBSS/ Dnase-I solution. 10 ml of EBS/Dnase-I solution was added and allowed to settle for 10 minutes. 10 ml of supernatant was removed into a 50 ml tube carefully then 2 ml of EBS/Dnase-I was added to the tissue and triturated again along with 10 ml of EBS/Dnase-I solution. The supernatant was removed after 10 minutes and added to first and is repeated one more time then spun at 250 g / 5 minutes. The pellet was resuspended in 10 ml of DMEM and cells were counted.

Seeding density

1x 10^4 cells /T –75 flask, each flask contained 5 ml medium (DMEM Delbucco`s modified eagle medium) with 10% FCS and 50U/ ml penicillin-streptomycin, cultures were incubated at 37^0C and 5% CO_2 in humidified incubator.

2.8 Isolation and culture of cerebral cortical neurons from embryonic day-18 rat pups

Embryonic-day 18 rat pups were removed from pregnant rat and Cerebral cortices were isolated (L. J. Martin et al.,2001 neuroscience) and dissociated by 10% (v/v) trypsin digestion and trituration with a fire-polished Pasteur pipette. Plated onto 35 mm tissue culture dishes, Tissue culture dishes were coated with 33mg/mL poly-D-lysine. The cells were plated in Neurobasal medium (Gibco) supplemented with B27 (Gibco), 300 mM glutamine (Gibco), and streptomycin/ amphotericin B (Gibco). Three days after plating, 50% of the medium was changed and subsequently the medium was changed every 4 days. Neuronal cultures were maintained for up to 30 days *in vitro*

2.9 Preparation of cell extracts

Cells were harvested by trypsinization with 0.25% of trypsin and 1 mM EDTA. Trypsin was inactivated by 20% serum and the cells were pelleted at 300 xg for 10 minutes and washed twice with PBS. Cells were lysed in RIPA (50 mM Tris-HCl (PH;7.5), 1% Triton-X-100, 1% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 150 mM NaCl, 1 mM PMSF, 1% Aprotenine, 10 μg/ml pepstatin and leupeptin)

buffer incubated in ice for 1hour and centrifuged at 3000g for 30 minutes. Supernatant was used for the protein analysis.

2.10 Cell viability assay

Reduction of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) is chosen as an optimal end point of cell viability measurement [Mosmann, 1983; Cole, 1986; Alley et al.,1988]. Cells ($0.2x10^6$ cells per well) were seeded in 96-well plates. Increasing concentrations of compound were added to the cells and incubated at 37°C for 14 h in a CO_2 Incubator with 5% CO^2. The media was replaced with a fresh growth medium along with 20 µl of 5mg/ml 3-(4,5-dimethylthiozol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma). After incubation for 4 h in a humidified atmosphere, the media was removed and 200 µl of 0.1N acidic isopropyl alcohol was added to the wells to dissolve the MTT-formazan crystals. The absorbance was recorded at 570 nm, immediately after the development of purple colour. Each experiment was conducted in triplicate and the data are represented as average, with standard deviation. Percent viability of the cells was computed with reference to the absorbance of reduced MTT in the experiments conducted in absence of any compound.

2.11 siRNA synthesis

siRNA synthesis was done as described in Donze and Picard, (2002). In brief, desalted DNA oligonucleotides were ordered from Integrated DNA techonologies USA. (1) T7Promoter sequence 2) Alpha sense SiAs: 3) Alpha antisense SiAas: 4) Beta sense SiBs: 5) Beta antisense Si Bas: (1) is T7 promoter sequence. (2) and (3) are the Topoisomerase II alpha sense and antisense Oligos and (4) and 5 are Topoisomerase II beta sense and antisense oligos.

In each transcription reaction, 1 nM of each oligonucleotide was annealed in 50µl of TE buffer (10 mM Tris-HClpH8.0 and 1 mM EDTA) by heating at 95°C; after 2 min, the heating block was switched off and allowed to cool down slowly to obtain dsDNA. Transcription was performed in 50 ul of transcription buffer (40 mM Tris-HCl pH 7.9, 6 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl and 2 mMs permidine) 1 mM rNTPs, 0.1 U yeast pyrophosphatase 40 U RNAse out (Life technologies) and

100 U T7RNA polymerase containing 200 pM of the dsDNA as template. After incubation at 37°C for 2 hours, 1U RNase free DNAse I was added at 37°C for 15 min. Sense and antisense RNAs generated in separate reactions were annealed by mixing both crude transcription reactions heating at 95°C for 5 minutes followed by 1 h at 37°C to obtain T7 RNA polymerase synthesized small interfering double-stranded RNA (T7 siRNA). This mixture (100ul) was then adjusted to 0.2M Sodium acetate pH5.2 and precipitated with 2.5 vol. of ethanol. After centrifugation, the pellet was washed once with 70% ethanol, dried and resuspended in 50 μl of water.

Figure 2.1

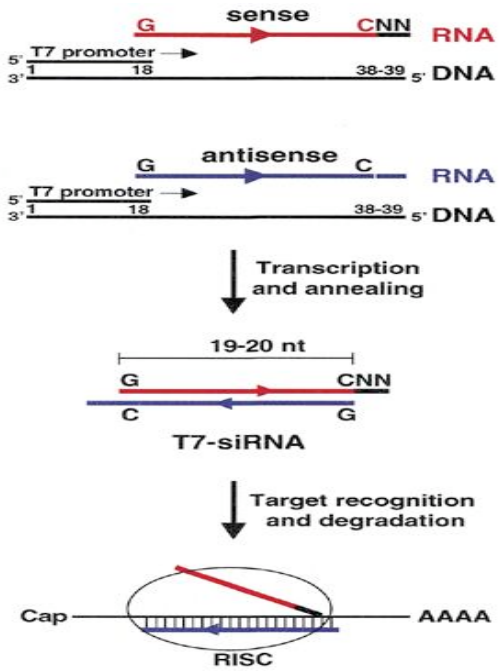


Fig. 2.1. Strategy to generate T7 siRNAs: The sequence of the gene of interest is shown in red (sense) or blue (antisense), while the two unrelated nucleotides are in black. RISC stands for the RNA-induced silencing complex that targets the mRNA for cleavage (Taken from Donzé and Picard, 2002).

2.12 siRNA transfection

$1x10^6$ million cells were transfected by using Lipofectamine-2000 (Invitrogen) with 0.5μm of non-silencing TopoIIα and β separately, TopoIIα and β siRNA separately and with both TopoIIα and β siRNA. The analysis was performed after 48 and 72 hours.

2.13 Neutral comet assay

Neutral comet assay was done as described in (Hazlehurst et al.1995). Briefly, cells (5000-10,000) were centrifuged and resuspended in 500 ml cold PBS, and 1.5 ml of 1% agarose was added to each sample. The agarose-cell suspension was gently layered on a frosted-glass microscope slide, allowed to solidify for 5 min, and then placed immediately in ice-cold lysis buffer containing 30 mM disodium ethylenediamine tetraacetic acid (EDTA, pH 8.0), 0.5% sodium dodecyl sulfate

(SDS) and 0.25 mg/ml proteinase K (Sigma). The samples were lysed for 1 h at 4 _C and then kept at 37 _C for 12–16 h. After cell lysis and digestion of protein–DNA complexes with proteinase K, the agar slides were re-equilibrated in TBE (90 mM Tris–HCl, 90 mM boric acid and 2 mM EDTA, pH 8.0) for 2 h, with a change of buffer every 15 min. The samples were electrophoresed with TBE buffer for 20 min at 25 V. The DNA was then stained with 20 lg/ml of ethidium bromide (Sigma) for 20 min and slides were washed twice for 5 min in TBE. To ensure random sampling, 50 images/slide were captured and, in some experiments, the observer was blinded to the conditions. The images were captured on a Confocal microscope (Lieca) and quantified by using Comet-IV software (Perceptive Instruments, UK). The comet moment was calculated by using the following equation described by Kent et al.1995: comet moment $\sum_{0-n}$ ((intensity of DNA at distance X)x (distance)/(intensity of total DNA). The mean comet-moment value obtained from control samples was subtracted from the mean comet moment value for each treated dosage. Data shown are the means and SD values from three independent experiments (50 images for each dose of each independent experiment).

2.14 Annexin-V assay

Annexin V staining of apoptotic cells was used to determine whether reducing the levels of either TopoIIα or β increased the sensitivity of SK-N-SH and 1321N1 cells to H_2O_2 induced cell death. 48 hours after transfection with appropriate siRNA constructs, cells were treated with 1 mM H_2O_2 for 24h and then placed in drug free media for an additional 48 h then peroxide induced apoptosis was measured by annexin V staining (Invitrogen kit) and FACS analysis.

2.15 FACS analysis

The FACS analysis was done according to Bruce *et al.,* (1990) briefly to prepare cells for fluorescence-activated cell sorter (FACS) analysis, adherent cells were removed from plastic tissue culture flasks by trypsinization for 8 min at 37^0C. Fetal calf serum was added to 25% to block trypsin activity. The cells were then filtered through nylon mesh to remove clumps, counted, and distributed to a 96-well tray

($5x10^5$ cells/well) in 50 µl of RPMI 1640 medium containing 0.01M NaN_3, washed twice with RPMI-azide, and then incubated with mouse anti-human monoclonal antibodies (1:50 dilution) CD4, CXCR4 and CCR5 in 50 µl of RPMI-azide for 1 h at 4^0C. After two washes with RPMI-azide, cells were incubated in 150 µl of 1:180 dilution of fluorescein isothiocyanate (FITC)- conjugated goat anti-mouse immunoglobulin in RPMI-azide for 1 h at 4^0C. after two washes with RPMI-azide, the cells were suspended in 1 ml of PBS containing 1% formaldehyde. The stained and fixed cells were analyzed on a FACSTAR cell sorter (Becton Dickinson, Mountain View, Calif.) with an argon ion laser emitting 488-nm light at 200 mW.

2.16 Infection assay

5x 10^6 cells were seeded into the 60 mm dishes one-day before the experiment, the cells were challenged with HIV-1 (20 ng/ml) in a medium containing 2% serum, and after 2 h post-infection, serum was increased to 10%. The infection was stopped at 4 and 24 h the time at which TopoIIα and β and TNFα show significant expression. For the p24 quantification the medium is aspirated out completely 48 h post-infection and cells were washed twice with the medium and supplemented with complete medium containing 10% FBS. HIV-1 was quantified in terms of p24 after 96 hours of post-infection. HIV-1.

2.17 Infection in presence of NDGA and peptides

One-day before the experiment, 5 x 10^6 cells were cultured in the 60 mm dishes. Drug and peptides were added to the cells at indicated concentrations and incubated for 10 min. These cells were challenged with HIV-1 as explained in infection assay.

2.18 Anti-HIV activity

Anti-HIV activity was assayed out as explained in Kondapi *et al.,* 2002. In brief, SupT-1 cells ($0.2x10^6$) in RPMI1640, 10%FCS was seeded in 96-well plate. Increasing concentrations of ferrocene derivatives (20µl) were added to the cells and were challenged with HIV-$1_{93IN101}$ at a final concentration of 2 ng of p24 per ml (20µl). In one set, the infected cells were incubated at 37°C for 96 h in a CO_2 incubator with 5% CO_2. In the second set, the virus and drug were incubated for 5 h

and cells were washed twice and re-cultured in fresh medium without drug and virus and incubated for 96 h at 37°C in a CO_2 incubator. The cells were analyzed for the viability using MTT as explained in the cytotoxicity assay. The supernatants were collected at 96 h post-infection and analyzed using p24 antigen capture assay kit (SAIC Frederic). The inhibition of infection, in the absence of the drug was considered to be 0% and the percent inhibition of HIV replication was calculated based on this control. Each compound was analyzed in triplicates at increasing concentrations on a logarithmic scale and standard deviation was also shown. In control infection study, in the absence of drugs, the virus replicated was 10 ng/ml of p24 equivalent. Azidothymidine (AZT) and Etoposide were used as positive controls.

2.19 Preparation of cell extracts from HIV-1 infected cells

Human SupT1 cells ($2x10^6$ cells/ml) were challenged with HIV-$1_{93IN101}$ (10 ng of p24 viral core protein per ml). Infected cells were washed twice with buffer A (20 mM Hepes, pH 7.4/150 mM KCl/5 mM $MgCl_2$/1 mM dithiothreitol/2 mM aprotinin) at 5 h p.i. and permeabilized with 0.025%Triton X-100. Cells were incubated for 10 min at room temperature, and then lysate was centrifuged at 1000*g* for 10 min at room temperature. The supernatant was clarified by centrifugation at 8000*g* for 3min.

2.20 Isolation of viral cDNA

SupT1 cells ($0.4x10^6$) were challenged with HIV-$1_{93IN101}$ (100 pg of p24 viral core protein) in the presence of increasing concentrations of drugs as indicated at 5% CO_2 and 37°C. The cells were harvested at 5h. post-infection and washed with phosphate-buffered saline. They were then lysed with 50 µl lysis buffer containing 10x Solution A (1M KCl, 100 mM Tris–HCl (pH 8.3), 2.5 mM $MgCl_2$), 10x Solution B (100 mM Tris–HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.45% Tween 20, 0.45% Nonidet P 40, and 50 mM NaCl). The cell lysates were treated with RNase I (10 µg/ml) and incubated at 37°C for 30 min. Proteinase K (60 µg/ml) was added to the lysates and incubated at 56°C for 2 h followed by the inactivation of Proteinase K

at 95°C for 10min. The lysates were then stored at 20°C, for their subsequent use for PCR analysis.

2.21 PCR analysis of viral cDNA

The cell lysates were added to the 50 μl of reaction mixture comprising of 10x PCR buffer, 0.2 mM of each deoxynucleotidetriphosphates (dNTPs), 2.5 mM $MgCl_2$, 0.40 μM SK38 (5′-ATAATCCACCTATCCCAGTAGGAGAAA T-3′), SK39 (5′-TTTGGTCCTTGTCTTATGTCCAGAA TGC-3′) primers (Saiki et al., 1988) and 0.5 U of *Taq* DNA Polymerase (Saiki et al., 1988; Sonza et al., 1996). The mixtures were heated to 94°C for denaturation for 2min and then subjected to amplification for 30 cycles of PCR (1min 94°C, 1.30min 60°C, and 2min 72°C), and a final step for extension at 72°C for 5min. Control amplification was done using β-actin specific primers (Forward: 5′-GGCCCAGAGCAAGAGAGGTAT CC-3′, Reverse-5′-ACGCACGATTTCCC TCTCAG C-3′) (Kwan et al., 2001). The products were resolved on 2% agarose gel electrophoresis, ethidium bromide stained, and photographed.

2.22 Data Analysis

All of the numerical data are presented as means ± SEM. Statistical significance was calculated using Student's t-test to determine whether compared groups are distinct. Differences were considered significantly different if $p < 0.05$.

CHAPTER 3

Analysis of the function of Topoisomerase IIα and β in neuronal development

DNA Topoisomerase II (Topo II) is a nuclear enzyme that catalyses the transport of one of the two DNA double helices through the other. Thus, its enzymatic activity is essential for chromosome segregation, DNA replication, Chromosomal condensation, genetic recombination (Wang *et al.,* 1996). The two mammalian Topo II isoforms, 170 kDa TopoIIα and 180 kDa TopoIIβ share about 72% identity in their primary sequences and possess the same enzymatic activity *in-vitro* (Austin *et al.,* 1993; Drake *et al.,* 1989; Jenkins *et al.,* 1992; Tsai-Pflugfelder *et al.,* 1988). However, the two enzymes are regulated very differently. For example, immunohistochemical studies have shown that TopoIIα is only present in proliferating tissues, including tumors, while TopoIIβ is present in all tissues, including terminally differentiated tissues (Bauman *et al.,* 1997; Hsiang *et al.,* 1988; Tsutsui *et al.,* 2001a). Furthermore, the level of TopoIIα rises during S phase, reaching its peak at the G2/M phase of the cell cycle (Heck and Earnshaw, 1986; Niimi *et al.,* 2001; Woessner *et al.,* 1991). TopoIIα, but not TopoIIβ, has been shown to be located in replication foci in S phase, possibly through its interaction with PCNA (Earnshaw *et al.,* 1985). In addition, TopoIIα, identified as the major chromosome scaffold protein Sc1, forms the structural scaffold, together with condensing in mitotic chromosomes (Maeshima and Laemmli, 2003; Sumner, 1996; Swedlow and Hirano, 2003; Taagepera *et al.,* 1993). Thus, accumulating evidence has suggested that TopoIIα plays an important role in cell cycle events such as DNA replication and chromosome condensation/ segregation (Cuvier and Hirano, 2003; Grue *et al.,* 1998; Lyu and Wang, 2003; Wang, 2002). In contrast, TopoIIβ is present in all cells, and its level is not significantly changed during the cell cycle (Feister *et al.,* 1997; Liu and Wang, 1987; Lyu and Wang, 2003; Tsao *et al.,* 1989; Tsutsui *et al.,* 2001b; Watanabe *et al.,* 1994; Yang *et al.,* 2000).

In the embryonic stage, TopoIIβ in the brain is a nucleoplasmic enzyme showing higher levels of expression in the differentiating neurons (Tsutsui *et al.,* 2001). Both the isoforms show different patterns of tissue distribution. TopoIIα is shown to be higher in testes, spleen, bone marrow and liver. TopoIIβ is detected in high levels in differentiated tissue like brain (Jurenke and Holden, 1993; Capranico *et al.,* 1992). The abundance of TopoIIβ in terminally differentiated cells suggests that it may play a role in DNA metabolism other than, or in addition to, DNA

replication and chromosome condensation/ segregation. However, the precise function of TopoIIβ remains elusive. A number of recent studies have suggested that TopoIIβ plays a role in neuronal differentiation. First, studies of whole-body TopoIIβ knockout mice have demonstrated a prenatal death phenotype. It has been demonstrated that motor neuron axons fail to innervate the diaphragm muscles and sensory neuron axons fail to enter the spinal cord, suggesting a role of TopoIIβ in axon growth and/or guidance (Yang *et al.,* 2000). Studies of brain-specific TopoIIβ knockout mice have also revealed a major defect in corticogenesis during brain development (Lyu and Wang, 2003). Second, studies of developing rat cerebellum have demonstrated that post-mitotic granule cells in the external germinal layer show an abrupt transition of expressed Topo II isoforms from TopoIIα to TopoIIβ, again suggesting an association between TopoIIβ and neuronal differentiation (Kondapi *et al.,* 2004; Lyu and Wang, 2003; Tsutsui *et al.,* 2001). Kamal *et al.,* (2007) showed that TopoIIβ knockout mice failed in formation of growth cone and axon development. In this chapter we have studied the role of TopoIIβ in neuronal development and differentiation.

Results

In the present study, the levels of Topo II isoforms were analyzed in the three regions of developing rat brain viz. cerebellum, cerebral cortex and midbrain. TopoIIα and β present in the extracts prepared from different parts of brain was immunoprecipitated using monoclonal human TopoIIα and β antibodies. The activity of the TopoIIα and β in immunoprecipitate was analyzed using enzyme catalyzed relaxation of supercoiled pRYG plasmid DNA. The products were analyzed on 1% agarose gels and stained with ethidium bromide. The protein levels of TopoIIα and β were monitored through Western blot analysis of the Protein using mouse anti-human TopoIIα and β monoclonal antibodies.

Granule and purkinje neurons are the major population of neuronal cells in cerebellum, they mainly involve in coordination and movement. Neuronal death in cerebellum leads to neurodegenerative diseases like Ataxia, autism and nystagmus type of disorders. Cortical neurons are mainly involved in cognitive functions, neuronal death in cortical cells leads to cognitive dysfunction, Alzheimer's and

schizophrenia etc... These neurons have been used in various studies for understanding mechanism of neuronal development. In the present study we have investigated the role of Topo II in neuronal development and differentiation using cortical, granule and purkinje neurons.

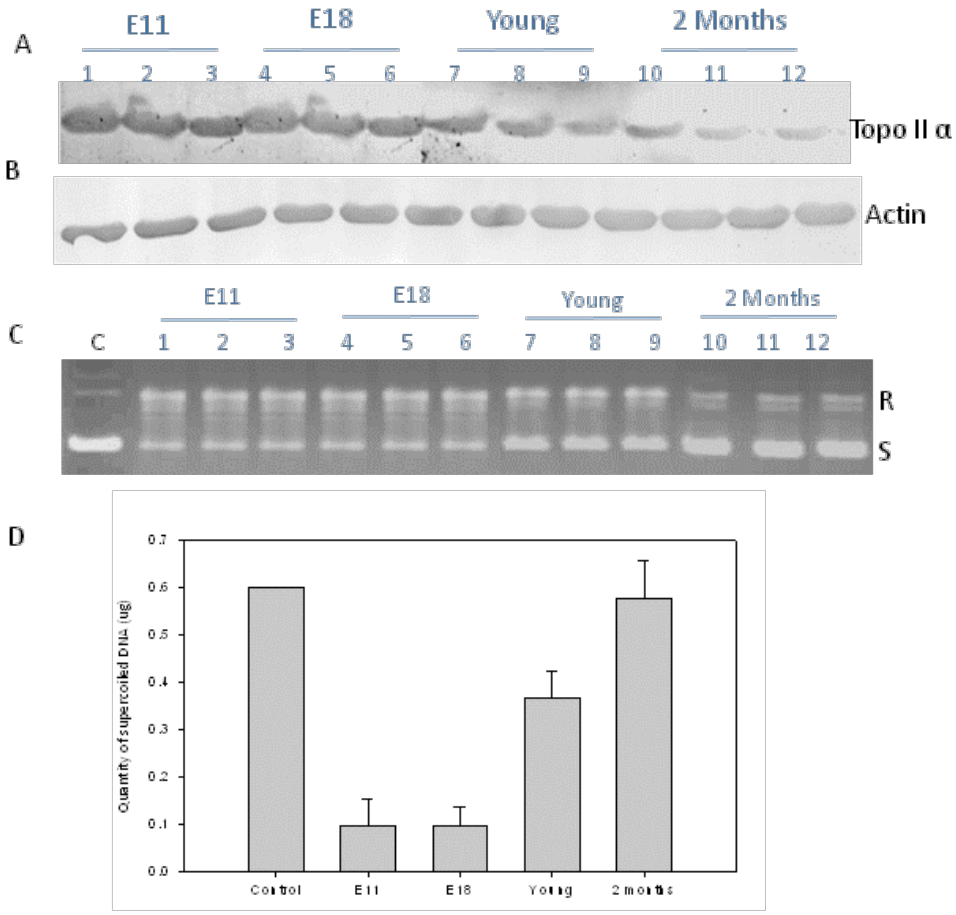
Topoisomerase II α during brain development

The levels of TopoIIα were analyzed in embryonic day 11 (E11), embryonic day 18 (E18), young and 2 months old rat cerebellum, cerebral cortex and mid brain. Results show that TopoIIα is high in cerebellum of E11 and E18 embryos but in post-natal day 1 pups shows significant decrease and become negligible in 2 months age rat cerebellum. Comparative analysis of TopoIIα show same expression profile in cerebral cortex and mid brain, while shows low expression in mid brain than cerebellum and cerebral cortex (Fig.3.1A, 3.3A and 3.5A). Actin was used as loading control (Fig.3.1B, 3.3B and 3.5B).This observation was correlated well with the catalytic activity of TopoIIα (Fig.3.1C, 3.3C and 3.5C). Supercoiled DNA in enzymatic assay was quantified and measured as bar diagram (Fig 3.1D, 3.3D and 3.5D). This analysis indicates that TopoIIα level is high in proliferating tissues like developing rat embryonic brain.

Topoisomerase II β during brain development

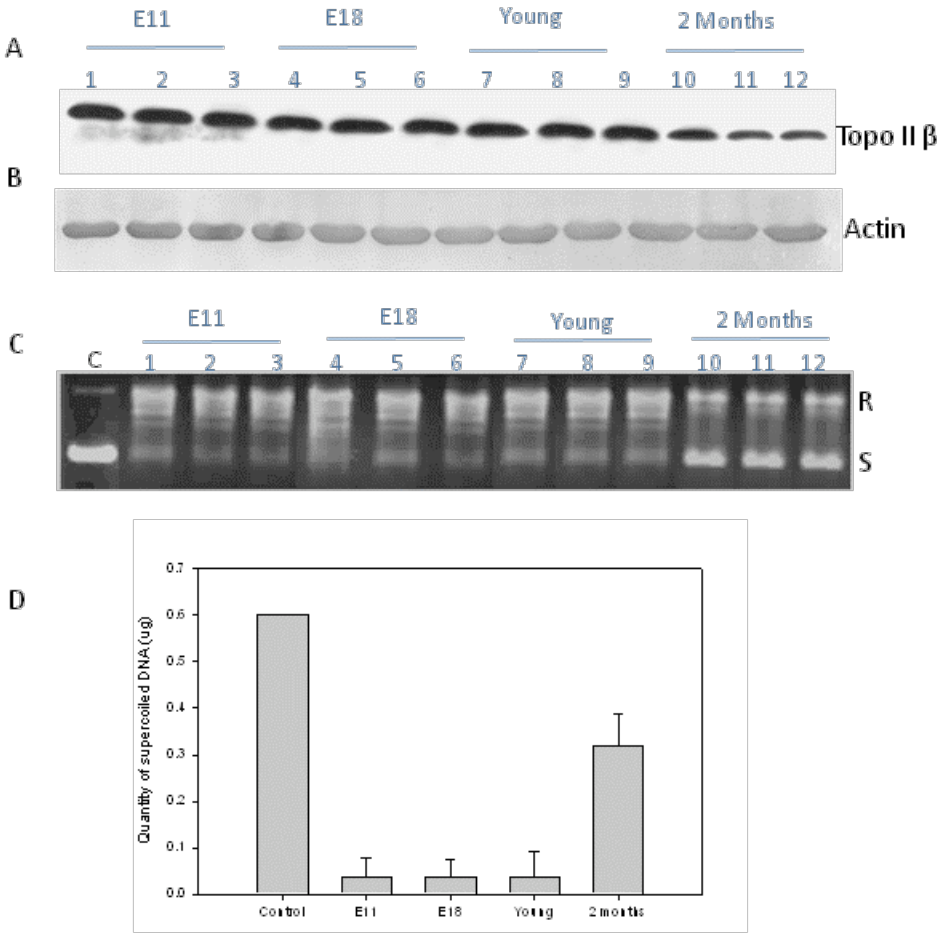
The levels of TopoIIβ were analyzed in E11, E18, young and 2 months old rat cerebellum, cerebral cortex and mid brain. The results show higher levels of TopoIIβ through out the all stages of development (E11, E18 and young), while a moderate decrease was observed in 2 months old rat cerebellum, where the neuronal cells are completely differentiated. Comparative study of TopoIIβ expression profile in cerebellum, cerebral cortex and mid brain, show that its expression is low in mid brain compared to cerebellum and cerebral cortex (Fig. 3.2A, 3.4A and 3.6A). Actin loading control was showed in Fig 3.2B, 3.4B and 3.6B. These results were closely correlated with catalytic activity of TopoIIβ (Fig. 3.2C, 3.4C and 3.6C). Supercoiled DNA after relaxation assay was quantified and showed as bar diagram (Fig 3.2D, 3.4D and 3.6D). Expression of TopoIIβ both in developmental and post-natal stages suggests that it has synergistic role during embryogenesis, development and differentiation of neurons.

Figure 3.1



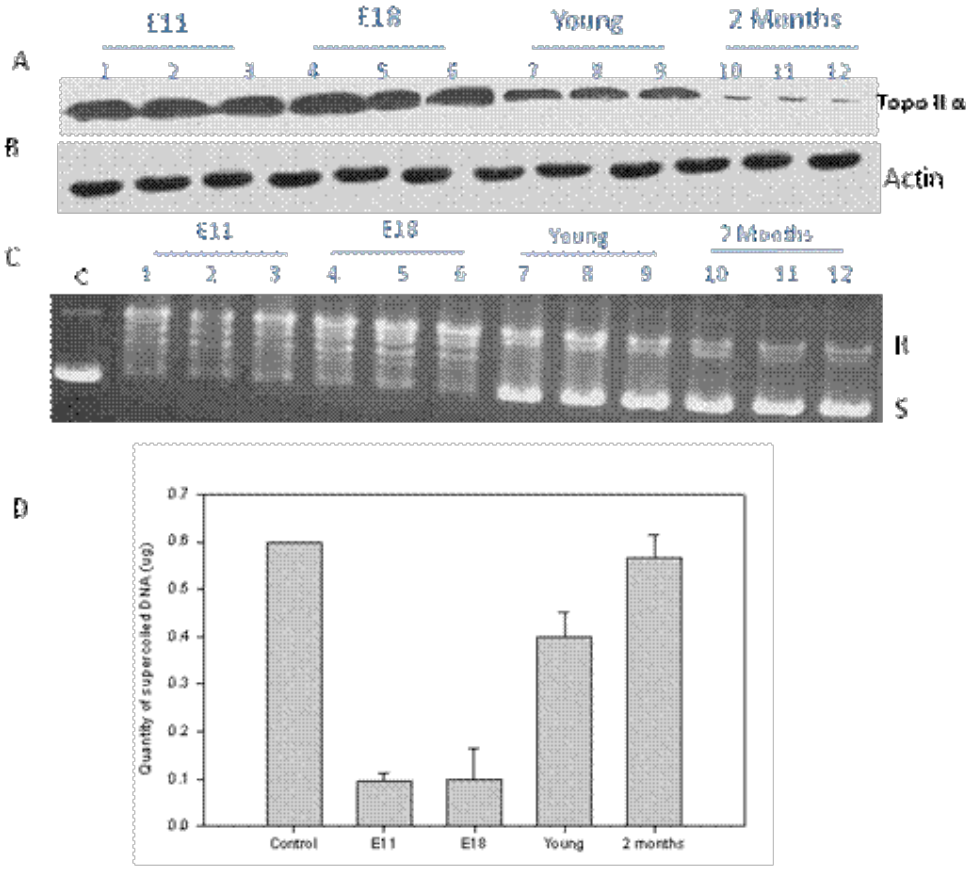
TopoIIα protein levels and enzymatic activity in cerebellum during different stages of rat brain development: Tissue extracts were prepared from cerebellum of E11 (lanes 1-3), E18 (lanes 4-6), young (lanes 7-9) and 2 months (lanes 10-12) old rat brain. Total protein was estimated by Bradford method. 40 µg of protein extract was separated on 7.5% SDS PAGE and Western transferred and probed with monoclonal Abs against TopoIIα and blots were developed with ECL (panel A), Actin was used as loading control (panel B). TopoIIα activity was measured in 100 µg protein of cerebellum extracts (panel C) through relaxation assay with pRYG plasmid DNA, lane c shows ~ 0.6 µg of pRYG plasmid DNA. Corresponding quantified supercoiled DNA was shown in panel D.

Figure 3.2



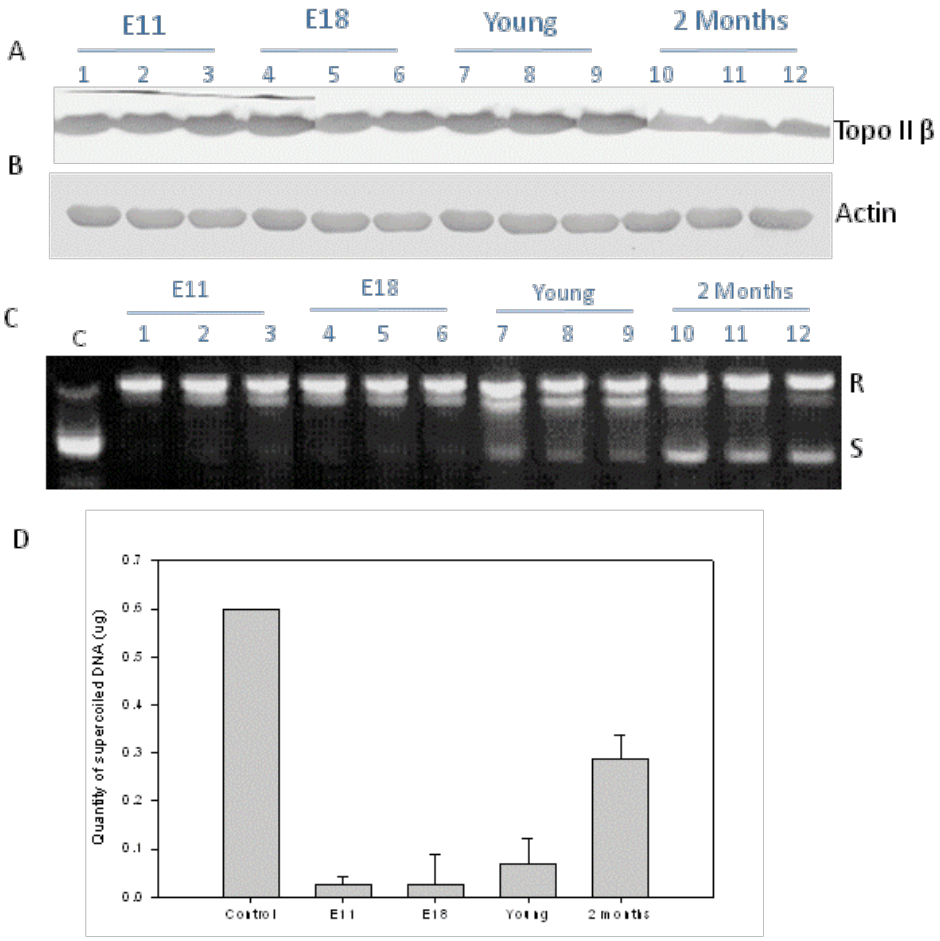
TopoIIβ protein levels and enzymatic activity in cerebellum during different stages of rat brain development: Tissue extracts were prepared from cerebellum of E11 (lanes 1-3), E18 (lanes 4-6), young (lanes 7-9) and 2 months (lanes 10-12) old rat brain. Total protein was estimated by Bradford method. 40 µg of protein extract was separated on 7.5% SDS PAGE and Western transferred and probed with monoclonal Abs against TopoIIβ and blots were developed with ECL (panel A), Actin was used as loading control (panel B). TopoIIβ activity was measured in 100 µg protein of cerebellum extracts (panel C) through relaxation assay with pRYG plasmid DNA, lane c shows ~ 0.6 µg of pRYG plasmid DNA. Corresponding quantified supercoiled DNA was shown in panel D.

Figure 3.3



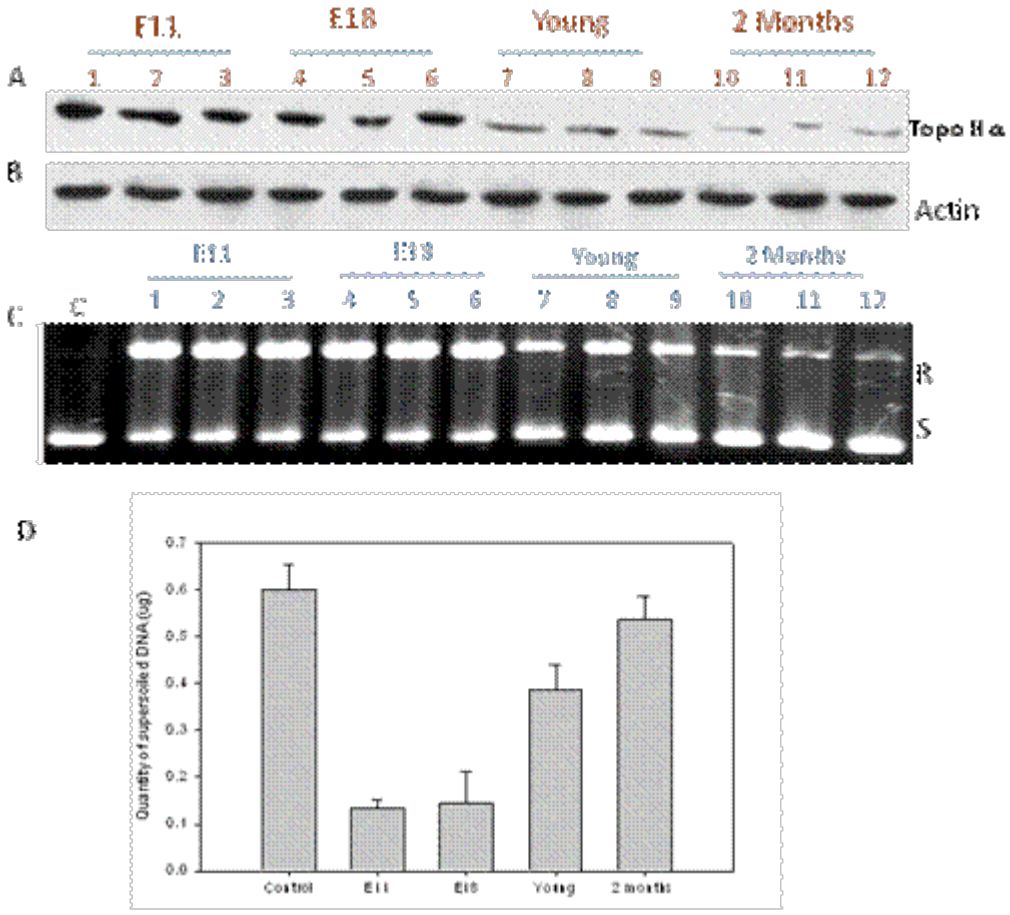
TopoIIα protein levels and enzymatic activity in cerebral cortex during different stages of rat brain development: Tissue extracts were prepared from cerebral cortex of E11 (lanes 1-3), E18 (lanes 4-6), young (lanes 7-9) and 2 months (lanes 10-12) old rat brain. Total protein was estimated by Bradford method. 40 µg of protein extract was separated on 7.5% SDS PAGE and Western transferred and probed with monoclonal Abs against TopoIIα and blots were developed with ECL (panel A), Actin was used as loading control (panel B). TopoIIα activity was measured in 100 µg protein of cerebral cortex extracts (panel C) through relaxation assay with pRYG plasmid DNA, lane c shows ~ 0.6 µg of pRYG plasmid DNA. Corresponding quantified supercoiled DNA was shown in panel D

Figure 3.4



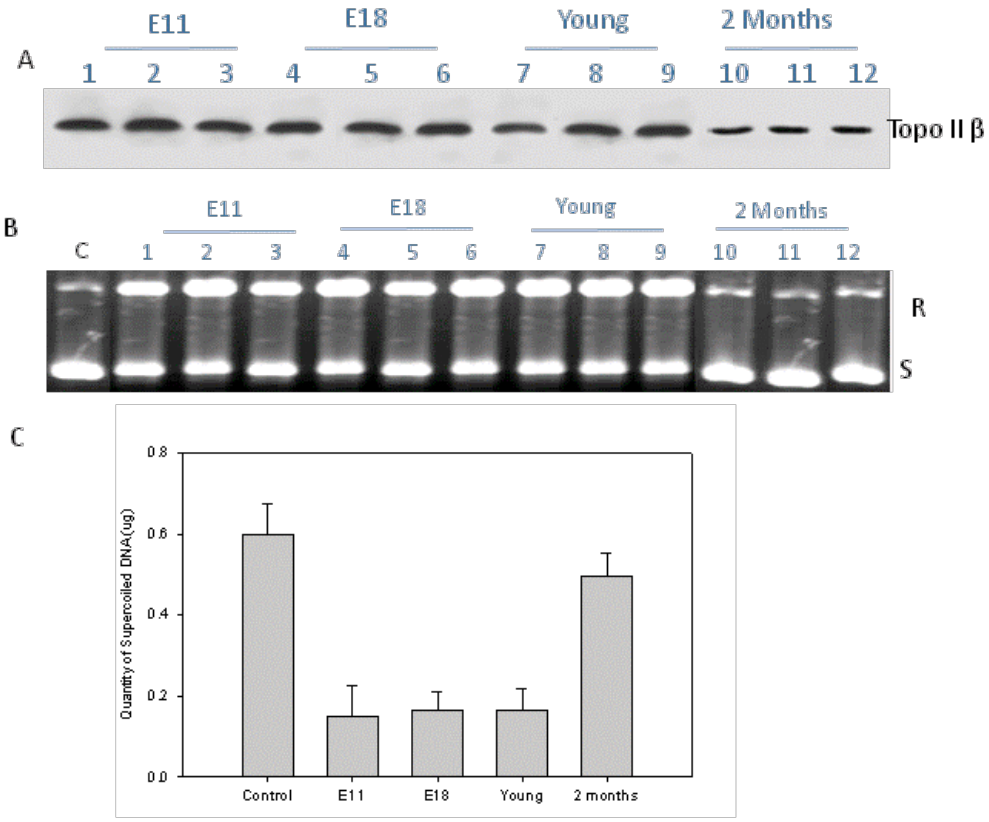
TopoIIβ protein levels and enzymatic activity in cerebral cortex during different stages of rat brain development: Tissue extracts were prepared from cerebral cortex of E11 (lanes 1-3), E18 (lanes 4-6), young (lanes 7-9) and 2 months (lanes 10-12) old rat brain. Total protein was estimated by Bradford method. 40 µg of protein extract was separated on 7.5% SDS PAGE and Western transferred and probed with monoclonal Abs against TopoIIβ and blots were developed with ECL (panel A), Actin was used as loading control (panel B). TopoIIβ activity was measured in 100 µg protein of cerebral cortex extracts (panel C) through relaxation assay with pRYG plasmid DNA, lane c shows ~ 0.6 µg of pRYG plasmid DNA. Corresponding quantified supercoiled DNA was shown in panel D.

Figure 3.5



TopoIIα protein levels and enzymatic activity in mid brain during different stages of rat brain development: Tissue extracts were prepared from mid brain of E11 (lanes 1-3), E18 (lanes 4-6), young (lanes 7-9) and 2 months (lanes 10-12) old rat brain. Total protein was estimated by Bradford method. 40 µg of protein extract was separated on 7.5% SDS PAGE and Western transferred and probed with monoclonal Abs against TopoIIα and blots were developed with ECL (panel A), Actin was used as loading control (panel B). TopoIIα activity was measured in 100 µg protein of mid brain extracts (panel C) through relaxation assay with pRYG plasmid DNA, lane c shows ~ 0.6 µg of pRYG plasmid DNA. Corresponding quantified supercoiled DNA was shown in panel D.

Figure 3.6



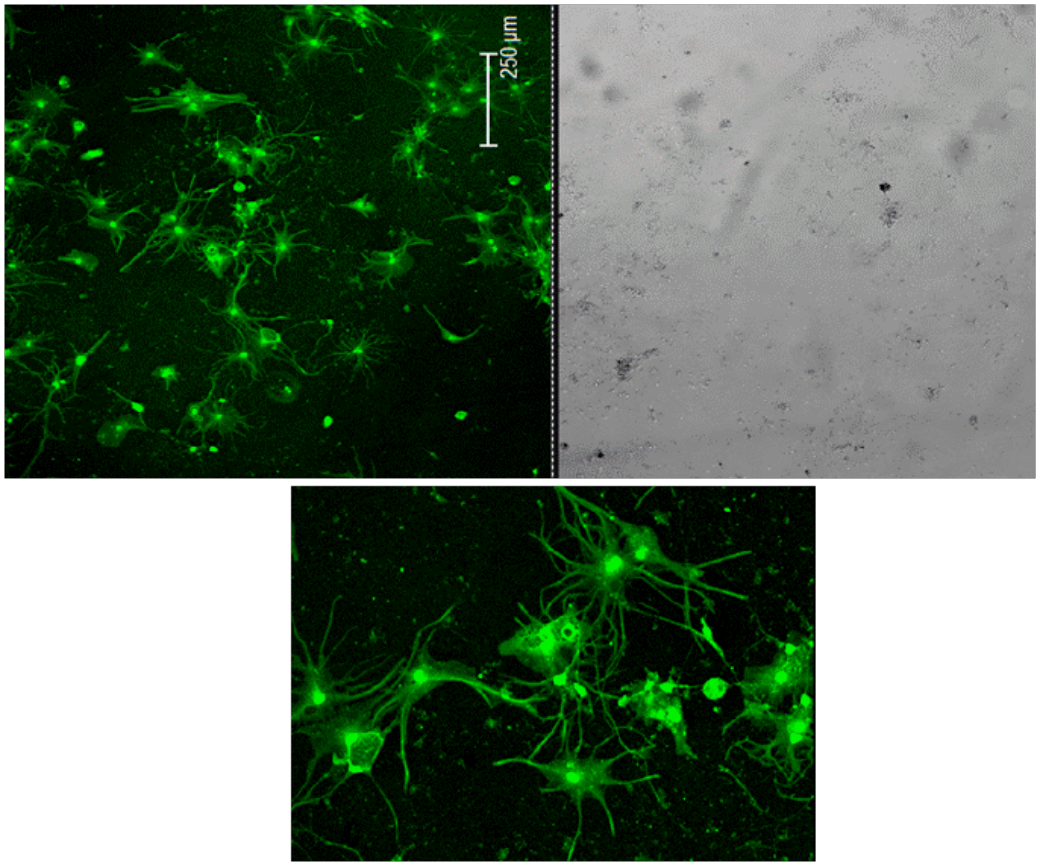
TopoIIβ protein levels and enzymatic activity in mid brain during different stages of rat brain development: Tissue extracts were prepared from mid brain of E11 (lanes 1-3), E18 (lanes 4-6), young (lanes 7-9) and 2 months (lanes 10-12) old rat brain. Total protein was estimated by Bradford method. 40 µg of protein extract was separated on 7.5% SDS PAGE and Western transferred and probed with monoclonal Abs against TopoIIβ and blots were developed with ECL (panel A), TopoIIβ activity was measured in 100 µg protein of mid brain extracts (panel B) through relaxation assay with pRYG plasmid DNA, lane c shows ~ 0.6 µg of pRYG plasmid DNA. Corresponding quantified supercoiled DNA was shown in panel C.

We have isolated different cell types (granule neurons, purkinje neurons and astrocytes from cerebellum and cortical neurons from cerebral cortex) from rat brain tissue to examine the levels of TopoIIα and β in neuronal cells and cultured in suitable media. Granule neurons were isolated from 8 day-old rat cerebellum and maintained healthy for 20 days, 10µm of cytosine-arabinoside was added after 24 hrs to remove proliferating cell like glial cells. Granule neurons were stained with neuronal specific enolase (NSE) (Fig 3.8). Purkinje neurons and cortical neurons were isolated from E18 rat embryos brain and maintained healthy for 30 days, 10µm of cytosine-arabinoside was added after 2 days to remove proliferating cell like glial cells. Purkinje neurons were immunostained with calbindin D 28k antibody (Fig 3.7 and Fig 3.9) and the cortical neurons were immunostained with NSE. Astrocytes were isolated from 6-day-old rat pups cerebellum and maintained for 30 days in culture, cells were confirmed by immunostaining with glial fibrillary acidic protein (GFAP) antibody (Fig. 3.10). The homogeneity of cultures were checked by immunoblotting with NSE for neuronal cells and GFAP for astrocytes. The results show that all cultures have more than 90% homogeneity (Fig 3.11), these cultures were used for further analysis.

Expression profile of TopoIIα and β in different cell types of the brain

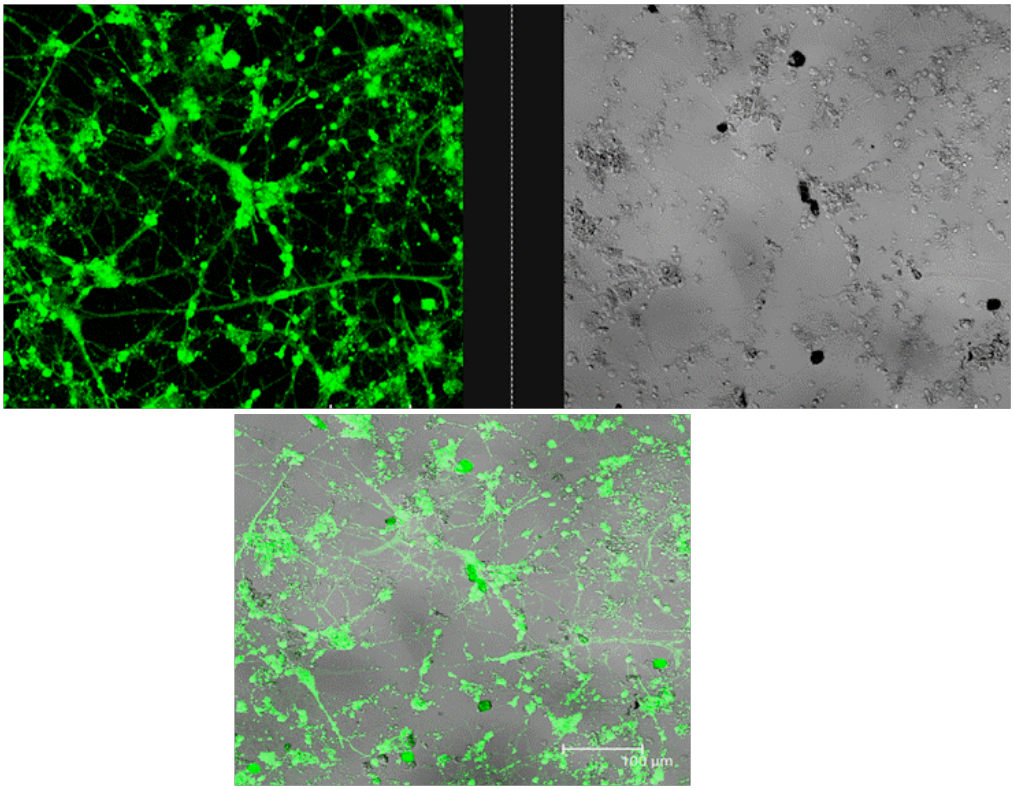
TopoIIα and β levels were analyzed in cultured granule neurons (4 days culture), purkinje neurons (6 days culture), cortical neurons (6 days culture) and astrocytes (10 days culture). The results show that TopoIIα expression is high in astrocytes (dividing cells), while the expression of TopoIIα was negligible in differentiated neurons. Whereas, TopoIIβ expression is high in neurons, while show moderate expression in astrocytes (Fig 3. 12). These results suggest that TopoIIα is associated with cell proliferation, but TopoIIβ is associated with cell differentiation and maturation.

Figure 3.7



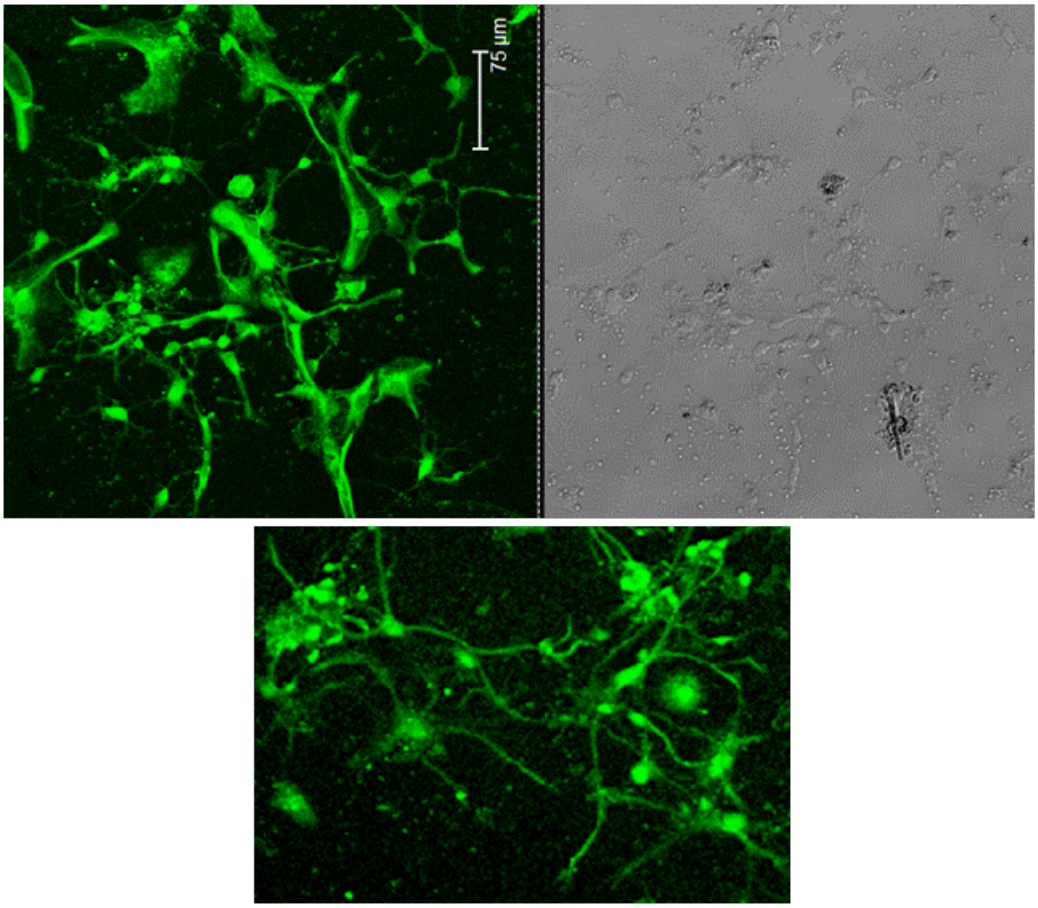
Purkinje neurons in culture: Purkinje neurons were isolated from the 18 days rat embryo cerebellum and cultured in DMEM/F12 containing N3 supplement, glutamine, thyroxin and 1% FBS. Cells were immunostained with calbindin-D28k monoclonal antibodies and detected with FITC conjugated secondary antibodies. Stained cells were captured under confocal microscope (Lieca)

Figure 3.8



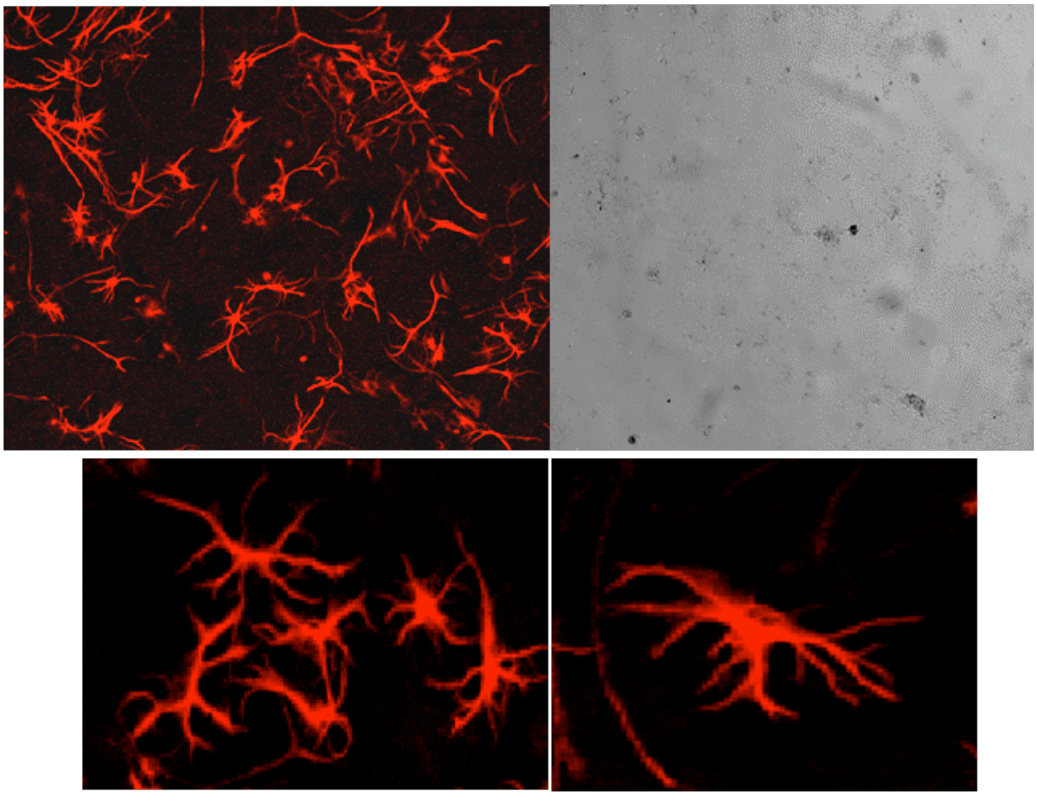
Granule neurons in culture: Granule neurons were isolated from the 8 day old rat pups cerebellum and cultured in EMEM containing glucose, glutamine, KCl and 10% FBS. Cells were immunostained with neuronal specific enolase (NSE) polyclonal antibodies and detected with FITC conjugated secondary antibodies. Stained cells were captured under confocal microscope (Lieca)

Figure 3.9



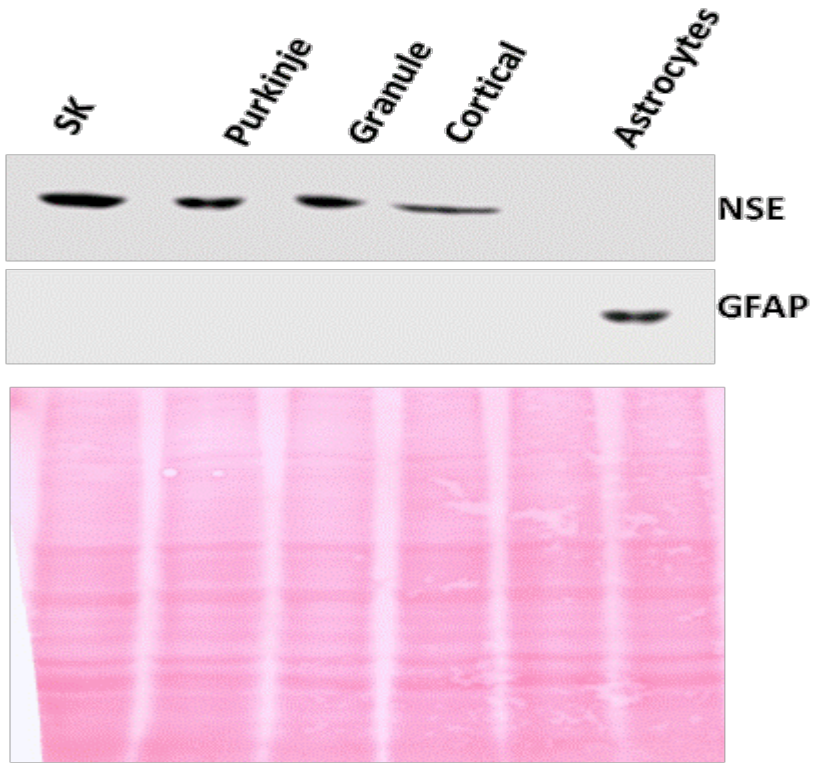
Cortical neurons in culture: Cortical neurons were isolated from the 18 days rat embryos cerebral cortex and cultured in neurobasal medium containing , glutamine, and B12 supplement. Cells were immunostained with neuronal specific enolase (NSE) polyclonal antibodies and detected with FITC conjugated secondary antibodies. Stained cells were captured under confocal microscope (Lieca)

Figure 3.10



Astrocytes in culture: Astrocytes were isolated from the 6 day old rat pups cerebellum and cultured in DMEM/F12 containing D/L valine and 10% FBS. Cells were immunostained with glial fibrillary acidic protein (GFAP) monoclonal antibodies and detected with TRITC conjugated secondary antibodies. Stained cells were captured under confocal microscope (Lieca)

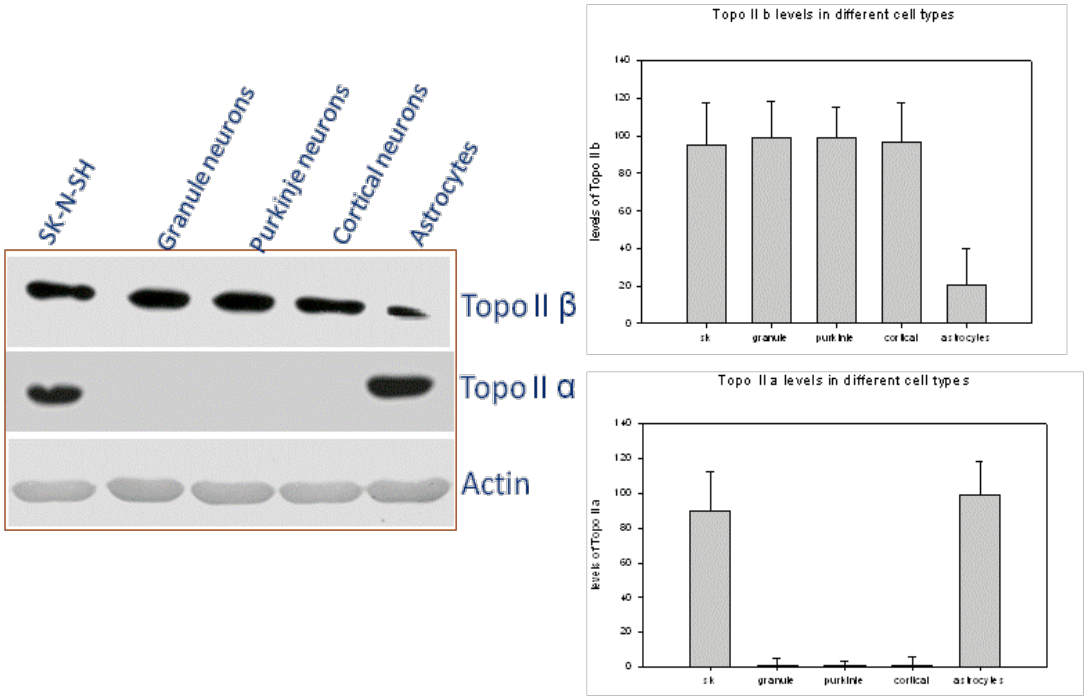
Figure 3.11



Homogeneity of primary cultures isolated from brain

Purkinje, granule neurons and astrocytes were isolated from cerebellum and cortical neurons were isolated from cerebral cortex of rat brain and cultured in corresponding mediums. Neuronal cells were treated with 10µM cytosine arabinoside to remove glial cells. Glial cells were cultured in D-valine containing medium to remove fibroblasts and neuronal cells. Cell extracts were prepared from the individual cultures and 40µg of total protein was resolved on SDS-PAGE and probed with NSE to check neuronal cells purity and GFAP for glial cell purity. SK-N-SH neuroblastoma used as neuronal control.

Figure 3.12



TopoIIα and β in different primary cells: Purkinje, granule neurons and astrocytes were isolated from cerebellum and cortical neurons were isolated from cerebral cortex of rat brain and cultured in corresponding mediums. Cells were harvested by trypsinization and lysed in lysis buffer. 40µg of total protein from each cell type was resolved on SDS-PAGE and transferred to nitrocellulose membrane. Probed with isoform specific monoclonal antibodies and detected with ECL Reagent. Bands were quantified using NIH-Image J software.

Down regulation of TopoIIβ in neuronal cells

TopoIIα and β were down regulated in neurons using siRNA technique. siRNA was synthesized with corresponding DNA templates (Table.1) by *in-vitro* transfection method. Lipofectamine -2000 was used for the transfection of neurons. The results from these experiments show that, TopoIIβ was down-regulated more than 70% in three types of neurons (Fig 3.13). Corresponding densitometry plots were showed in Fig 3.13. These TopoIIβ down regulated cells were used for further study of neuronal development

Role of TopoIIβ in neurite out growth and neuronal development

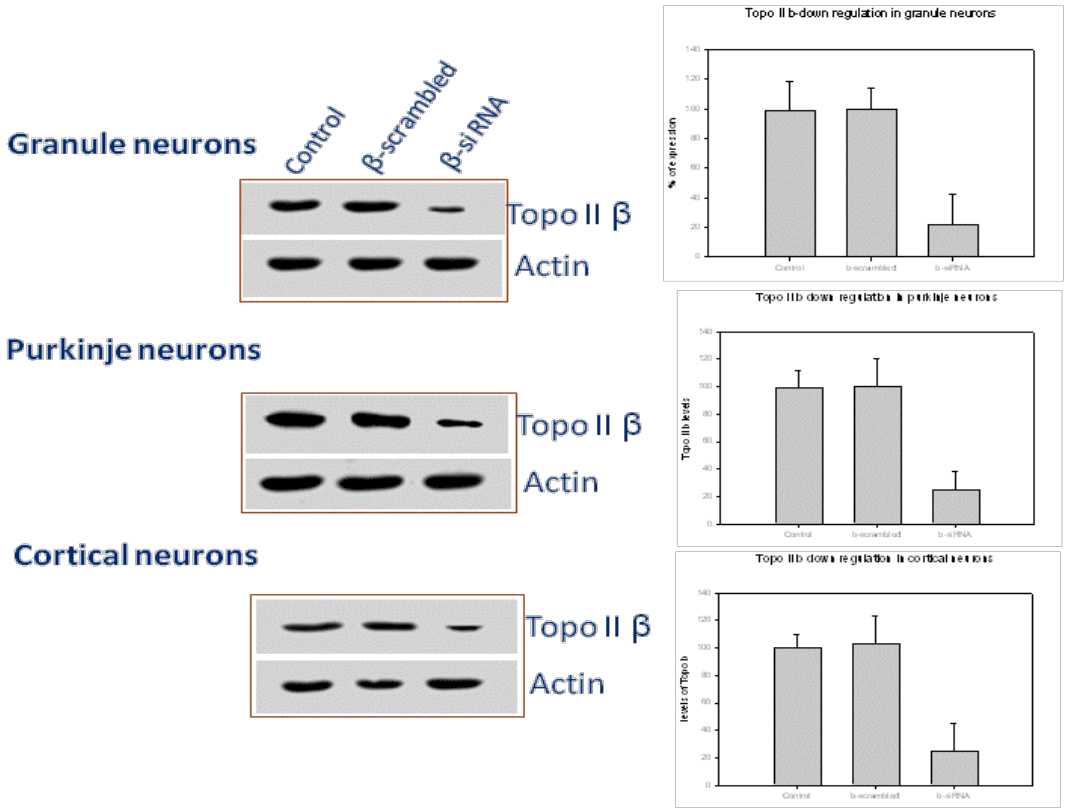
TopoIIβ role in neurite out growth was studied in granule neurons, cortical neurons and Purkinje neurons by down regulation of TopoIIβ as well as by inhibition of TopoIIβ using ICRF-193. Neurons were isolated from rat pups and were cultured *in-vitro* on poly-L-lysine coated plates or cover-slips to induce neurite outgrowth. As shown in Fig. 3.12, TopoIIα not showing any expression in cultured neurons. By contrast, the expression of TopoIIβ was significantly higher in cultured neuronal cells (Fig. 3.12). Neurons were isolated as described in methodology and cultured for 2 hrs in corresponding media, after 2 hrs media was changed and neurons were transfected with TopoIIβ specific siRNA. Neurons were allowed to grow for 48 hrs after the incubation neurite out growth in each neuronal cell type was measured using NIH Image J software. Average of 25 cells from each neuronal cell type was plotted on y-axis. The results from these experiments shows that TopoIIβ down regulated neurons fail to form neurite out growth (Fig 3.16, 3.17 and 3.18). The experiments were repeated with catalytic inhibitor of TopoIIβ, ICRF-193, is known to down-regulate TopoIIβ by activating a 26S proteasome pathway (Mao *et al.,* 2001; Xiao *et al.,* 2003 and Kamal *et al.,* 2007). To demonstrate that ICRF- 193 indeed inhibited TopoIIβ in neurons, the level of TopoIIβ in ICRF-193-treated neurons were monitored. As shown in Fig. 3.15, ICRF-193 effectively down-regulated TopoIIβ in neurons in a dose dependent manner, suggesting that ICRF-193 is effective in inhibiting TopoIIβ in neurons. We studied the cytotoxic activity of ICRF-193 in neurons. We did not find any significant cytotoxic activity of ICRF-193 at or below 100 μM in neurons (Fig 3.14). In order to evaluate the role of

TopoIIβ in neuronal differentiation, the Topo II inhibitor ICRF-193 was employed in neurite outgrowth assays. As shown in Fig. 3.16, 3.17 and 3.18 ICRF-193 (50 μM) effectively inhibited neurite outgrowth (60% to 80% reduction) of neurons, which indicates that TopoIIβ is required for neuronal development and maturation

Regulation of neuronal genes expression by TopoIIβ

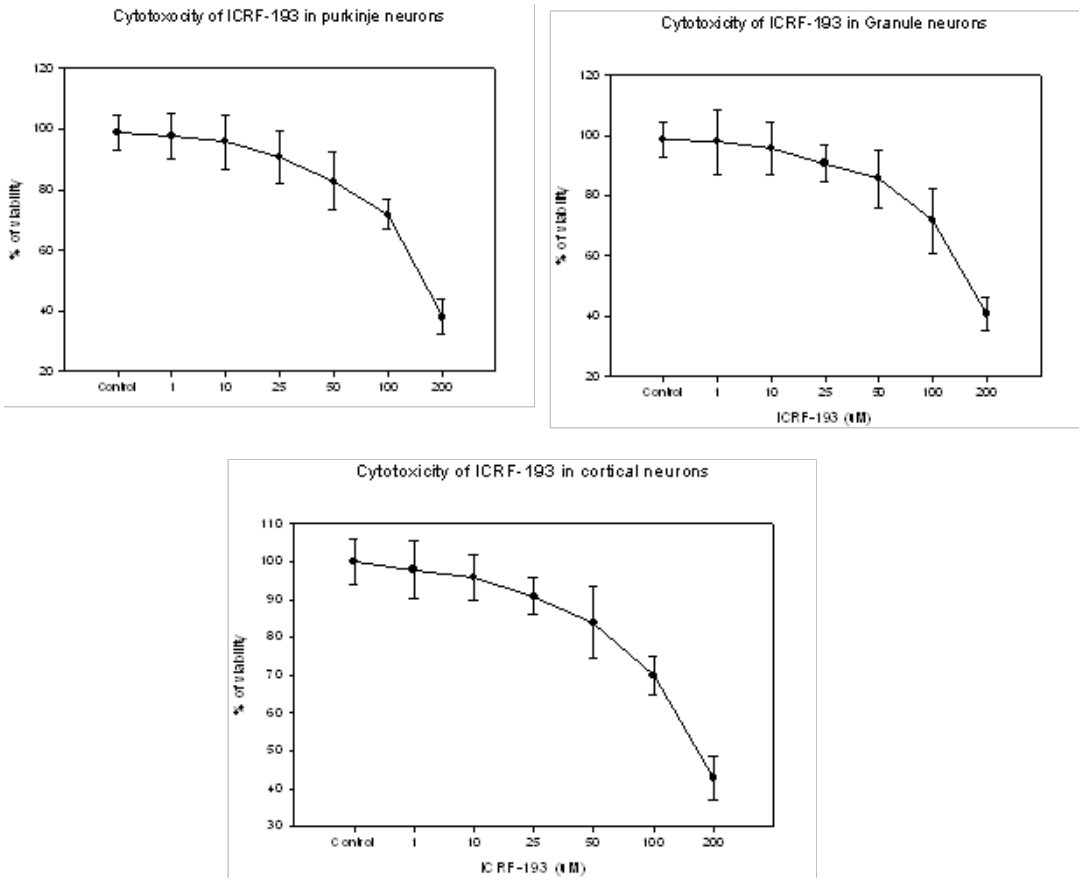
Neurite outgrowth in cultured neurons has been shown to require newly made proteins through transcription and translation.. One possible explanation for a role of TopoIIβ in neurite outgrowth is that TopoIIβ may involved in the regulation of the expression of certain genes required for neurite outgrowth, for this we have analyzed expression levels of reline, synaptophysin, β-catanin and β actin in TopoIIβ down regulated cells. The results show that expression levels of reline and synaptophysin decreased in TopoIIβ down-regulated cultured neuronal cells, when compared to normal neuronal cells. But there is no significant change in β-catenin and β-actin levels in TopoIIβ down regulated neurons (Fig 3. 19). These results suggest that TopoIIβ may affect neurite outgrowth through its regulation of the expression of certain neuronal genes. Alternatively TopoIIβ may activate a factor that promotes the expression of genes those are involved in neuronal differentiation.

Figure 3.13



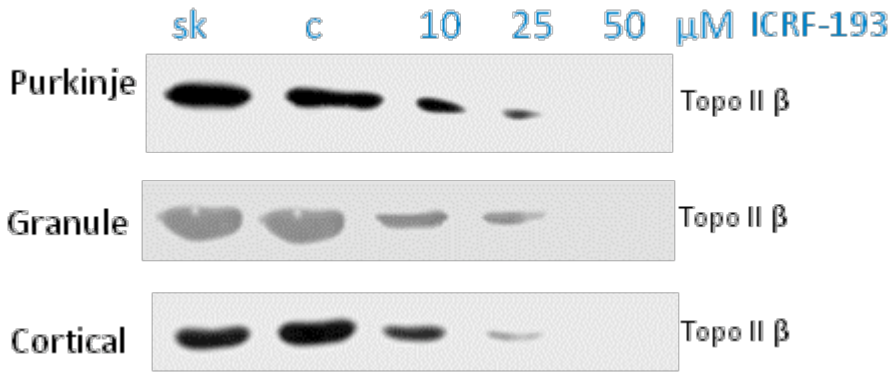
Down regulation of TopoIIβ in neurons: purkinje neurons, granule neurons and cortical neurons were transfected with TopoIIβ siRNA (0.5mM) and scrambled siRNA (negative control) by lipofection (lipofectamine 2000) method. Cells were harvested after 36 hrs and lysed in lysis buffer. 40µg of protein was resolved on SDS-PAGE and transferred to NC memebrane. Membrane was probed with TopoIIβ specific antibodies and detected using ECL reagent. Bands were quantified using NIH-Image J software.

Figure 3.14



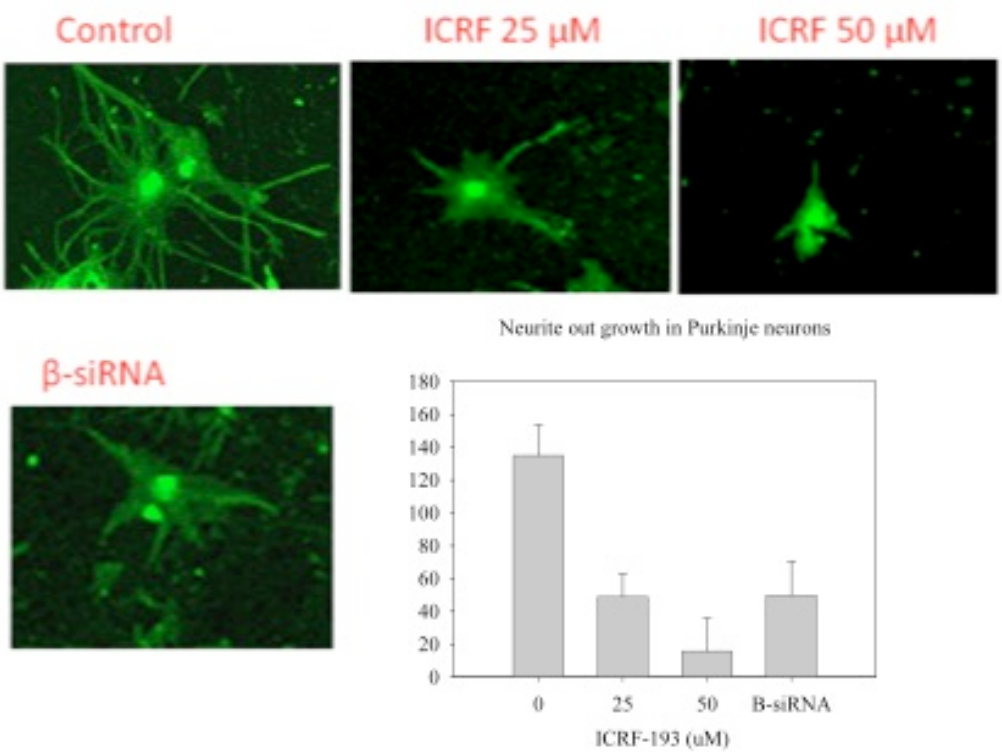
Cytotoxicity assay for ICRF-193 in neuronal cells: Purkinje neurons, granule neurons and cortical neurons were cultured in 96 well plate and treated with increasing concentrations of ICRF-193. Incubated for 16 hrs at 37^0C (5% CO2) and followed by MTT (5mg/ml) incubation for 4 hrs, reaction was stopped with acidic isopropanol (1N HCl in isopropanol) readings were taken at 570nm using ELISA reader.

Figure 3.15



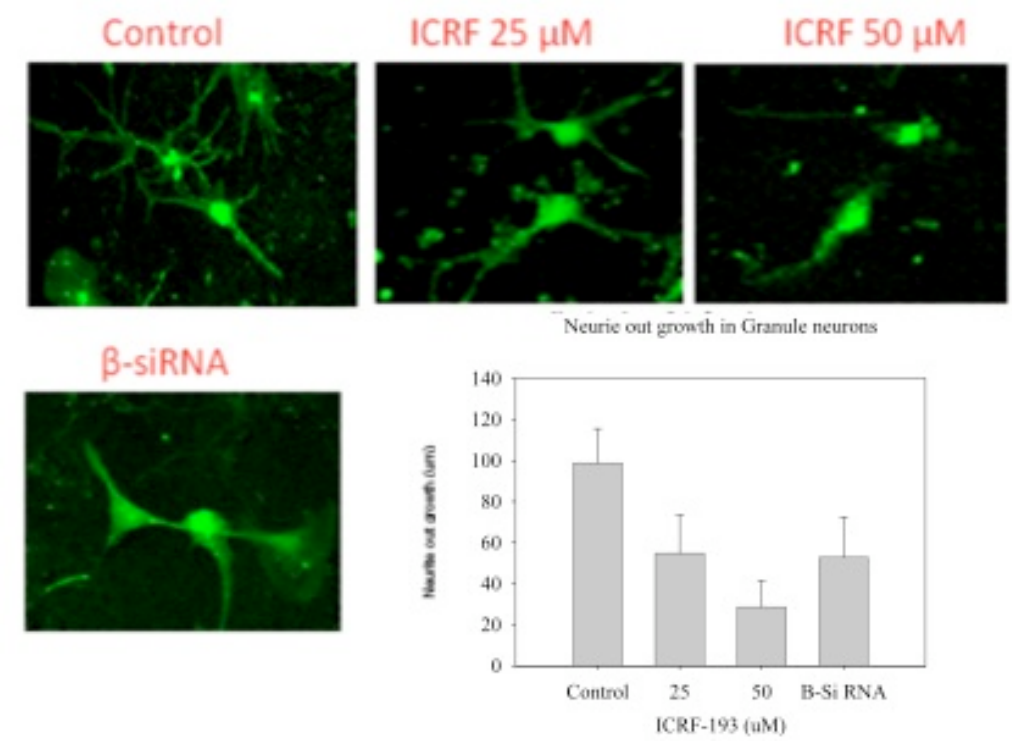
Inhibition of TopoIIβ expression by ICRF-193: Neuronal cells were treated with increasing concentration of ICRF-193 for 12 hrs and cell extracts were prepared into the lysis buffer. 40µg protein was resolved on SDS-PAGE and transferred to NC membrane. Then probed with TopoIIβ specific monoclonal antibodies and detected with ECL reagent

Figure 3.16



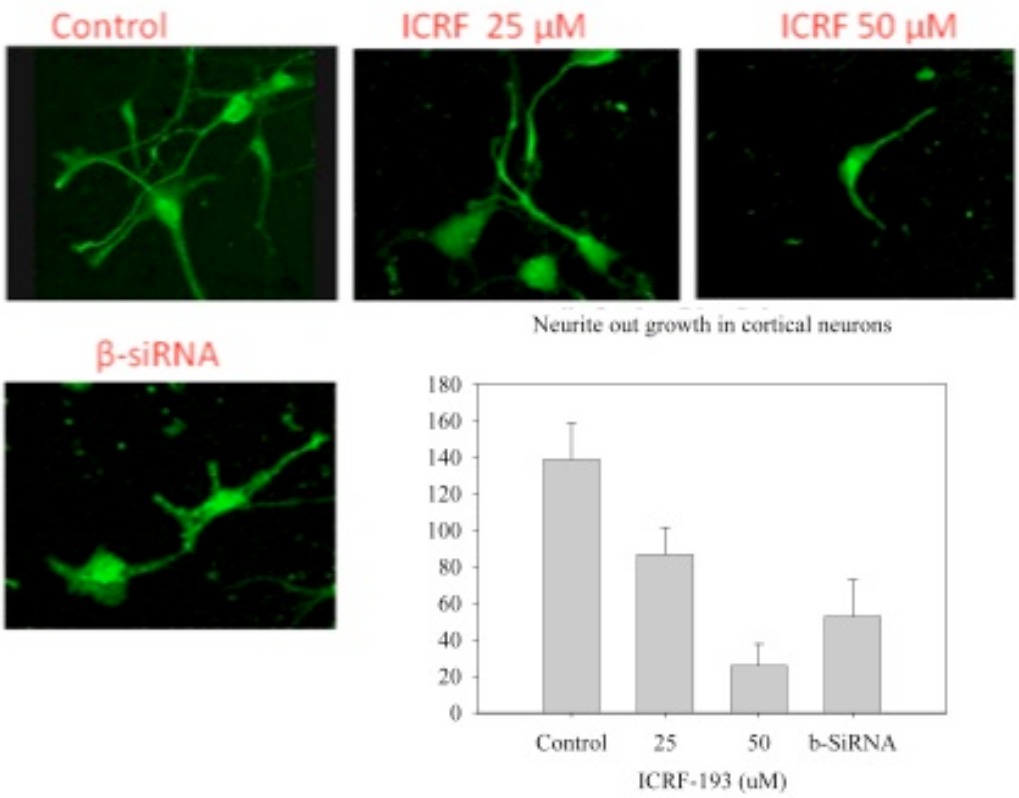
Inhibition of neurite out growth in TopoIIβ inhibited and down-regulated purkije neurons: Purkinje neurons were isolated from 18 days embryonic rat cerebellum and cultured in specific medium. After 2h neurons were transfected with TopoIIβ specific siRNA. Neurite out growth was measured after 48 hrs using NIH Image J software. For ICRF-193 neurons were treated for 12hrs with 25µM and 50µM ICRF-193, then cultured in fresh medium for 48hrs and neurite out growth was measured after 48 hrs. Neurite length was plotted on y-axis in µm

Figure 3.17



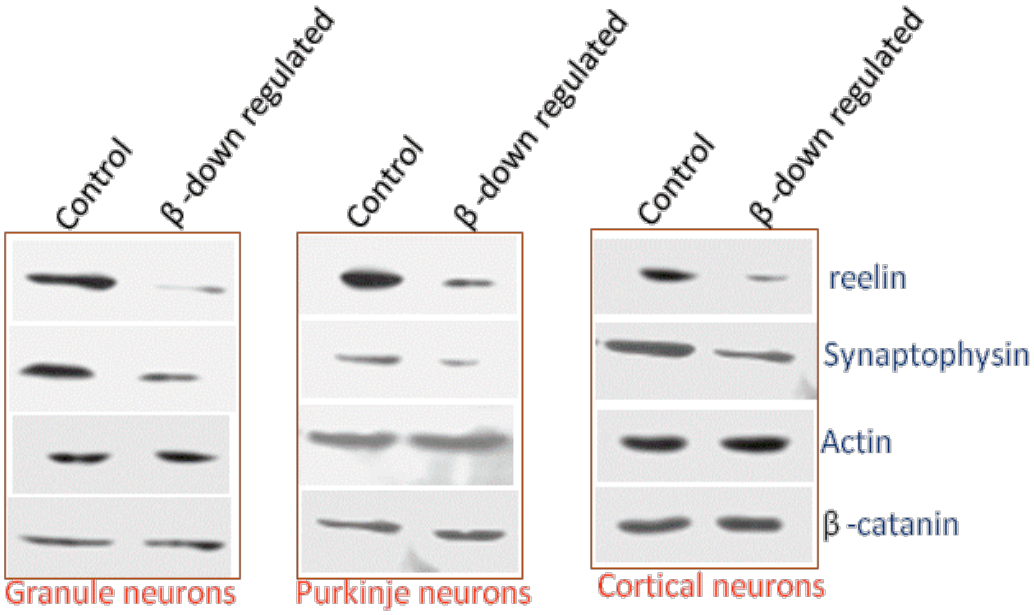
Inhibition of neurite out growth in TopoIIβ inhibited and down-regulated granule neurons: granule neurons were isolated from 8 day old rat pups cerebellum and cultured in specific medium. After 2h neurons were transfected with TopoIIβ specific siRNA. Neurite out growth was measured after 48 hrs using NIH Image J software. For ICRF-193 neurons were treated for 12hrs with 25µM and 50µM ICRF-193, then cultured in fresh medium for 48hrs and neurite out growth was measured after 48 hrs. Neurite length was plotted on y-axis in µm.

Figure 3.18



Inhibition of neurite out growth in TopoIIβ inhibited and down-regulated cortical neurons: cortical neurons were isolated from 18 days embryonic rat cerebral cortex and cultured in specific medium. After 2h neurons were transfected with TopoIIβ specific siRNA. Neurite out growth was measured after 48 hrs using NIH Image J software. For ICRF-193 neurons were treated for 12hrs with 25µM and 50µM ICRF-193, then cultured in fresh medium for 48hrs and neurite out growth was measured after 48 hrs. Neurite length was plotted on y-axis in µm

Figure 3.19



Expression profile of neuronal genes in TopoIIβ down regulated neurons: Neuronal cells were transfected with TopoIIβ specific siRNA and incubated for 36 hrs and cell extracts were prepared in the lysis buffer. 40µg protein was resolved on SDS-PAGE and transferred to NC membrane. Then probed with indicated protein specific monoclonal antibodies and detected with ECL reagent

Discussion

Development of neuronal cells is a complex process that requires transient expression of various genes responsible for neuronal development and differentiation (Cheung and Briscoe, 2003; Ferhat *et al.,* 1996; Shewan *et al.,* 1996) and formation of functional contacts with effector neurons/cells (Marangos and Schmechel, 1987; Nishikawa *et al.,* 1999; Vannucchi and Faussone-Pellegrini, 2000). Neurite outgrowth and axon guidance are critical neuronal differentiation events in the development of neuronal networks. Outgrowth and guidance are both directed by the movement of growth cones (Keynes and Cook, 1995a,b; Tessier-Lavigne and Goodman, 1996). For example, growth cones can sense external stimuli and regulate the rearrangement of the actin cytoskeleton. Such reorganization of the cytoskeleton can lead to the extension of axons toward chemoattractants, their retraction from chemorepellents (Tanaka and Sabry, 1995), or the extension of longer or shorter neuritis (scalar, neurite outgrowth events). Examples of external stimuli that regulate neurite outgrowth and guidance include extracellular matrix molecules and fragments derived from extracellular matrix molecules (Meiners and Mercado, 2003). Convincing evidence is now available suggesting that the Rho family GTPases (Rho, Rac, and Cdc42) play crucial roles in integrating signals generated from extracellular matrix and reorganization of the actin cytoskeleton in neurons, thereby regulating the morphology of neurites and growth cones (Kranenburg *et al.,* 1999; Lundquist, 2003; Luo *et al.,* 1997). It is believed that a dynamic change in neuronal network requires strict control of gene expression involved in these processes

The results of TopoIIα and β in brain and different cell types shows that, the activity of TopoIIα is involved during early stages of development, where cells undergoing rapid cell division, then its level drastically decreased in differentiated brain tissue, it suggests that TopoIIα is associated with cell. The higher levels of TopoIIβ during development and post-natal stages suggest that β isoform is essential for neuronal development and maturation. Further we have analyzed the TopoIIβ activity in neurite out growth through down-regulation of TopoIIβ. Results show that TopoIIβ deficient and inhibited (ICRF-193) neurons failed to form axon out growth (60-80% inhibition in neurite out growth) and failed to differentiate

completely. Previous studies have been indicated that TopoIIβ is required for growth cone formation and neuronal development (Kamal *et al.,* 2007 and Lyu *et al.,* 2006). In the present studies the observed residual neurite outgrowth (20% to 30%) could be due to low expression of TopoIIβ. It has been shown that neurite outgrowth is stimulated by various factors including NGF, BDNF, laminin, fibronectin, tenascin-C, and FGF-2 (Chiquet and Wehrle-Haller, 1994; Deumens *et al.,* 2006; Edgar, 1985; Grothe *et al.,* 2006)

The effect of the inhibition of translation and transcription on neuronal maturation including neurite outgrowth has remained controversial. Several reports have indicated that inhibition of translation and transcription stimulates neurite outgrowth (Burstein and Greene, 1978; Louis *et al.,* 1994). In contrast to these observations, there is strong evidence demonstrating that inhibition of translation and transcription inhibits neurite outgrowth (Kang and Schuman, 1996; Schacher and Wu, 2002). In fact, a cDNA macroarray analysis of rat CGNs treated with IRCF-193 has suggested that TopoIIβ is required for the expression of about one third of the genes that are induced during differentiation, but not overall gene expression (Tsutsui *et al.,* 2001b). Studies in TopoIIβ knockout (cerebral cortical) neurons have indeed showed that the expression levels of certain neuronal genes (i.e. Robo1, catenin alpha 2, cadherin 8) are down-regulated. However, the expression levels of actin and cadherin-13 are not significantly affected (Kamal *et al.,* 2007). Consistent with these studies, transcription profiling and chromatin immunoprecipitation studies have suggested that TopoIIβ is specifically involved in the transcription of many neuronal genes such as those involved in cell migration/ cell adhesion/axon guidance, but not general house keeping genes (Lyu *et al.,* 2006). Indeed, a role of TopoIIβ in hormone induced gene expression has been demonstrated in a recent study (Ju *et al.,* 2006). Our analysis of some of the proteins which are critical for neuronal migration and neurite out growth shows that the levels of some proteins decreased in TopoIIβ down regulated neurons. Reeline and synaptophysine levels were decreased in TopoIIβ down regulated cells but there is no change in β-catenin and β-actin levels. Reelin is crucial for regulating the processes of neuronal migration and positioning in the developing brain (Weeber et al.2002, Arcangelo 2005). It stimulates dendrite development and

regulates the migration of neuroblasts generated during adult neurogenesis such as sub-ventricular and sub-granular zones (Niu *et al.,* 2004). Synaptophysine a major integral transmembrane protein of synaptic vesicles. This membrane protein is specific for the synaptic vesicles in the central and peripheral nervous system and responsible for synapsis development and neurotransmission (Sun *et al.,* 2006 and Kamal *et al.,* 2007)

In summary TopoIIβ is essential for neuronal development and differentiation. It regulates the growth cone formation and axon development through neurite out growth by regulating expression of genes, which are involved in neuronal differentiation.

CHAPTER 4

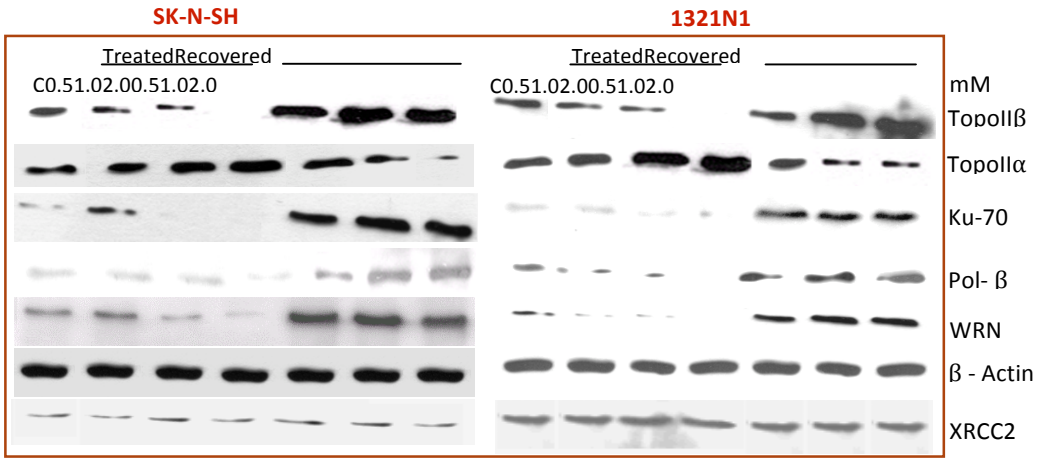
Investigation of the role of Topoisomerase II α and β in peroxide-induced DNA damage and repair in neurons

Human Topoisomerase II present in two isoforms, 170 kDa TopoIIα and 180 kDa TopoIIβ, catalytically show similar activity, but their distinct functional activity is not yet understood (Jenkins *et al.,* 1992, Drake *et al.,* 1989 and Sakaguchi *et al.,* 2004). While TopoIIα is found to be present in proliferating cells (Turley *et al.,* 1997), TopoIIb is present in all cell types, with a predominant localization in brain especially in neurons (Kondapi *et al.,* 2004, Chaly *et al.,* 1996, Tsutsui *et al.,* 2001 and Pierre *et al.,* 2002). The decreasing activity of TopoIIβ with aging suggests its possible role in DNA repair activity in neurons during aging (Kondapi *et al.,* 2004). The frequently occurring insults to the DNA in an aging brain are due to enhanced oxidative metabolism and insufficient antioxidant pool that causes reduction in the removal rate of the reactive oxidative intermediates (Lenaz *et al.,* 2000 and Lu *et al.,* 2005). Exposure of chromosomal DNA to reactive oxidative intermediates can cause double stand breaks damage to DNA. While higher repair competent cells can correct such damaged DNA, the repair deficient cells are prone to increased DNA damage leading to a wide spectrum of cellular dysfunctions (Sage *et al.,* 1996 and Waldstein *et al.,* 1982). Hydrogen peroxide is one such frequently released intermediates of oxidative metabolism; it induces double stand breaks (DSBs) in chromosomal DNA (Amatore *et al.,* 2001, Slupphaug *et al.,* 2003, Arbault *et al.,* 2004 and Tandara *et al.,* 2006). It has been reported that several DNA damaging agents and TopoII poisons can enhance the activity of DNA repair enzymes (Wang *et al.,* 2002). In absence of isoform-specificity of poison employed, the studies could not address the differential activities of alpha and β isoforms. Emmons *et al.,* (2006) studied activities of TopoIIα and β in repair of melphalan-induced cross links in the cellular DNA of K562 Leukemia and U937 histocytic lymphoma cells, the results could correlate TopoIIβ levels with the repair of cross links, but could not clearly address the associated repair pathways as well as the activity of alpha isoform in DNA damage and repair. In the present investigation, using H_2O_2 mediated DNA damage as a model; we studied the DSBs repair efficiency of neuroblastoma and astrocytoma and primary neuronal (Granule neurons) cells in the presence as well as absence of Topoisomerase II isoforms. The results clearly bring out the distinctly different functional activity pattern of these two Topoisomerase II isoforms.

Results

The activity of TopoIIα and β in DNA damage and DSBs repair was studied using Human neuroblastoma cell lines SK-N-SH, astrocytoma 1321N1 and Granule neurons were exposed for 24 hrs to increasing concentrations of H_2O_2. The resulting DNA damage was analyzed by single cell neutral comet assay using confocal microscope (Leica). The results show that H_2O_2 can synergistically damage the DNA (Figure 4.2A and B). The damaged DNA could be repaired when cells were recultured in fresh media for 72 hours. Western blot analysis showed (Fig 4.1) enhanced levels of TopoIIα with increasing DNA damage, while the TopoIIβ levels remained unaltered (Fig 4.1). The levels of TopoIIα were found down regulated to negligible, while the levels of TopoIIβ were enhanced along with Ku70, WRN helicase and pol-β, during recovery. XRCC2 did not show any expression in this pathway (Fig 4.1). Double strandbreaks quantification by neutral comet assay shows that H_2O_2 can synergistically damage the DNA in SH-N-SH and 1321N1 cells (Fig 4.2A and 4.2B). The results thus point out to a distinct role of TopoIIα in promoting H_2O_2 mediated DNA damage and to an active involvement of TopoIIβ in DNA DSBs repair activity, in Ku70-mediated Non Homologous End-Joining (NHEJ) repair pathway.

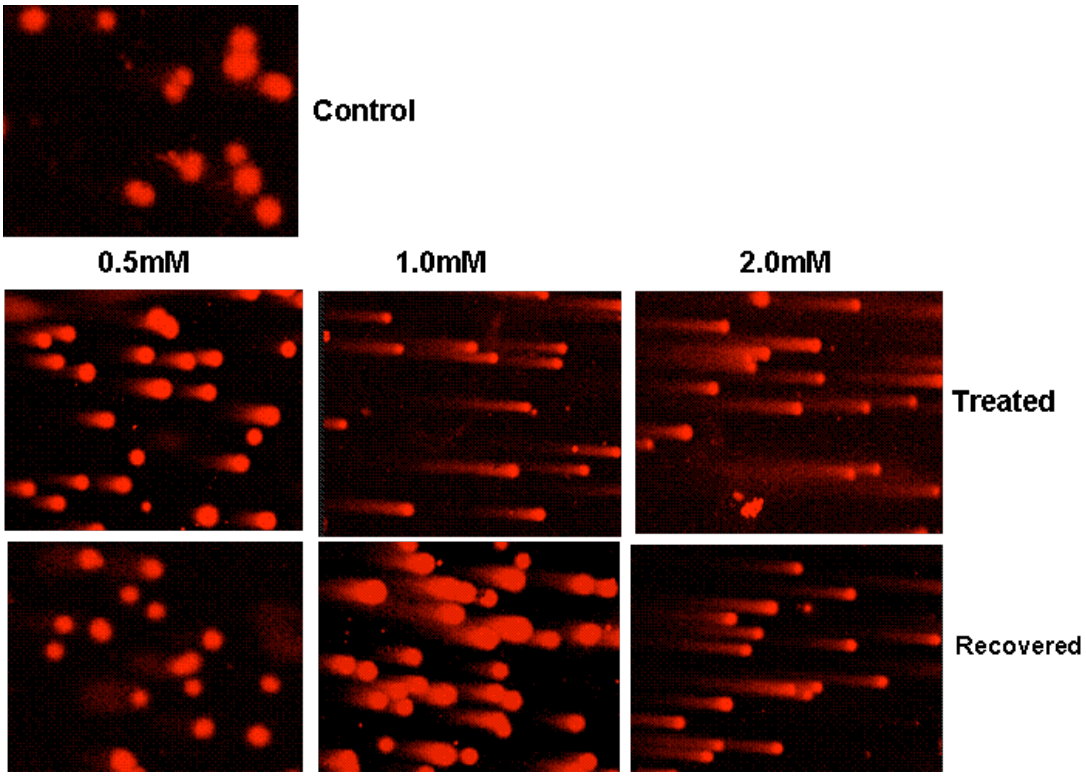
Figure 4.1



Panel A: The expression profile of TopoIIα, TopoIIβ and repair proteins during H_2O_2 mediated DNA damage and repair: The SK-NSH and 1321N1 cells were incubated in the presence of indicated concentrations of H_2O_2 for 24 hours (Treatment). After 24 hours the cells were washed with fresh medium and recultured in complete medium for 72 hours. The proteins in whole cell extract (100μg) analyzed by Western blot analysis and probed with corresponding antibodies. The control lane (C) is in absence of H_2O_2 treatment (n = 3).

Figure 4.2A

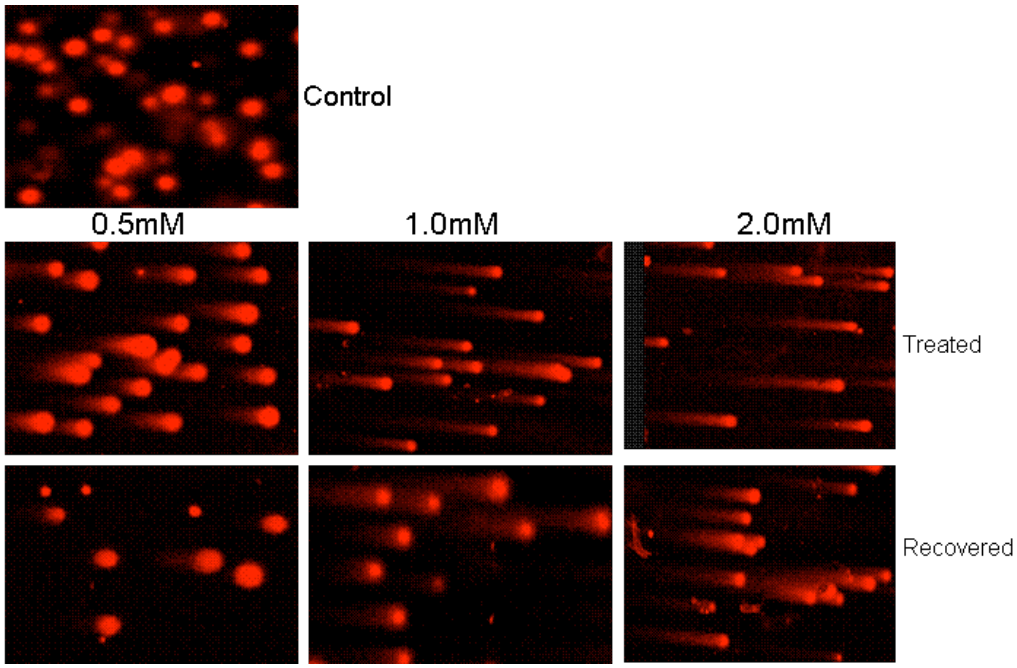
SK-N-SH



Quantification of DNA double strand breaks by neutral comet assay: SK-N-SH cells were treated with indicated concentrations of peroxide. Double strand breaks were quantified in treated (24h) and recovered (72h) cells using neutral comet assay (n = 3).

Figure 4.2B

1321N1



Quantification of DNA double strand breaks by neutral comet assay: 1321N1 cells were treated with indicated concentrations of peroxide. Double strand breaks were quantified in treated (24h) and recovered (72h) cells using neutral comet assay (n = 3).

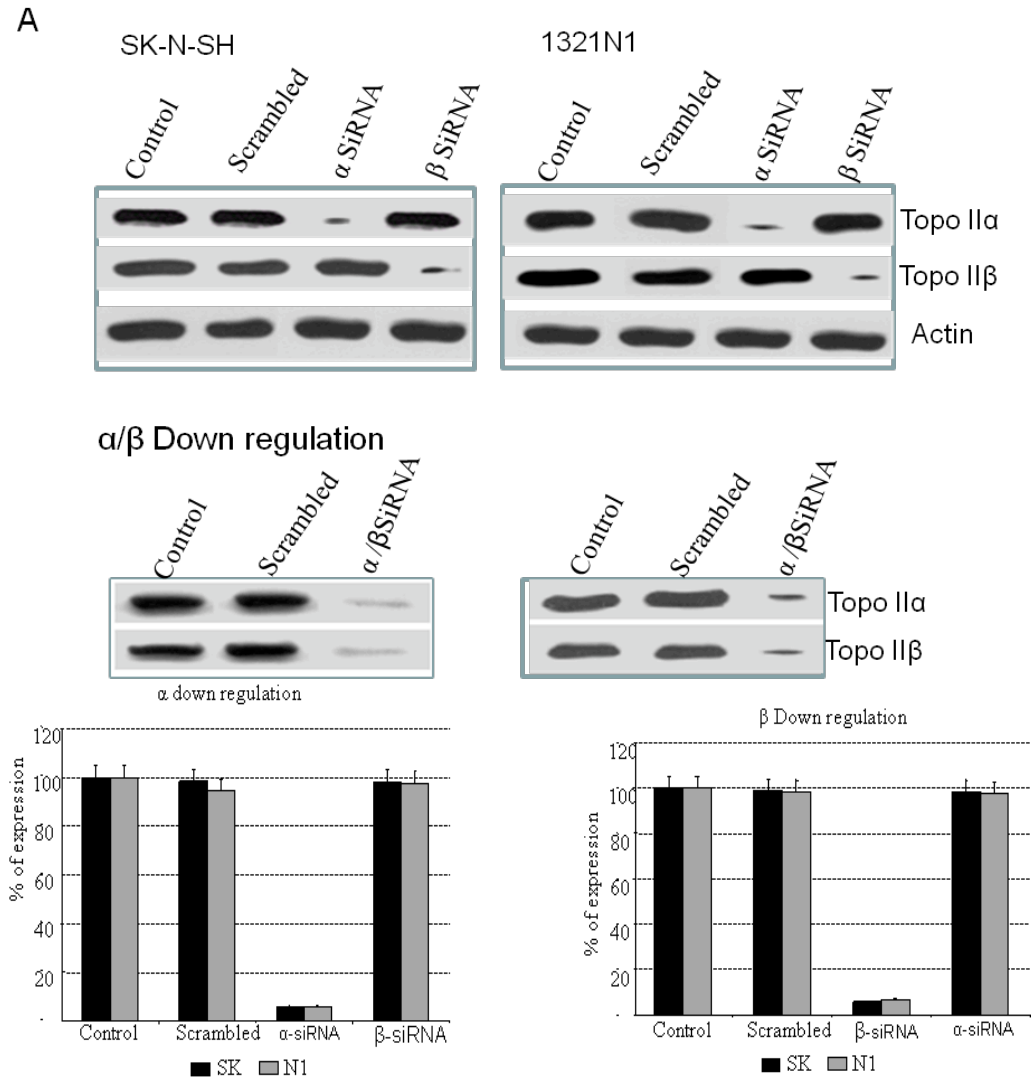
Down regulation of TopoIIα and β

To confirm the role of TopoIIα and TopoIIβ, further investigations were carried out through siRNA-mediated knock down experiments involving down regulation of TopoIIα and TopoIIβ isoforms. The siRNA sequences for TopoIIα and TopoIIβ were designed as per standard protocols. The cells were transfected with TopoIIα and TopoIIβ specific siRNA (Table 1) and the protein levels were analyzed using Western blots. The results showed an efficient down regulation of these isoforms in both SK-N-SH and 1321N1 cell lines. Scrambled sequences were used as negative controls (Fig: 4.3).

Table 1: TopoII siRNA templates for *In-vitro* transcription

Name	siRNA sense template sequence	siRNA anti-sense template sequence
α scramble	5`ACCTCGACTGAG CAATATGTT3`	5`CATATTGCTCA GTCGAGGTTT3`
α siRNA	5`ACTGAATAATCA GGCTCGCTT3`	5`GCGAGCCTGAT TATTCAGTTT3`
β scramble	5`ACACTCGATCAAT CCAGTGTT3`	5`CACTGGATTGA TCGAGTGTTT3`
β siRNA	5`GCTTAACAATCA AGCCCGTTT3`	5`ACGGGCTTGAT TGTTAAGCTT3`
T7 promoter	5`TAATACGACTCACTATAG3`	5`ATTATGCTGAG TGATATC3`

Figure 4.3



siRNA mediated down regulation of TopoIIα and β in SK-N-SH and 1321N1 cells: TopoIIα and β specific siRNA (Table1) was synthesized by *in-vitro* transfection and was transfected into the cells at 0.5μm/1x10^6 cells. The cells were incubated for 48 hours. The down regulation of TopoIIα and β was analyzed by western blot analysis. Experiments were repeated three times and corresponding densitometry error bars were showed (n = 3).

TopoIIα accelerated the formation of DSBs, while TopoIIβ promotes DSBs repair

The functional role of TopoIIα and TopoIIβ in H_2O_2 mediated DNA damage and DSBs repair was monitored by challenging TopoIIα and TopoIIβ deficient cells with H_2O_2 and the extent of double strand breaks were quantified by single cell neutral comet assay. The results showed that the TopoIIa$^-$TopoIIb$^+$ cells (Fig.4.4A,4.4B and Fig 4.5A and 4.5B) were resistant to H_2O_2 mediated DNA damage, while TopoIIa$^+$TopoIIb$^-$ cells were more than two fold sensitive to the H_2O_2 mediated DNA damage suggesting that TopoIIβ present in TopoIIα deficient cells could protect them from H_2O_2 mediated DNA damage. TopoIIa$^+$TopoIIb$^+$ (normal cells) and TopoIIa$^-$TopoIIb$^-$ cells show similar sensitivity to H_2O_2. Alternatively while α isoform accelerates the damage of DNA, a two fold increase in sensitivity of TopoIIa$^+$TopoIIb$^-$ cells to H_2O_2 indeed demonstrated the important role of TopoIIβ in promoting the DSBs repair of damaged DNA. In the absence of β isoform, H_2O_2 mediated over expression of TopoIIα accelerates DNA damage (Fig.4.4A, 4.4B and Fig 4.5A and 4.5B). Further, knocking down of both TopoIIα and TopoIIβ did not effect the H_2O_2 mediated DNA damage suggesting that the resistance of TopoIIα deficient cells to H_2O_2 is either due to constitutively expressed TopoIIβ and / or due to repair-signaling associated with the up-regulation of TopoIIβ. These results clearly demonstrate the distinct functions of these two isoforms, the TopoIIα being the accelerator of DNA damage and TopoIIβ, the promoter of the repair of damaged DNA.

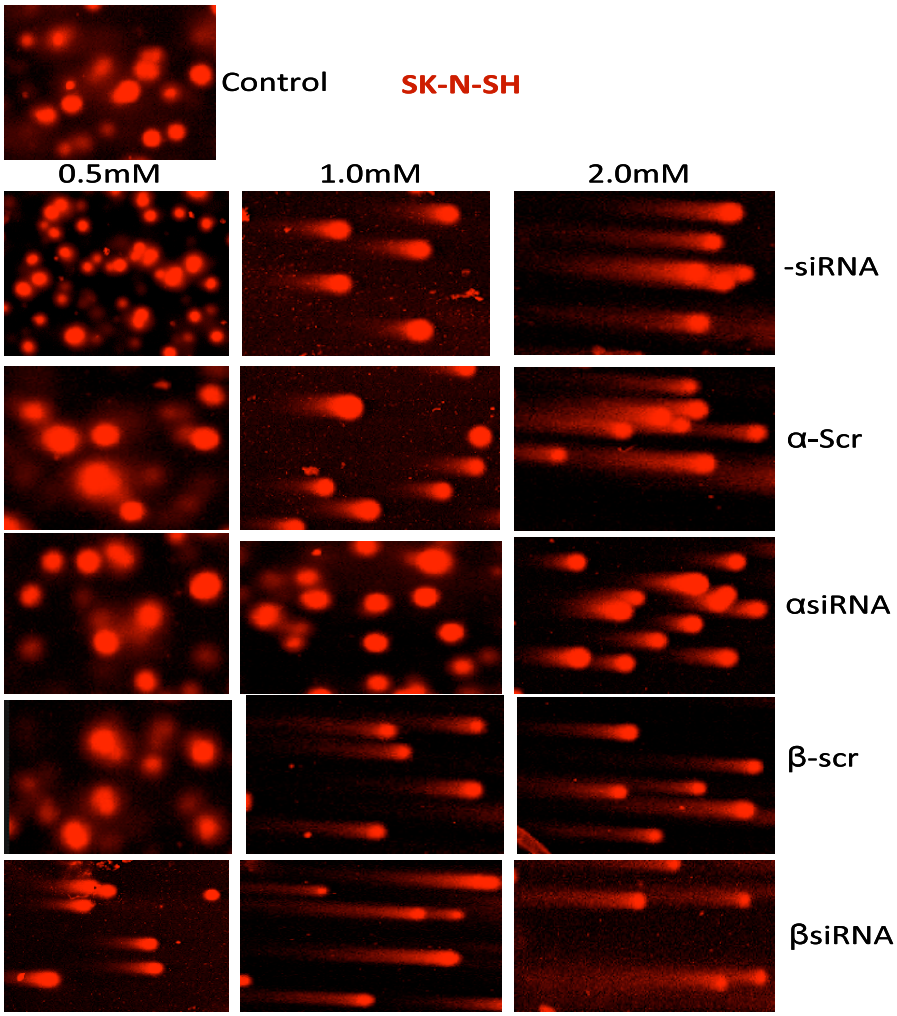
TopoIIβ deficient cells are very sensitive to peroxide mediated apoptotic cell death

These results are further confirmed by analysis of survival of TopoIIα$^+$TopoIIβ$^+$, TopoIIα$^-$TopoIIβ$^-$, TopoIIα$^+$TopoIIβ$^-$ and TopoIIα$^-$TopoIIβ$^+$. The results of these experiments clearly demonstrate that the TopoIIα deficient cells can survive from the insults of reactive oxidative intermediates, while TopoIIβ deficient cells show more sensitivity to this damage (Fig 4.7). The results showed that TopoIIα$^-$ TopoIIβ$^+$ cells are resistant to H_2O_2 induced oxidative DNA damage compared to

TopoIIα^{+}TopoIIβ^{-} and TopoIIα^{+}TopoIIβ^{+} cells confirming the repairing potential of TopoIIβ and the damaging function of TopoIIα.

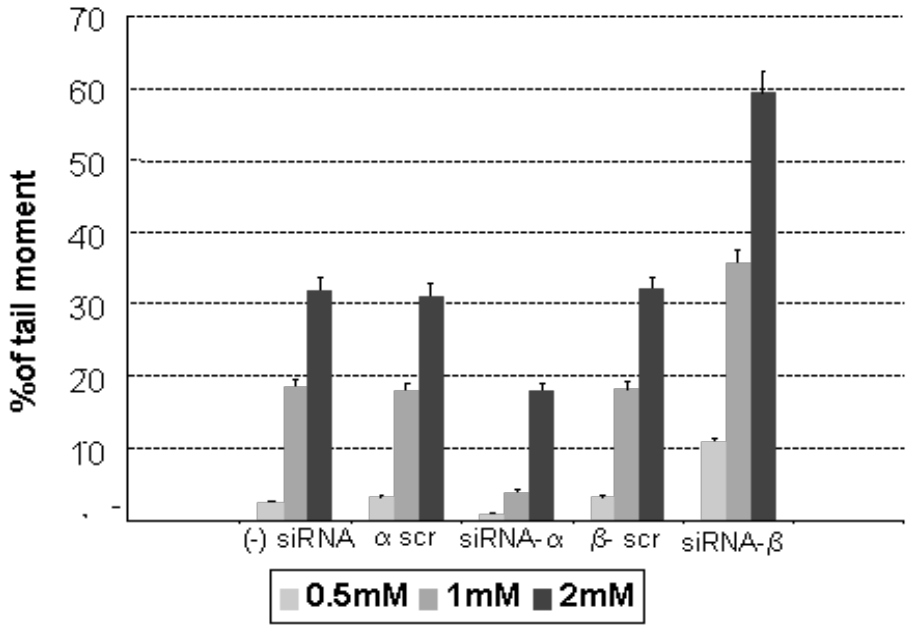
Further more, large proportion of TopoIIα^{+}TopoIIβ^{-} cells were annexin-V positive (Fig 4.6) suggesting that they undergo a rapid apoptotic cell death in the presence of H_2O_2, thus pointing out to an important cell survival function of TopoIIβ in promoting DNA repair and promoting cellular recovery form H_2O_2 mediated insult. The absence of TopoIIα coupled with the presence of TopoIIβ can help cells to become repair efficient and hence show resistance to H_2O_2 mediated DNA damage due to the dominance of DNA repair process. Furthermore, the endogenous expression of TopoIIβ is thus essential for cell survival, in promoting DNA repair process against insults from oxidative reactive intermediates.

Figure 4.4A



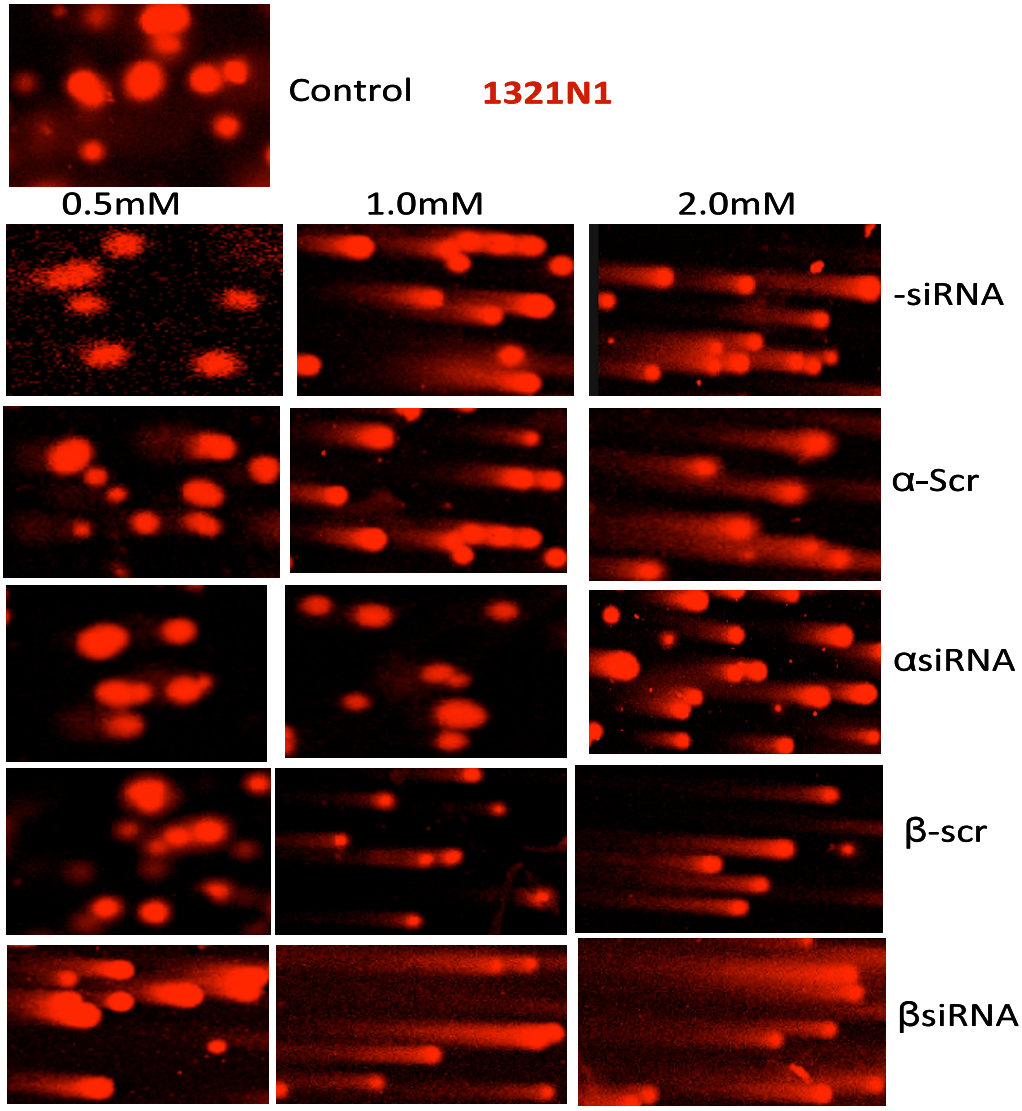
TopoIIα and β down regulation drastically effect the double strand breaks formation: The TopoIIα and β down regulated SK-N-SH cells were incubated with indicated concentrations of H_2O_2 for 24 hours and followed 72 hours recovery. The extent of DNA damage and recovery was analyzed by single cell neutral comet assay. (Scr-Scrambled)

Figure 4.4B



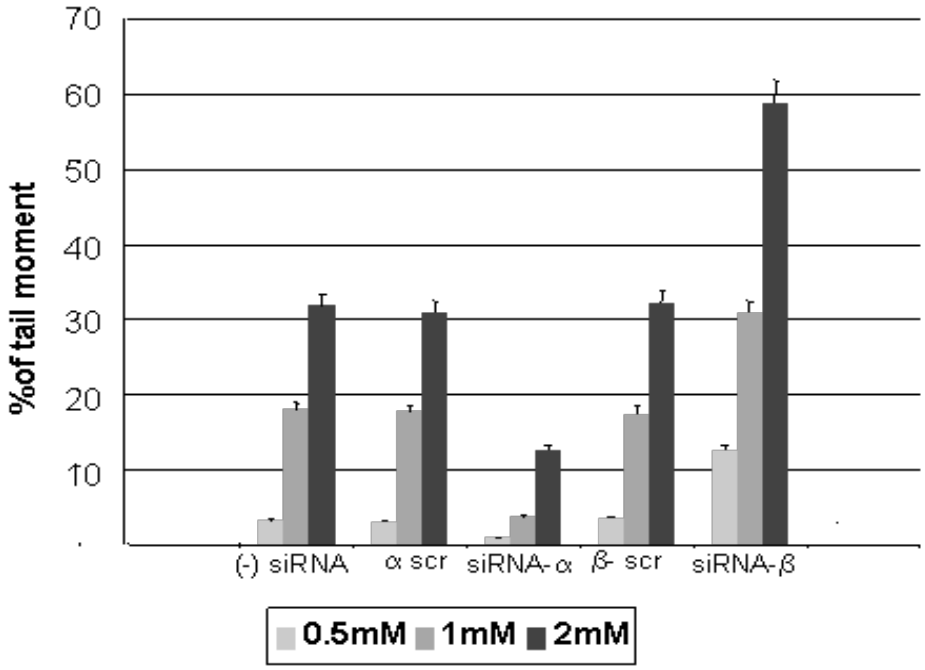
TopoIIα and β down regulation drastically effect the double strand breaks formation: The TopoIIα and β down regulated SK-N-SH cells were incubated with indicated concentrations of H_2O_2 for 24 hours and followed 72 hours recovery. The extent of DNA damage and recovery was analyzed by single cell neutral comet assay (Fig 3A). The percentage of tail length was measured by using Comet-IV software (Perceptive Instruments UK) and the extent of DNA damage was plotted on y-axis (Fig 3B). Experiments were repeated three times and mean ± standard error was plotted ($p < 0.05$, $n = 3$).

Figure 4.5A



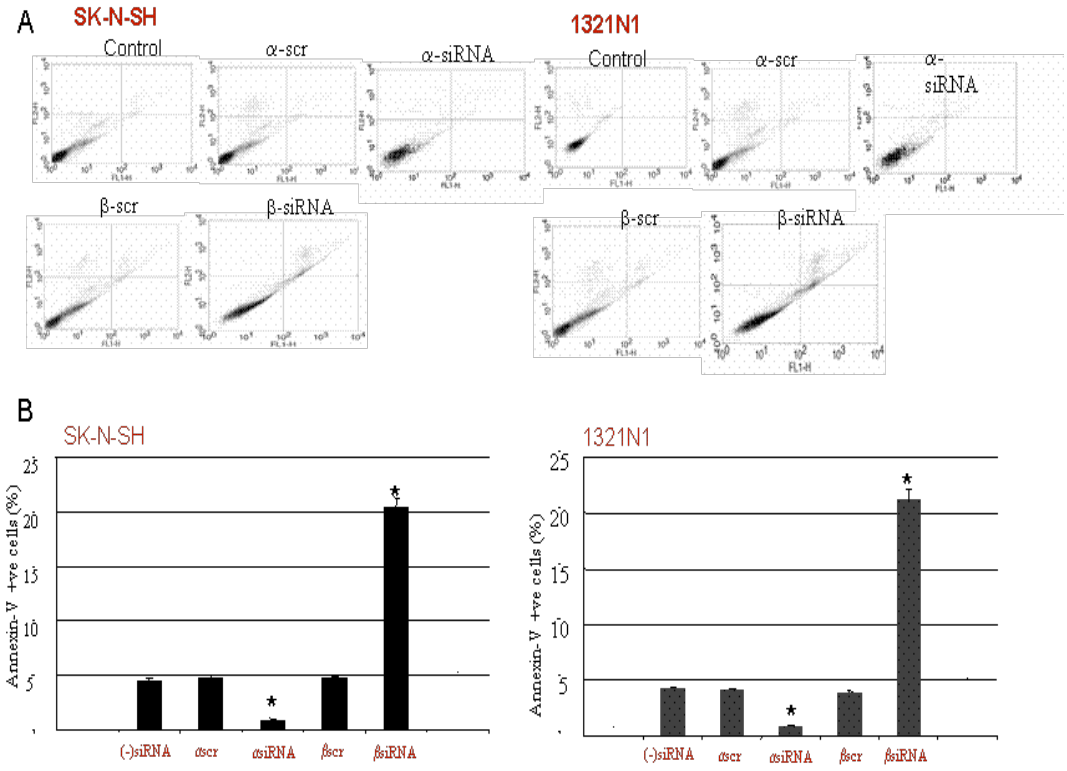
TopoIIα and β down regulation drastically effect the double strand breaks formation: The TopoIIα and β down regulated 1321N1 cells were incubated with indicated concentrations of H_2O_2 for 24 hours and followed 72 hours recovery. The extent of DNA damage and recovery was analyzed by single cell neutral comet assay.

Figure 4.5B



TopoIIα and β down regulation drastically effect the double strand breaks formation: The TopoIIα and β down regulated 1321N1 cells were incubated with indicated concentrations of H_2O_2 for 24 hours and followed 72 hours recovery. The extent of DNA damage and recovery was analyzed by single cell neutral comet assay. The percentage of tail length was measured by using Comet-IV software (Perceptive Instruments UK) and the extent of DNA damage was plotted on y-axis. Experiments were repeated three times and mean ± standard error was plotted ($p < 0.05$, $n = 3$).

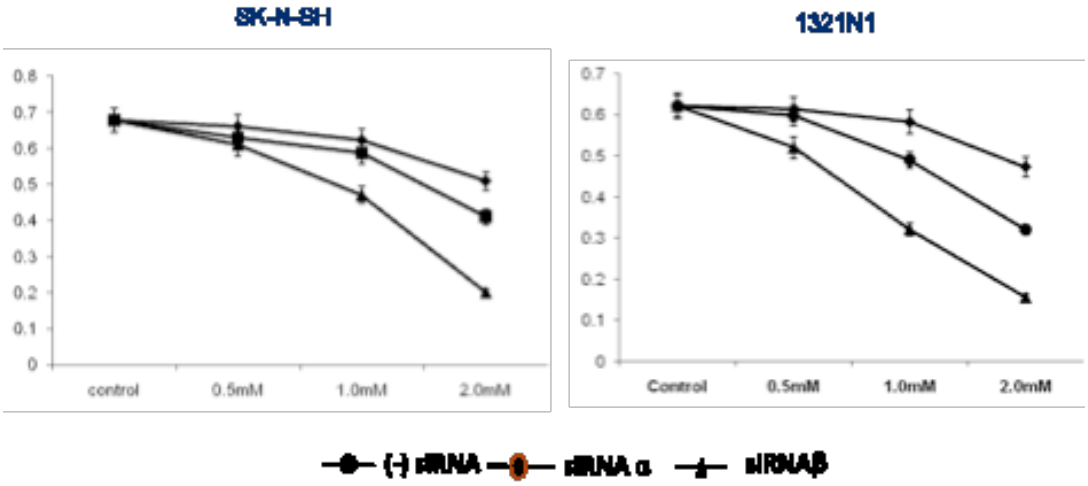
Figure 4.6



Apoptosis in TopoIIα and β down regulated cells during H_2O_2 treatment

Panel A: TopoIIα and β down regulated SK-N-SH and 1321 N1 cells were incubated with H_2O_2 for 24 hours and recultured for 72 hours in peroxide free medium. The cell death in TopoIIα and β down regulated SK-N-SH and 1321N1 cells was monitored with Annexin-V (FITC conjugated, Invitrogen) by Flow cytometry (BD FACS). Percent of Annexin-V positive cells were represented on Y-axis **(Panel B)** ($p < 0.01$, $n = 3$).

Figure 4.7



Analysis of cell survival in TopoIIα and β down regulated cells during H_2O_2 treatment: Panel A: TopoIIα and β down regulated SK-N-SH and 1321 N1 cells were incubated with H_2O_2 for 24 hours and cell survival was analyzed by MTT assay . Viable cells (in OD) were represented on Y-axis and the concentration of H_2O_2 on X-axis. Experiments were repeated three times and mean ± standard error was plotted ($p< 0.05$, $n = 3$).

Confirmation of TopoIIβ role in Double strand breaks repair through *In-vitro* Non-homologous end joining assay (NHEJ)

To conform the role of TopoIIβ in DSB`s repair we have conducted *in-vitro*-non homologous end joining assay with EcoRI digested pUC19 plasmid DNA using TopoIIα and β down-regulated cells nuclear extract and non-down regulated nuclear extracts. The results of this NHEJ experiment clearly demonstrated that TopoIIβ deficient nuclear extracts failed to form dimmers, trimers and multimers in NHEJ assay (Fig 4.8). Which indicates that TopoIIβ is required for the repair of double strand breaks through Non-homologous end joining path way. While TopoIIα deficient nuclear extracts showing equal NHEJ activity like TopoII isoforms non-down regulated nuclear extracts. It confirm that TopoIIβ playing crucial role in double strand breaks repair through non-homologous end joining path ways along with Ku-70 ang WRN helicase, but TopoIIα doesn't have any role in non-homologous end joining path way to repair DNA double strand breaks.

TopoIIβ required for DSB`s repair in neurons

DNA strand breaks accumulation in brain leads to the neurodegenerative diseases. Oxidative intermediates produced during general cell metabolism leads to formation of DNA single and double strand breaks. Here we have studied the role of TopoIIβ in peroxide induced DNA double strand breaks repair in Granule neurons (from rat cerebellum). Neurons were exposed for 24 hrs to increasing concentrations of H_2O_2. The resulting DNA damage was analyzed by single cell neutral comet assay using confocal microscope (Leica). The results show that H_2O_2 can synergistically damage the DNA (Figure 4.10). The damaged DNA could be repaired when cells were recultured in fresh media for 72 hours. Western blot analysis showed enhanced levels of TopoIIβ along with Ku70, WRN helicase and pol-β (Fig. 4.9) during recovery, while TopoIIα show negligible expression during DNA damage and recovery. Double strand breaks quantification by neutral comet assay shows that H_2O_2 can synergistically damage the DNA in granule neurons (Fig 4.10).The results suggesting that TopoIIα not playing any role in primary granule neurons either in DNA damage or repair, while TopoIIβ actively participates in

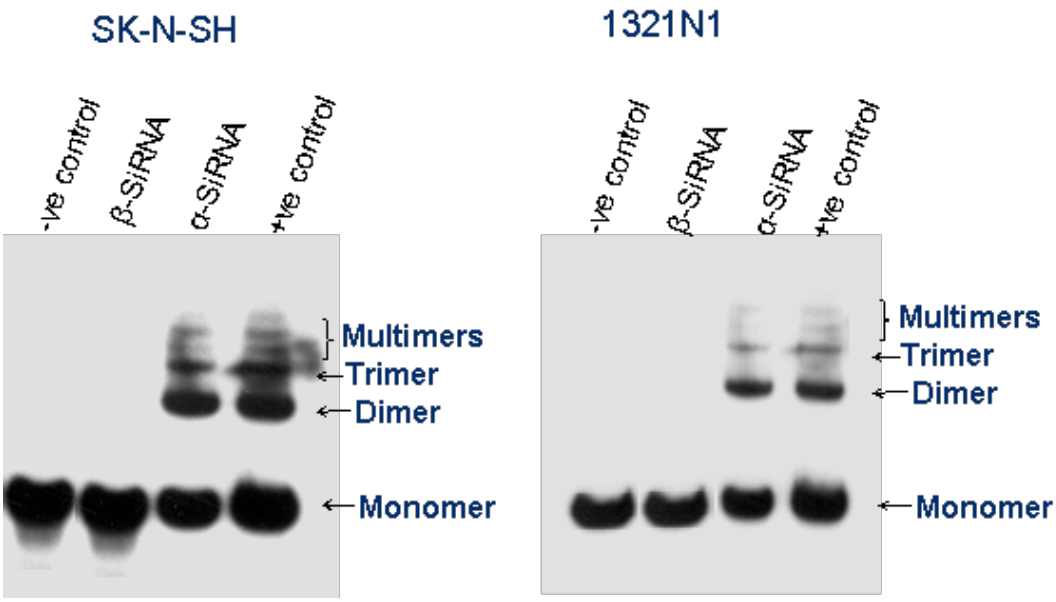
repair of DNA DSBs along with Ku70, WRN and pol-β through Non Homologous End-Joining (NHEJ) repair pathway.

Further we have analyzed the TopoIIβ activity in DSB`s repair in TopoIIβ down regulated neuronal cells. We have used *In-vitro* transcription method to synthesize siRNA and transfected by using lipofectamine to down regulate TopoIIβ in neuronal cells (As mentioned in materials and methods and Fig 3.10).

TopoIIβ down-regulated cells and non down-regulated cells were treated with peroxide and recultured for 72h in fresh medium. DNA double strand breaks were quantified by using neutral comet assay. The results shows that TopoIIβ down regulated cells more sensitive to the peroxide induced DNA damage when compared with normal cells. Its suggesting that TopoIIβ role in DSB`s repair (Fig 4.11A and 4.11B).

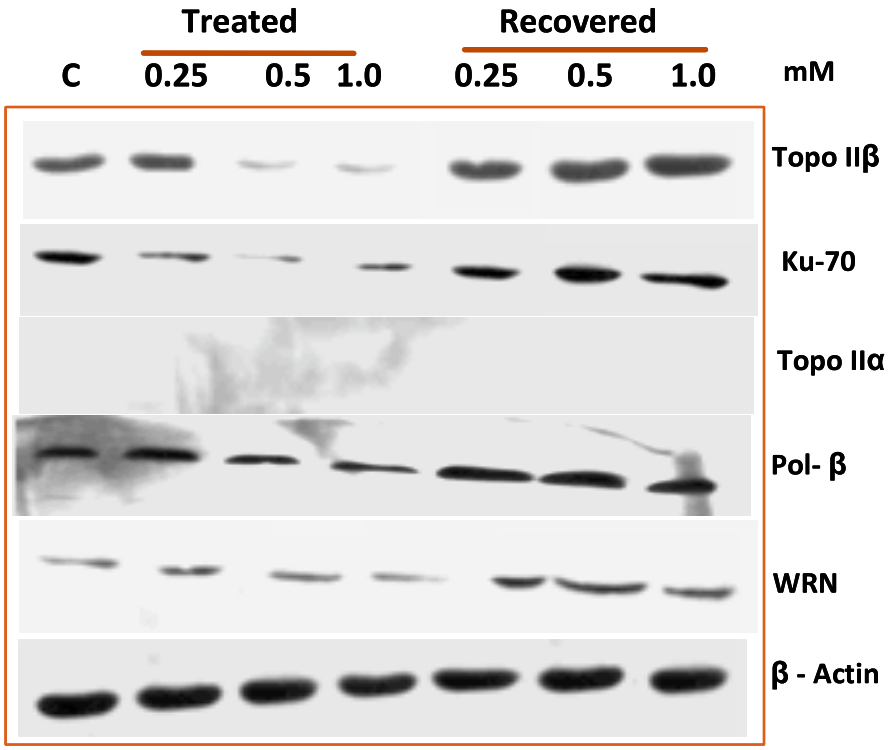
To conform the role of TopoIIβ in DSB`s repair we have conducted *in-vitro*-non homologous end joining assay with EcoRI digested pUC19 plasmid DNA using TopoIIα and β down-regulated cells nuclear extract and non-down regulated nuclear extracts. The results of this NHEJ experiment clearly demonstrated that TopoIIβ deficient nuclear extracts failed to form dimmers, trimers and multimers in NHEJ assay (Fig 4.12). Which indicates that TopoIIβ is required for the repair of double strand breaks through Non-homologous end joining path way. While TopoIIα deficient nuclear extracts showing equal NHEJ activity like TopoII isoforms non-down regulated nuclear extracts. It confirm that TopoIIβ playing crucial role in double strand breaks repair through non-homologous end joining path ways along with Ku-70 ang WRN helicase, but TopoIIα doesn't have any role in non-homologous end joining path way to repair DNA double strand breaks.

Figure 4.8



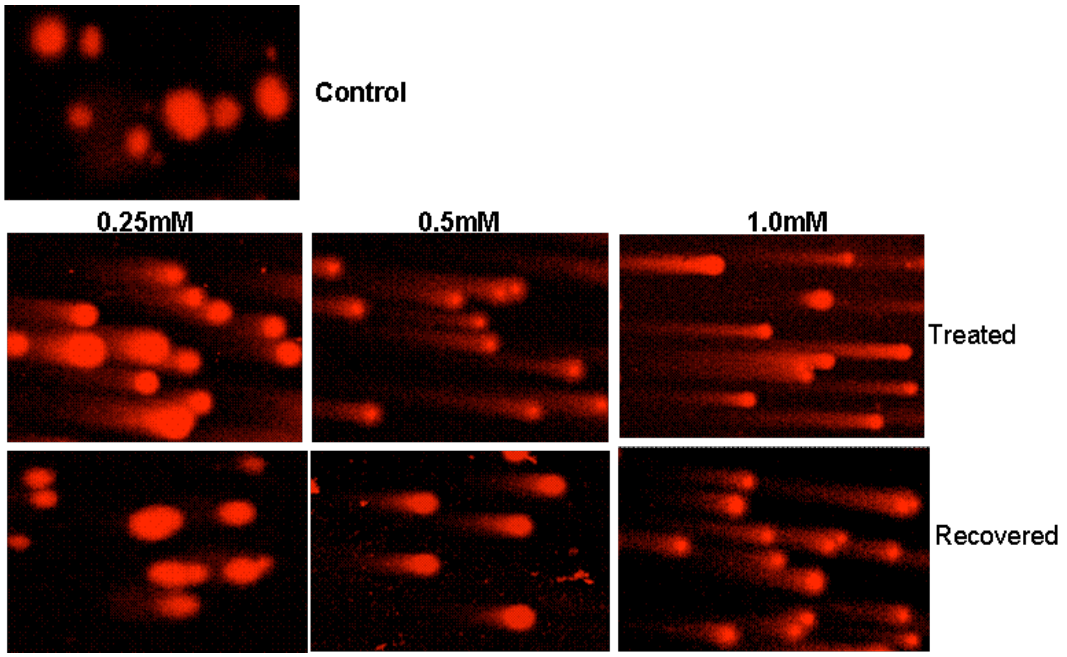
In-vitro **NHEJ assay to confirm TopoIIβ activity in DNA double strand breaks repair:** Nuclear extracts were prepared from Topo α and β down regulated and non down regulated SK-N-SH and 1321N1 cells.pUC19 plasmid DNA was digested with EcoRI and purified by phenol-chloroform method. Assay was conducted with 40µg of nuclear extract and 400ng of digested plasmid incubated for 2h at 25^0C. Products were resolved on 0.8% agarose gel and transferred on to nylon membrane and probed with randomly labeled (with α-p32 dCTP) linearized pUC19 plasmid DNA. Probed autorads were scanned by using Phosphorimager (Biorad).

Figure 4.9



The expression profile of TopoIIα, TopoIIβ and repair proteins during H_2O_2 mediated DNA damage and repair: Granule neurons were incubated in the presence of indicated concentrations of H_2O_2 for 24 hours (Treatment). After 24 hours the cells were washed with fresh medium and recultured in complete medium for 72 hours (Recovery). The proteins in whole cell extract (40μg) analyzed by Western blot analysis and probed with corresponding antibodies. The control lane (C) is in absence of H_2O_2 treatment.

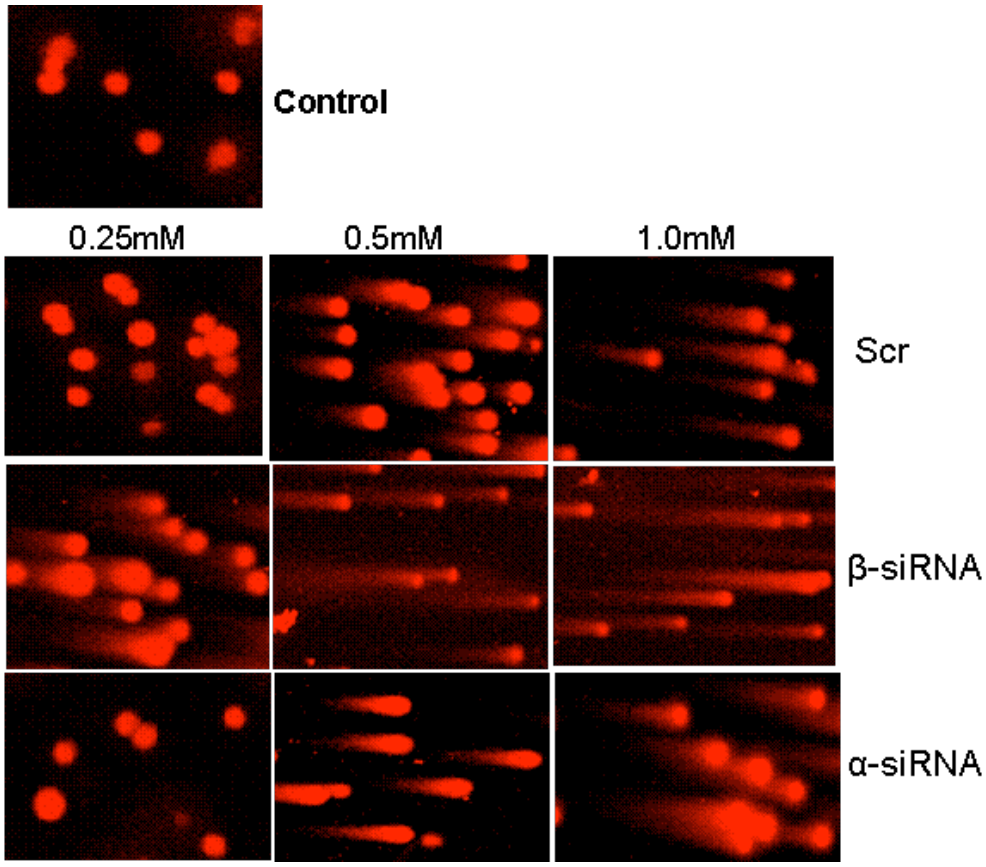
Figure 4.10



Quantification of H_2O_2 DNA double strand breaks in granule neurons by neutral comet assay:

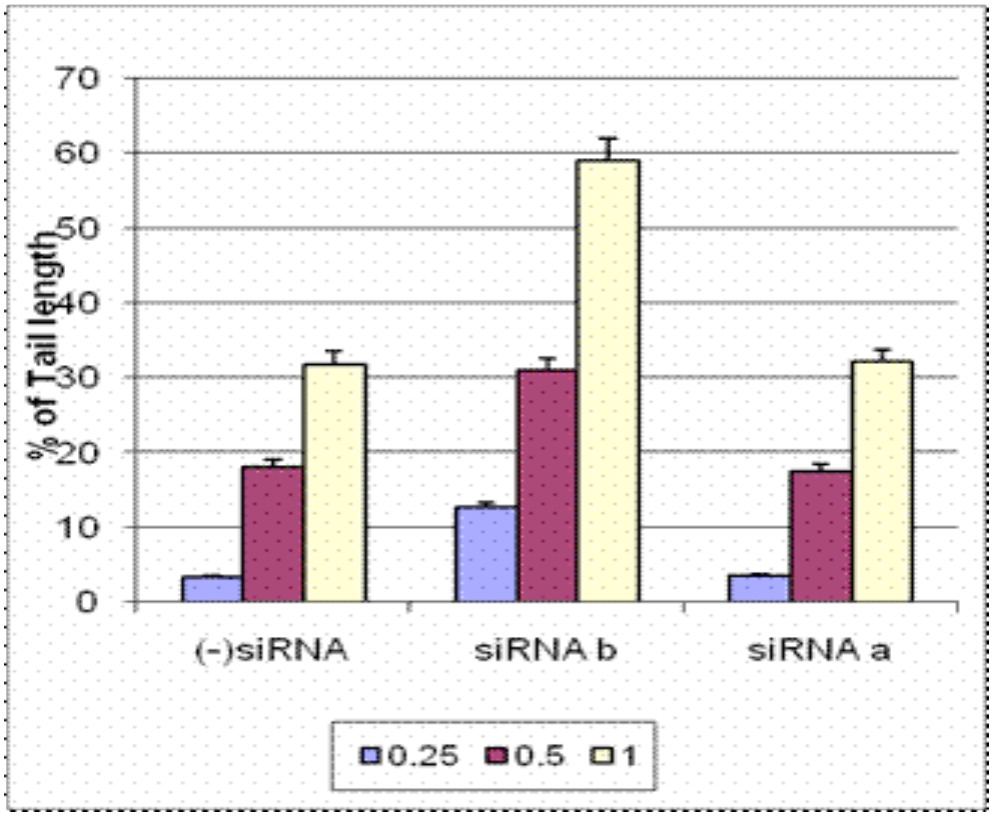
Granule neurons were treated with indicated concentrations of peroxide. Double strand breaks were quantified in treated (24h) and recovered (72h) cells using neutral comet assay (n = 3).

Figure 4.11A



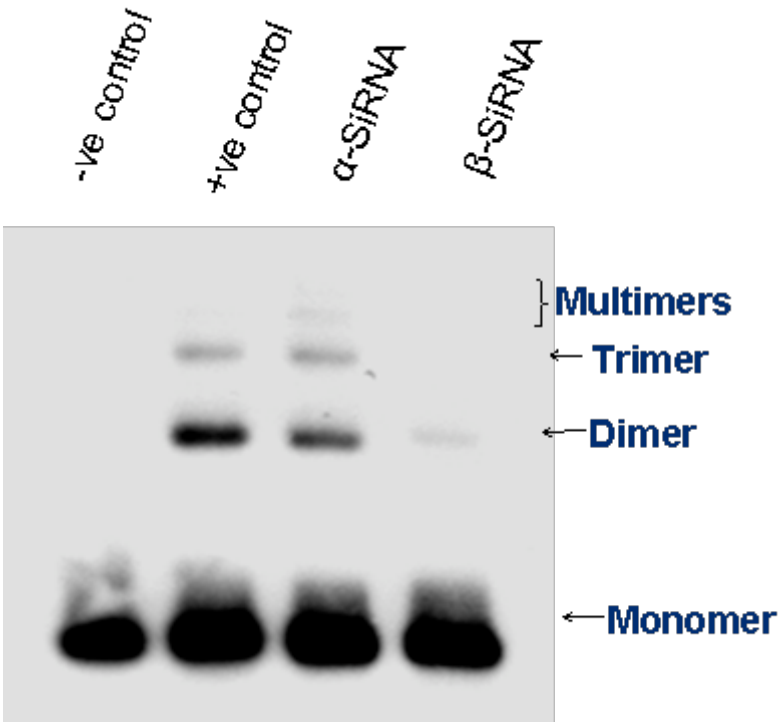
TopoIIα and β down regulation drastically effect the double strand breaks formation: The TopoIIα and β down regulated granule neurons were incubated with indicated concentrations of H_2O_2 for 24 hours and followed 72 hours recovery. The extent of DNA damage and recovery was analyzed by single cell neutral comet assay.

Figure 4.11B



TopoIIα and β down regulation drastically effect the double strand breaks formation: The TopoIIα and β down regulated granule neurons were incubated with indicated concentrations of H_2O_2 for 24 hours and followed 72 hours recovery. The extent of DNA damage and recovery was analyzed by single cell neutral comet assay. The percentage of tail length was measured by using Comet-IV software (Perceptive Instruments UK) and the extent of DNA damage was plotted on y-axis. Experiments were repeated three times and mean ± standard error was ploted ($p < 0.05$, $n = 3$).

Figure 4.12



In-vitro **NHEJ assay to confirm TopoIIβ activity in DNA double strand breaks repair:** Nuclear extracts were prepared from Topo α and β down regulated and non down regulated granule neurons cells. pUC19 plasmid DNA was digested with EcoRI and purified by phenol-chloroform method. Assay was conducted with 40µg of nuclear extract and 400ng of digested plasmid incubated for 2h at 25^0C. Products were resolved on 0.8% agarose gel and transferred on to nylon membrane and probed with randomly labeled (with α-p32 dCTP) linearized pUC19 plasmid DNA. Probed autorads were scanned by using Phosphorimager (Biorad).

Discussion

Topoisomerase II alpha known to localize in the nucleus and play important role during cell cycle, while β isoform reported be away from nucleus during cell cycle and show reticulocyte distribution during cell cycle (Chaly *et al.,* 1996 and Woessnar *et al.,* 1991). Various studies showed TopoII β uniformly distributed in all cell types, predominantly in neurons (Kondapi *et al.,* 2004). It has been shown to be involved in neuronal differentiation (Lyu *et al.,* 2003 and Tsutsui *et al.,* 2001), its deficiency reported to induce neuromuscular defects in mice (Yang *et al.,* 2000). These observations implicate the wide spectrum of housekeeping activities of TopoIIβ in cellular development and survival. Since all types cells are frequently encountered by various toxic agents leading to the damage of DNA followed by cellular dysfunctions. The signaling mechanisms of cells rescue the damaged cells and repair the DNA followed by restoration of cellular activity. The repair and recombination functions are highly conserved among all the cell types and very important for the non-dividing cells like neurons. Since Topoisomerase II β is uniformly distributed in all cell type, shown to be predominantly present in non-dividing neurons indicating possible role of this enzyme in DNA repair and recombination activities. This is further strengthened by our observation that topoII β decrease during ageing (Kondapi *et al.,* 2004), the aged cells reported to have deficiencies in DNA repair machinery. Furthermore, Emmons *et al.,* (2006) using alkaline Comet assay showed that Topoisomerase II β is required for single stand breaks repair of DNA cross links. In the present paper, we have addressed the important functions of Topoisomerase II β in double stand breaks repair. Further more, our results show that pro-apoptotic activity of TopoII alpha through enhancement of DNA double stand breaks in the presence of peroxide. This is further supported by the observations of Liu at al that H_2O_2 can enhance TopoIIα and DNA cross links suggesting the enhancement of DNA damage is indeed mediated by TopoIIα.. One can argue that the variation in TopoIIα and β levels is due to the presence of such cross links. Since we have extensively boiled with SDS, such a possibility can be ruled out. If such problem exist, one should not detect Ku70, which part of protein DNA complexes that are known to be formed during

DNA repair. These results further demonstrate that the DNA repair activities of TopoIIβ are conserved in both double strand and single breaks repair mechanisms.

TopoII has been shown to mediate DNA clustering and apoptosis in higher order chromatin fragmentation (Mirault *et al.,* 2006). It has been proposed that TopoIIα binding and cleavage at scaffold associated regions (SARs) or the regions with low energy level are vulnerable to damage due to genotoxic agents and serves as breakage sites (Zhang *et al.,* 2006). The TopoIIα may be stimulated through a double strand break (DSB)-dependent signaling pathway (Kwon *et al.,* 2000 and Ma *et al.,* 2005), with the activated TopoIIα promoting the unwinding of chromosomal DNA to expose it to reactive oxidative intermediates as well as to free radicals, leading to enhanced DNA damage. In contrast, the TopoIIβ may be activated through signaling proteins expressed during DNA repair. Repair process mediated by TopoIIβ may also promote repair of DSBs formed due to TopoIIα mediated DNA damage, through Non-Homologues End-Joining (NHEJ) repair pathway (Malik *et al.,* 2006) it has been shown that TopoIIβ is involved in regulation of transcription of protein involved in DNA repair (Ju *et al.,* 2006). It has been reported that TopoIIα and β interact distinctly with various forms of DNA repair activities *in-vitro*, these results addresses the importance of these enzymes in cellular damage and recovery against peroxide mediate-insult.. Thus, TopoIIβ may be involved in both in transcriptional activation of repair enzymes or in maintaining DNA topological changes during NHEJ repair process. The functional activity of TopoIIβ may occur through an association with DNA rearrangements during branch migration and resolution of crossover intermediates. This facilitates the release of torsional stress in order to maintain the topological integrity in DNA. In the absence of such an activity of TopoIIβ, the migration and forward movement of the repairing DNA ends will be inhibited, thereby blocking the progression of DNA repair. The most important finding of this study is thus to bring out that the two catalytically conserved isoforms of Topoisomerase II, exhibit distinctly different functions *in-vivo*. viz the TopoIIα acclerates the DNA damage and TopoIIβ participates in resolution of DNA rearrangements required for the DNA repair. This will be possible when the nature of substrate binding affinity of these enzymes is distinctly different. TopoII shown to bind to specific points of helix-helix juxtra

positions on negatively super coiled plasmid containing crossover junctions (Zechiedrich *et al.,* 1990) suggesting possible interaction of TopoII at DNA crossover. Furthermore, the cleavage reaction on double stranded DNA substrates contain nicks and deletions can cause the suicide to the substrates (Alsner *et al.,* 1996), especially apurinic sites are shown to stimulate DNA scission by 10 to 18 fold (Kingma *et al.,* 1997) as well as they are preferred compared to apyrimidine site in substrates that contain multiple lesions (39). These enzymes can use RNA containing substrates, which stimulates at 8 fold higher activity than deoxyribose (Kingma *et al.,* 2007) suggesting that TopoIIα and β recognize RNA, DNA and RNA/DNA hybrids as substrates (Wang *et al.,* 2000). Since TopoIIα displays higher affinity and cooperativity in binding to SARs (Bakshi *et al.,* 2001), association of alpha isoform at SAR may promote unwinding activity in chromosome leading to its exposure to reactive oxygen species that induce nicking and such nicks may stimulate TopoIIα activity and promotes strand cleavage and enhance double strand breaks. Since geometry and conformation of DNA is determining factor in TopoIIβ binding, it is possible that the conformation of DNA occur during the repairing complex formation may promote the TopoIIβ binding at the damaged DNA termini and progress the DNA cross-over (Zechiedrich *et al.,* 1990) during repair process. The activity of TopoIIβ at the repair ends may maintain the torsional equilibrium in the chromosome thus prevent the transmission of torsional stress to SARs, thus TopoIIα mediated unwinding process may not be activated.

Assessment of DNA repair activity of TopoII using TopoII poisons etoposide (Robson *et al.,* 1987) and Novobiocin may activate mixed signaling pathways viz. One due to the drug-mediated DNA damage, and the other due to drug-protein complex mediated signaling processes. In the present case, since such a problem does not arise, one can utilize the role of these enzymes themselves in understanding DNA damage and repair processes without such involvement of any non-specific signaling pathways. The result that TopoIIα accelerates DNA damage and TopoIIβ promotes DNA repair suggesting an important repair function of TopoIIβ in all cell types provides new directions towards understanding the important functional role of this isoform in aging brain.

Another important observation of these studies is that. The role of TopoIIβ in promotion of DNA repair activity highly conserved in primary neurons viz,

1. TopoIIβ expression elevates in the repair of H2O2 mediated DNA damage in granule neurons
2. TopoIIβ down regulated neurons are highly sensitive to H2O2 mediated DNA damage
3. Cell free extracts from TopoIIβ down-regulated granule neurons shows defective NHEJ activity.

The results indicate an important role of TopoIIβ in DNA repair in primary neurons. Absence of TopoIIα expression in both damage and repair show that TopoIIα has no significant role in neuronal functions. The observed expression of TopoIIα in replicating cells is due to the chromosomal junctions during replicative activities of TopoIIα, that expose DNA to ROS induced DNA damage. Further, the study point out that TopoIIβ function is important in terminally differentiated neurons.

CHAPTER 5

A study on the function of Topoisomerase IIα and β in HIV-1 induced inflammation

Neurological disorders are the first manifestation of symptomatic HIV infection in 10-20% of population, while about 60% of seropositive persons with advanced HIV disease will have clinically detectable neurological dysfunction (Levy *et al.,* 1985, Koppal *et al.,* 1985 and Snider *et al.,* 1983). The incidence of sub clinical neurological disease is even higher along with peripheral neuropathy and HIV associated cognitive dysfunction leading to HIV associated dementia (Catasti *et al.,* 1995 and Fischer-smith *et al.,* 2005) in early infection. HIV crosses the blood brain barrier (Malcolm et.al, 2004) and enters the neuronal system with concomitant internal systemic infection (Resnick *et al.,* 1988). HIV virus has been cultured from brain, nerve and cerebrospinal fluid (CSF) from patients during HIV infection (Rosnick *et al.,* 1985 and McArthur *et al.,* 1988). 50% of patients with AIDS eventually show neurological complications directly attributable to the infection of the brain by the HIV-1.While HIV-1 viral turnover in circulation is primarily due to infection of $CD4^+$ cells, specially T-lymphocytes and monocytes/macrophages, however, experimental evidence suggests that expression of the CD4 molecule is not the sole factor determined viral entry (Xu *et al.,* 2004). Additionally CD4 independent HIV-1 infection has been demonstrated *in-vitro* in several cell lines and in primary human cell cultures (Harous *et al.,* 1989). These observations indicate that other cell surface molecules function as co-receptors in the presence of CD4. Recent findings *in-vitro* indicated that $CD4^-$ cells in the brain, including astrocytes, endothelial cells and neurons harbor HIV-1 infection (Harouse *et al.,* 1996). But some neurological cell types are sensitive to virus, while others are resistant. Several theories have been proposed regarding HIV-1 mediated neuropathogenesis, such as the aberrant cytokine production by HIV infected microglial and glial cells, neurotoxicity of gene products of virus (gp120 and tat) and alteration of function of astrocytes by cytokines and toxins produced by HIV gene products (Kaul *et al.,* 2001 and Mohammad *et al.,* 2006).

The cytokines TNF-α and IFN-gamma have been implicated in the development and progression of multiple sclerosis (MS) and AIDS associated dementia complex. TNF α is a 17 kDa peptide produced by a wide range of cells (Tracy *et al.,* 1993). TNF α plays an important role in 1) inflammation, 2) the modulation of immunoresponse by affecting the expression of class I and class II MHC molecules

and adhesion molecules, 3) stimulation of cytokines such as IL-1, IL-6, IL-8 and IFN gamma. (Etty *et al.,* 1995**).** TNF α participates in the inflammatory reaction within the CNS. TNF α positive macrophages and astrocytes have been identified in the brain of MS patients, particularly in the plaque region (Hofman *et al.,* 1989). TNF α induction of IL-6 expression in astrocytes occurs by protein kinase C dependent pathway. Inflammatory markers shown to be enhanced in HIV-1 associated dementia, HIV-1 gp120 is shown to be involved in such response in neuroblastoma cells (Griffin *et al.,* 1994).

Topoisomerase II α (TopoIIα) and Topoisomerase II β (TopoIIβ) are 170 kDa and 180 kDa proteins; they promote the replication of viral DNA and chromosomal DNA (Wang *et al.,* 2002,). TopoIIα, is highly regulated during cell division, TopoIIβ is associated with non-proliferating function. The objective of the present investigation is to examine if systemic inflammation can regulate TopoIIα and β in neurons and astrocytes. The regulation of TopoIIα and β were analyzed based on the sensitivity of their expression to virus induced inflammation.

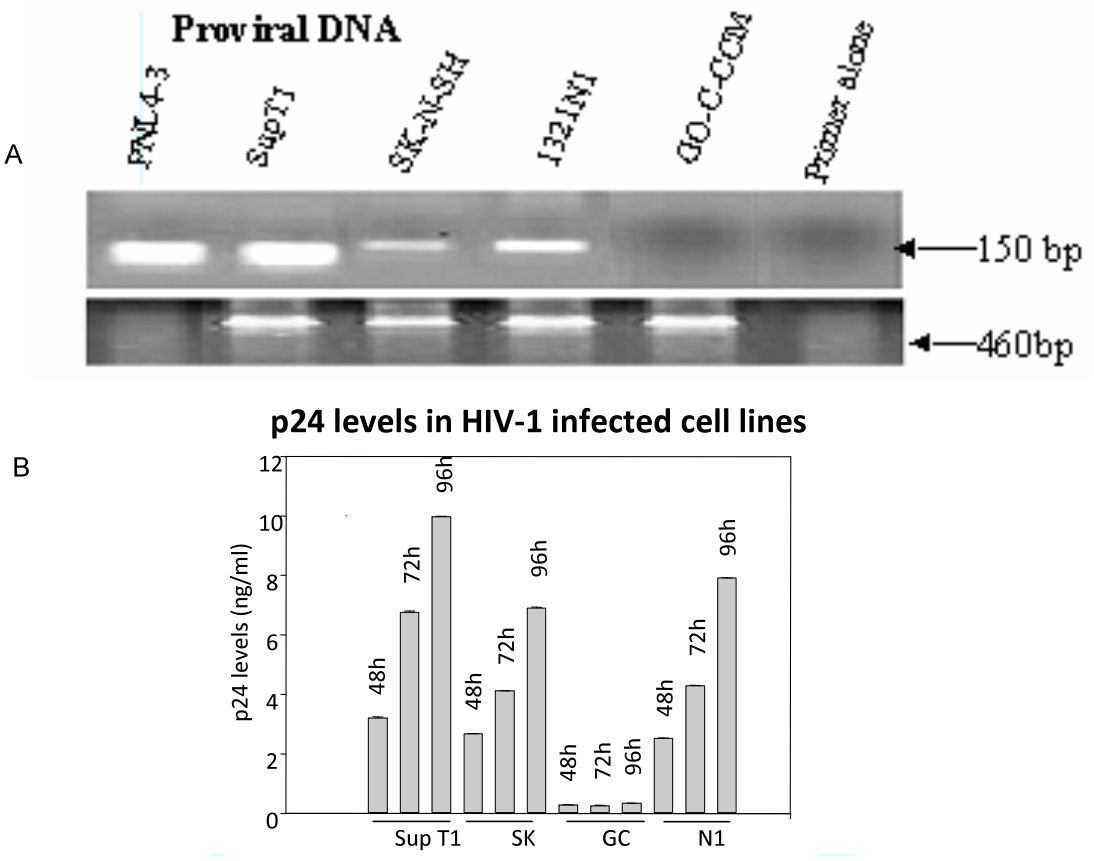
Results

The present study aims at understanding the HIV-1 induced inflammatory response mediated signaling pathway and the regulation of the expression of DNA Topoisomerase II (Topo II) isoforms. Further, the significance of anti-inflammatory compound NDGA and gp120 derived peptides in progression of viral replication in neurons and astrocytes is investigated. The neuroblastoma SK-N-SH (SK) was used as representative cell line for neurons, 1321N1 (N1) for astrocytes and GO-C-CCM (GO) for glial cells. Indian isolate HIV-1 $_{93IN101}$ was used in all experiments.

The sensitivity of SK, GO and N1 cell lines to HIV-1 infection

Neuroblastoma, astrocytoma, and glioblastoma, were challenged with HIV-1 $_{93IN101}$. Proviral DNA was analyzed after 5 hours of post-infection (Fig: 5.1A). The amount of virus replicated at day 4 was analyzed using p24 quantification through ELISA method, the results presented in Fig: 5.1B show that SK and N1 were sensitive to HIV-1 infection, while GO cell line is resistant to HIV-1 infection. Sup-T1 cells were used as a control cell line.

Figure 5.1



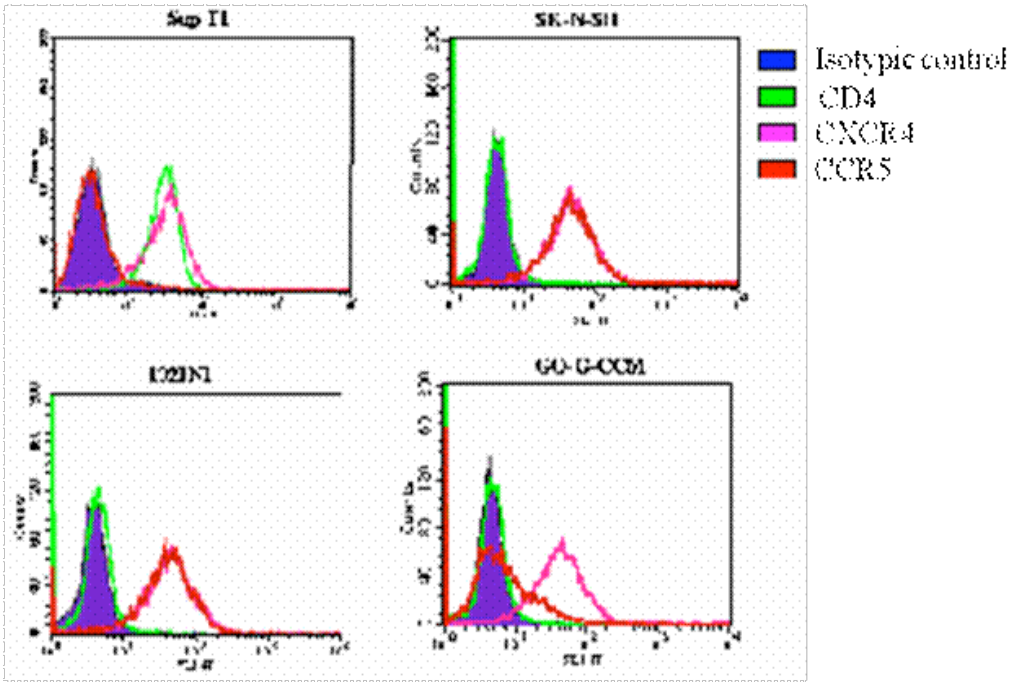
Sensitivity of neurons and astrocytes to HIV-1 infection

SK-N-SH (SK), 1321N1(N1), SupT1 and GO-G-CCM (GO) cell lines (5 · 106) were infected with 10 ng/ml of HIV-93IN101. Cell supernatant was collected at 48, 72 and 96 h of post-infection (p.i). HIV was estimated in terms of p24.SupT1 is used as positive control

(A) Proviral DNA in infected SK, N1, SupT1 and GO cell lines. The cells were harvested after 5 h of post-infection and proviral DNA was analyzed by amplification with gag specific SK38 and SK39 primers. Top panel is 150 bp gag specific product and the bottom panel is 450 bp of actin

(B).HIV-1 replication in terms of p24 on Y-axis and cell lines on the X-axis. Each experiment was repeated three times and data was plotted as an average of triplicates and standard deviation

Figure 5.1C



Expression of receptors in neurons and astrocytes

SupT1, SK, N1 and GO were probed with mouse anti-human CD4, CXCR4 and CCR5 antibodies, the bound antibody was probed by Goat anti-mouse IgG secondary antibody conjugated with FITC was sorted using FACS. The result was overlaid (Blue: isotypic control, green: CD4, pink: CXCR4 and red: CCR5)

Expression of CD4, CXCR4 and CCR5 receptors in SK, N1 and GO cell lines

Since SK and N1 cells are sensitive to HIV-1 infection, while GO cells are resistant, these cells are analyzed for the presence of primary receptor CD4, and co-receptors CXCR4 and CCR5 using Flow cytometry. The results show (Fig 5.1C) that the SK, N1 and GO cell lines are CD4 negative. Further, SK and N1 cell lines express both CXCR4 and CCR5 receptors, in contrast GO expressed only CXCR4, with very low expression of CCR5 and this could be the reason for its resistance to viral infection. These results suggest the importance of expression of CCR5 receptor in invasion of virus in neuronal cells.

Expression of TopoIIα and β and inflammatory cytokines in SK-N-SH, 1321N1 and GO-G-CCM cells in presence and absence of HIV-1

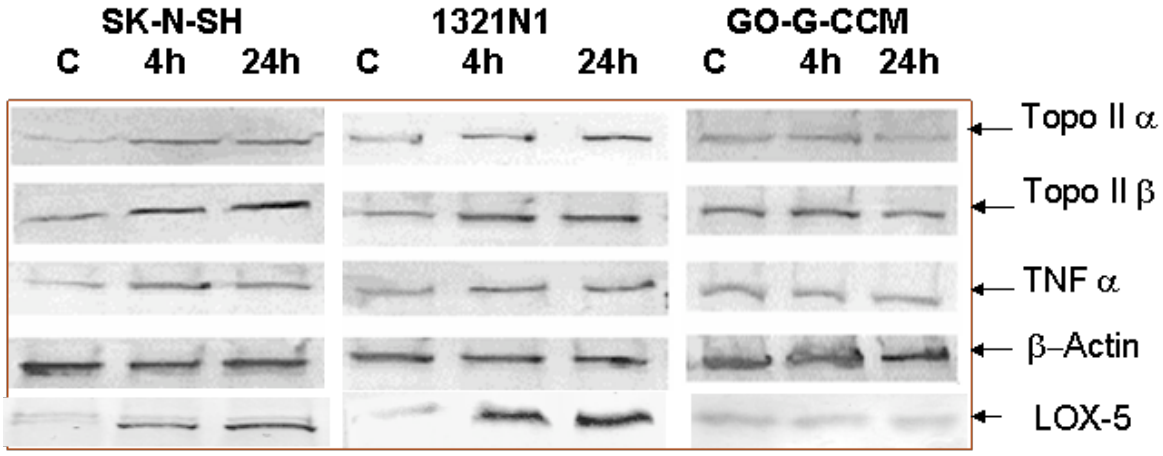
The cells were incubated with HIV-1 for 24hrs and the whole cell extract was analyzed for TNFα, LOX-5 and TopoIIα and β by western blot. The results show that TopoIIβ expression is enhanced along with TNFα, while TopoIIα expression increases concomitant with LOX-5 expression (Fig 5.2). This Suggests that TopoIIβ may follow TNFα mediated inflammatory pathway, while TopoIIα may be up regulated during stress response in the cells due to induction of activity of LOX-5, thus suggesting distinct modes of TopoIIα and β regulation.

Action of anti-inflammatory molecule NDGA during HIV-1 induced inflammation and viral replication

The cells were challenged in presence and absence of HIV-1 with increasing concentrations of NDGA. The results in uninfected cells show (Fig 5.3) that the Topo IIα and LOX-5 expressions are diminished at 10μm in SK and N1 (Fig: 5.3) cells, and there is no effect on the TopoII α expression in GO cell line (Fig: 5.3). The analysis of expression of TopoII β and TNFα shows that 50μm of NDGA is required to inhibit these proteins expression in SK, N1 cells, in contrast, the expression of TNFα and TopoIIβ are enhanced with increasing concentration of NDGA in GO cell line. Where as in infected cells (Fig 5.4) the effect of NDGA on TopoIIα and Lox-5 remains the same as that of uninfected cells (Fig 5.3) suggesting the regulation of TopoIIα is NDGA sensitive and follow similar pathways in both

infected and uninfected SK and N1 cells. Where as TopoIIβ and TNFα expression shows low sensitivity to NDGA in HIV infected SK cells (Fig 5.4) compared to uninfected SK cells suggesting HIV-1 infection induces an alternative NDGA resistance pathway for TopoIIβ and TNFα regulation in SK cells, while TopoIIβ and TNFα regulation remain same in both infected and uninfected N1 cells suggesting that the HIV-1 infection follow similar TopoIIβ and TNFα regulatory pathways in presence and absence of HIV infection.. Hence, the expression of Topo IIβ may be tightly linked with TNFα or it is possible that TNFα co- regulates TopoIIβ. These results suggest that under stress (virus infection) condition, the expression of TopoIIα is regulated in LOX-5 dependent pathway, while the expression of TopoIIβ is regulated in TNFα dependent path way. In HIV-1 resistant cells the regulation of TopoIIα and β may be distinctly different: For example in GO cell line, the levels of TopoIIα remain unaltered, while Topo IIβ and TNFα are enhanced by blocking NDGA-sensitive pathway.

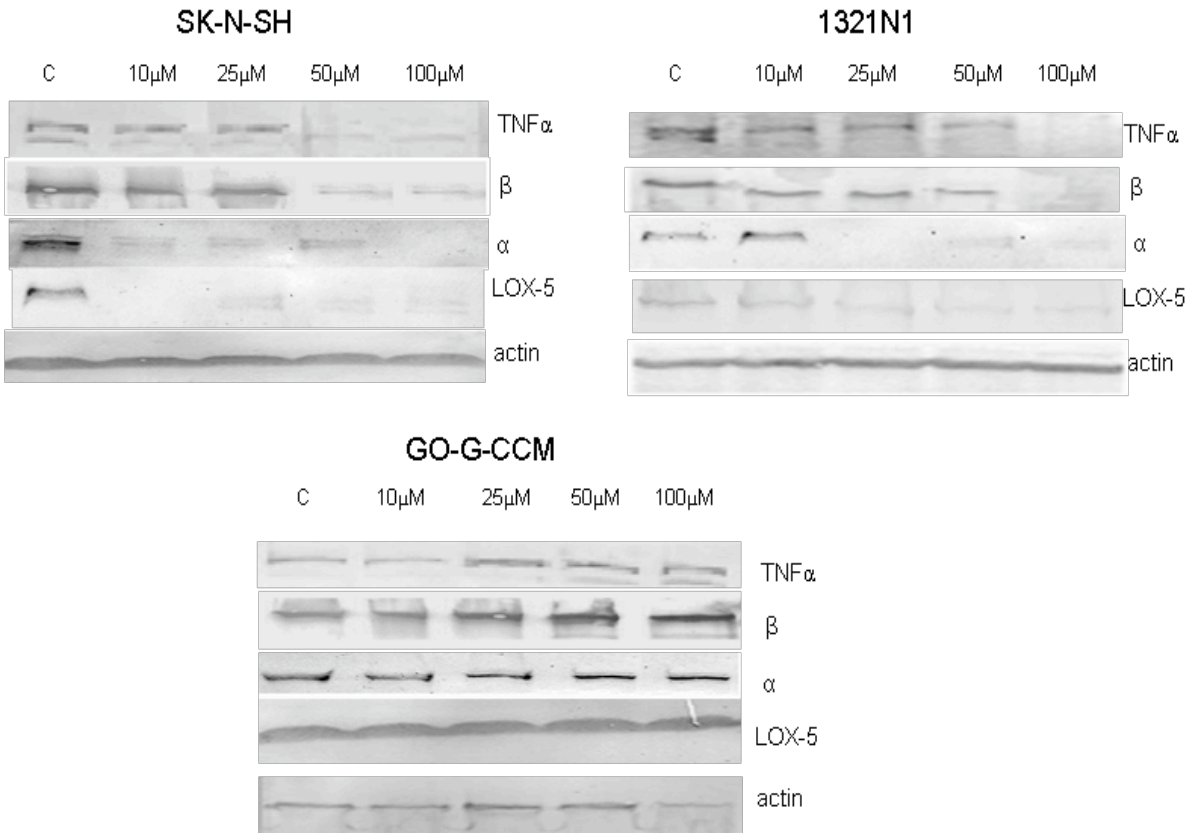
Figure 5.2



Cellular expression profile in neurons and astrocytes during HIV-1 infection:

SK-N-SH, 1321N1 and GO-G-CCM cell lines were infected with 10 ng/ml of HIV-193IN101. The cells were harvested at indicated time points of post- infection, and lysed. Total protein was estimated and 100 lg of total protein was loaded onto each well and separated on SDS–PAGE and western blotted. Then probed with indicated antigen specific antibodies and detected with ALP conjugated secondary antibodies.

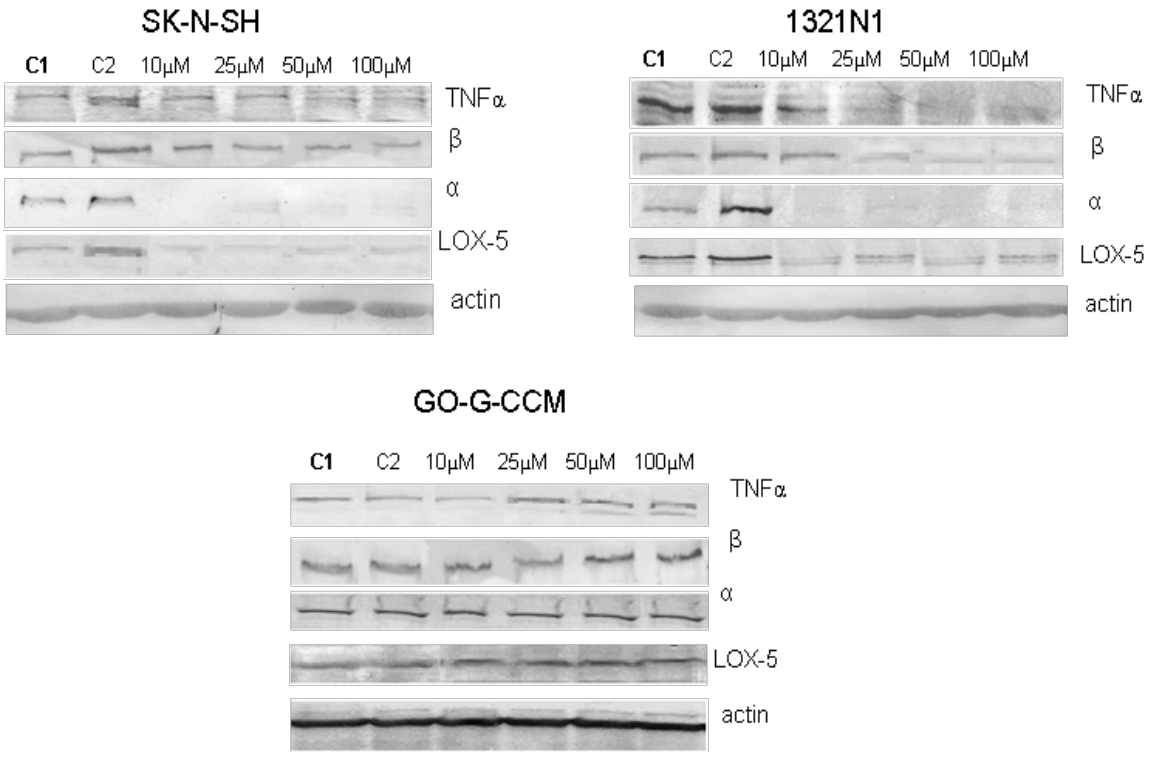
Figure: 5.3



NDGA sensitivity of Topo II a and b in SK, and N1 cells

SK-N-SH, 1321N1 and GO-G-CCM cells were incubated with increasing indicated concentration of NDGA in complete media and incubated for 24 h at 37^0C. Total protein (100 μg) was separated on SDS–PAGE and Western blotted and probed with antigen specific antibodies

Figure: 5.4

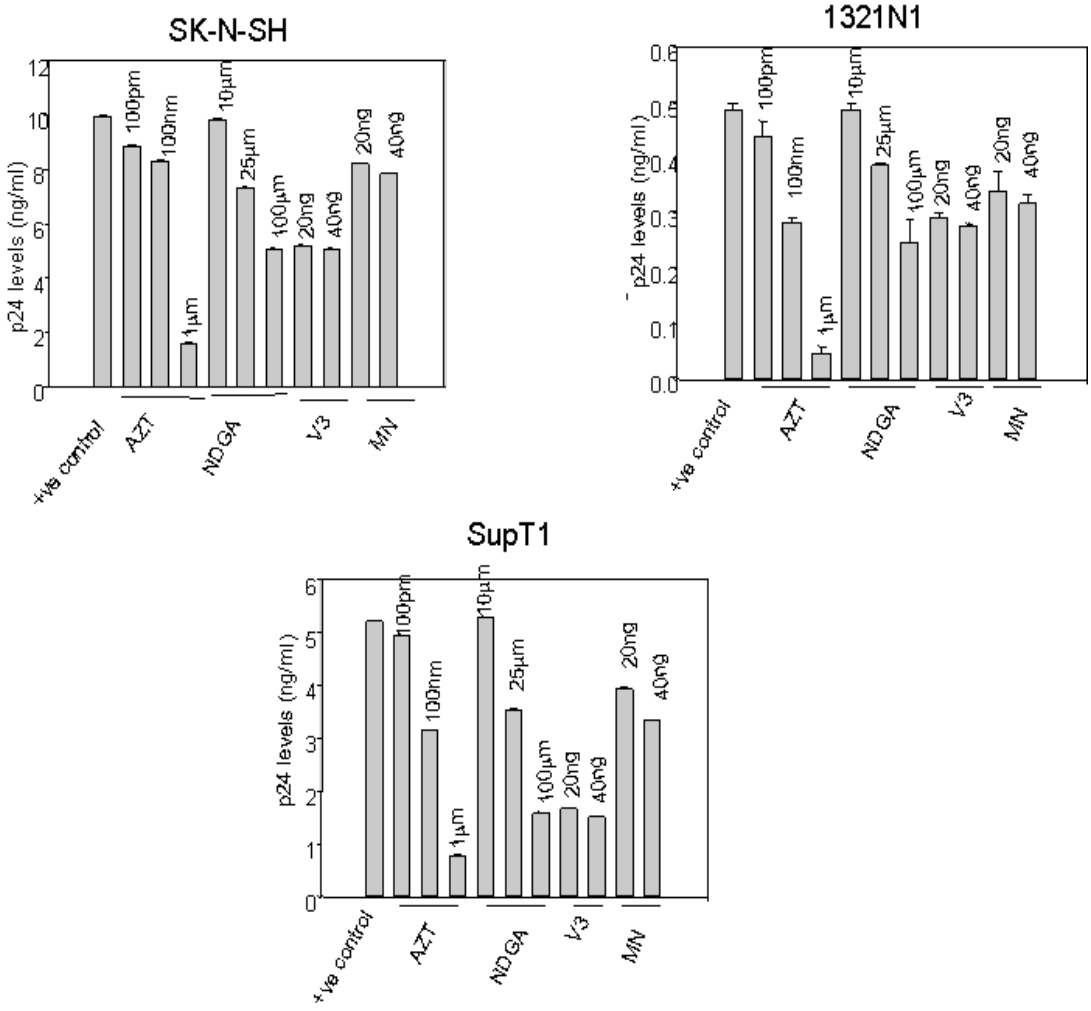


NDGA sensitivity of expression of Topo II a and b in HIV-1 infected SK, and N1 cells: SK-N-SH, 1321N1 and GO-G-CCM cells were incubated for 24 h in the presence of both HIV-1 and indicated concentration of NDGA at 37 ^{0}C. Total protein (100 μg) was separated on SDS–PAGE and western blotted and probed with antigen specific antibodies. C1: Uninfected cells without NDGA and C2: Infected cells without NDGA

Action of gp-120 derived peptides on replication

We have chosen two gp120 peptides namely V3 and MN peptides. These peptides inhibit viral entry *in-vitro* (data not shown). To monitor the involvement of gp-120 epitopes in virus induced inflammation, we have performed competitive experiments of HIV-1 infection with two gp-120 derived peptides. The peptides did not show any cytotoxicity to the cells. The cells were incubated in the presence of increasing concentrations of peptides and were challenged with the virus for 5h. The virus was washed and the cells were then recultured in fresh media and the amount of virus replicated after 4 days was estimated in SK and N1 (Fig 5.5) and proviral DNA was estimated after 4 hours (Fig 5.6). The results show both the peptides can compete with the virus interaction to the receptors in cell lines, suggesting that the interaction of virus in these cell types can be competed by the peptides. The analysis of TNFα, Topo IIα and TopoII β in the infection, conducted in the presence of peptides, show that these peptides can produce a 50% decrease in TNFα and TopoIIβ levels in SK and N1 (Fig 5.7) cells. While the levels remain unchanged in GO (Fig 5.7) cells. While the Topo IIα is significantly decreased in SK and N1 cells and no change was observed in TopoIIα in GO cells. The results suggest that the blockage of gp-120 mediated virus binding by the neutralizing peptides can inhibit the up-regulation of TNFα, Topo IIβ and TopoIIα. Hence gp120 mediated virus binding may be involved in the induction of inflammatory response and TopoIIα and β in neurons and astrocytes.

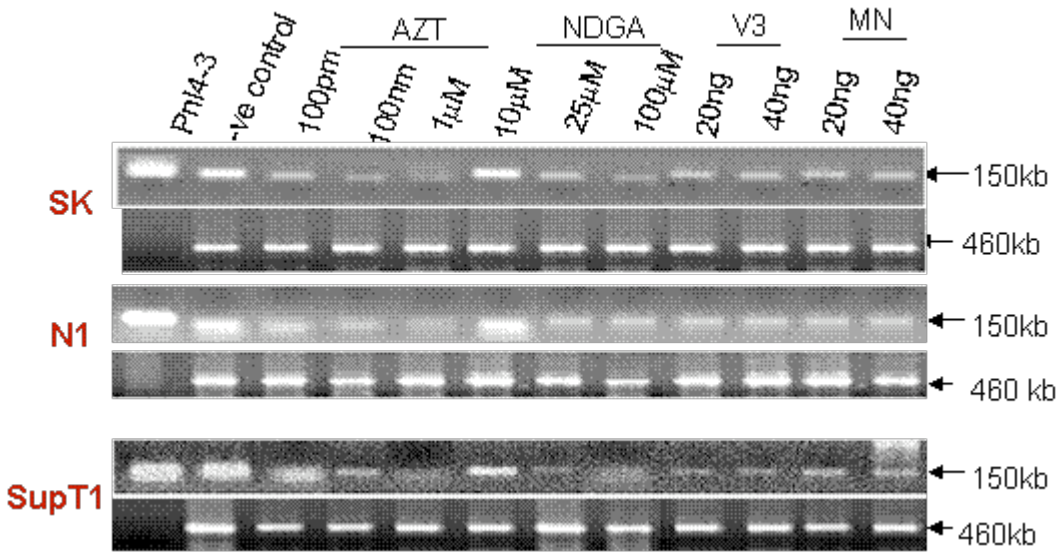
Figure 5.5



Action of AZT, NDGA, and peptides on HIV-1 replication

SK, N1 and SupT1 cells were challenged with virus in presence of AZT (100 pm, 100 nm and 1 lm), NDGA (10, 25 and 100 lm), V3 (20 and 40 ng) and MN (20 and 40 ng), the replicated virus was quantified in terms of p24 at day 4 of post-infection. p24 levels were plotted on Y-axis. The positive control was HIV-1 infected SupT1 cells in absence of agents

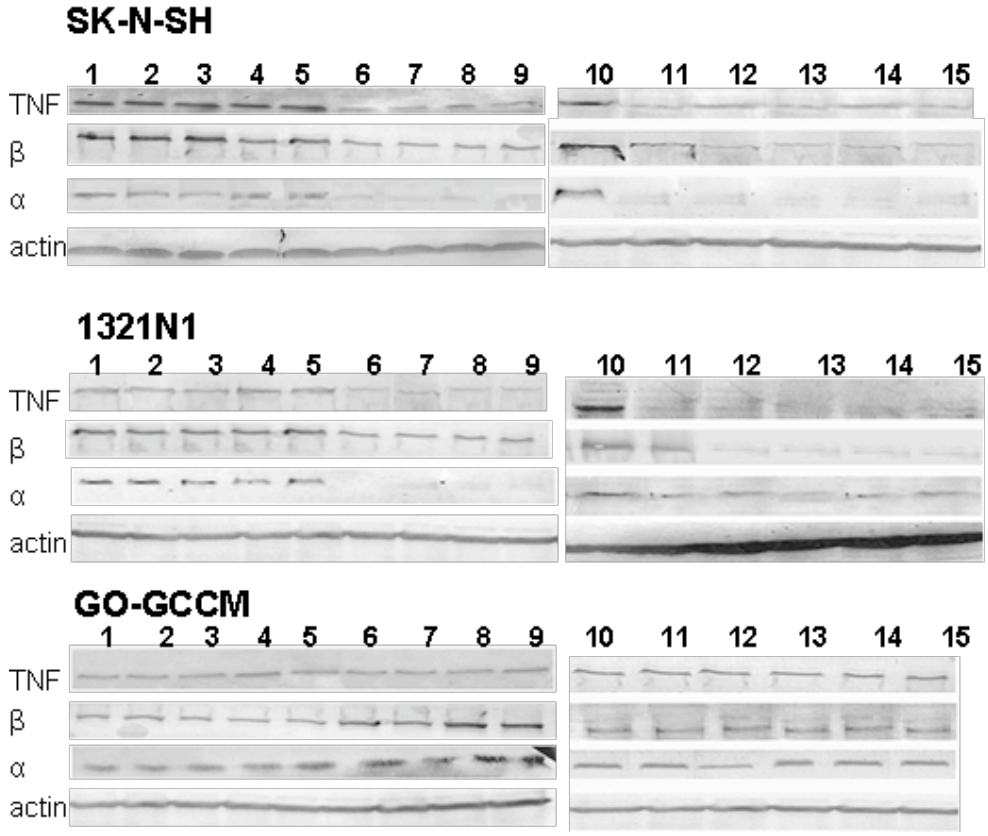
Figure 5.6



Proviral DNA synthesis in AZT, NDGA and peptides treated cells

SK, N1 and SupT1 cells were challenged with HIV-1 in presence of increased concentration of AZT, NDGA and peptides as indicated and cells were lysed and proviral DNA was analysed using gag specific SK38 and SK39 primers. The 150 bp band is gag specific amplification and 450 bp band is actin specific amplification

Figure 5.7



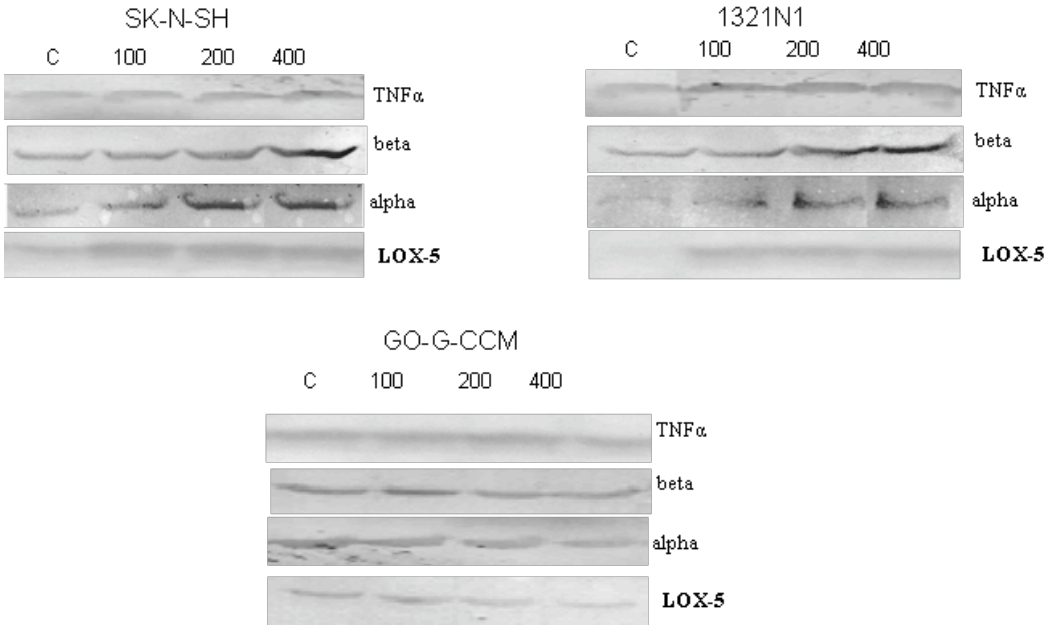
The sensitivity of cells to peptides in presence and absence of HIV-1

SK-N-SH, 1321N1 and GO-G-CCM cells were treated with peptides in presence and absence of HIV-1, cells were harvested after 24 h. One hundred micrograms of total protein was resolved on SDS–PAGE and Western blotted and probed with antigen specific antibodies. (#1: No peptide, # 2: 20 ng V3 and #3: 40 ng V3; # 4: 20 ng Mn #5: 40 ng Mn, # 2: 20 ng V3 and #3:40 ng V3; # 4: 20 ng Mn #5: 40 ng Mn; # 6: HIV+ 20 ng V3 and #7: HIV +40 ng V3; #8: HIV + 20 ng Mn #9: HIV+ 40 ng Mn, # 10: HIV alone, #11: HIV+ 25 lM NDGA; # 12: HIV+ 25 lM NDGA+ 20 ng V3; # 13: HIV+ 25 lM NDGA+ 40 ng V3; # 14: HIV+ 25 lM NDGA+ 20 ng Mn; # 15: HIV+ 25 lM NDGA+ 40 ng Mn

Both the Peptides inhibited gp120JR-FL mediated inflammation in SK-N-SH and 132N1 cells

SKN-SH neuroblastoma and 132N1 astrocytoma cells were incubated with increasing concentrations of gp120JR-FL, and the levels of TNFa, Topo II b, Topo II a and Cox 5 were monitored by Western blot analysis. The results in Fig. 5.8A show that the levels of Topo II a and b increased significantly along with TNFa and Cox 5 suggesting that gp120 induces inflammation of SN-N-SH and N1 cells, GO-G-CCM Gliobalstoma cells show an increase of Topo II a and b at 100 pM of gp120, while their levels decreased from 200 pM of gp120. While Cox-5 levels decreased with increasing concentrations of gp120. No change in the levels of TNFa has been observed thus suggesting that these cells are resistance to gp120-mediated inflammation. Analysis of activity of peptides on the gp120 mediated inflammatory activity show that both peptides inhibits gp120JR-FL mediated inflammation in SK-N-SH and 12N1 cells (Fig. 5.8B), where as the peptides did not show any effect on TNF and Cox 5 levels in GO cells (Fig. 5.8B). In addition, the results indicate that the peptides have distinct action on gp120-mediated Topo IIα and β regulation in GO cells.

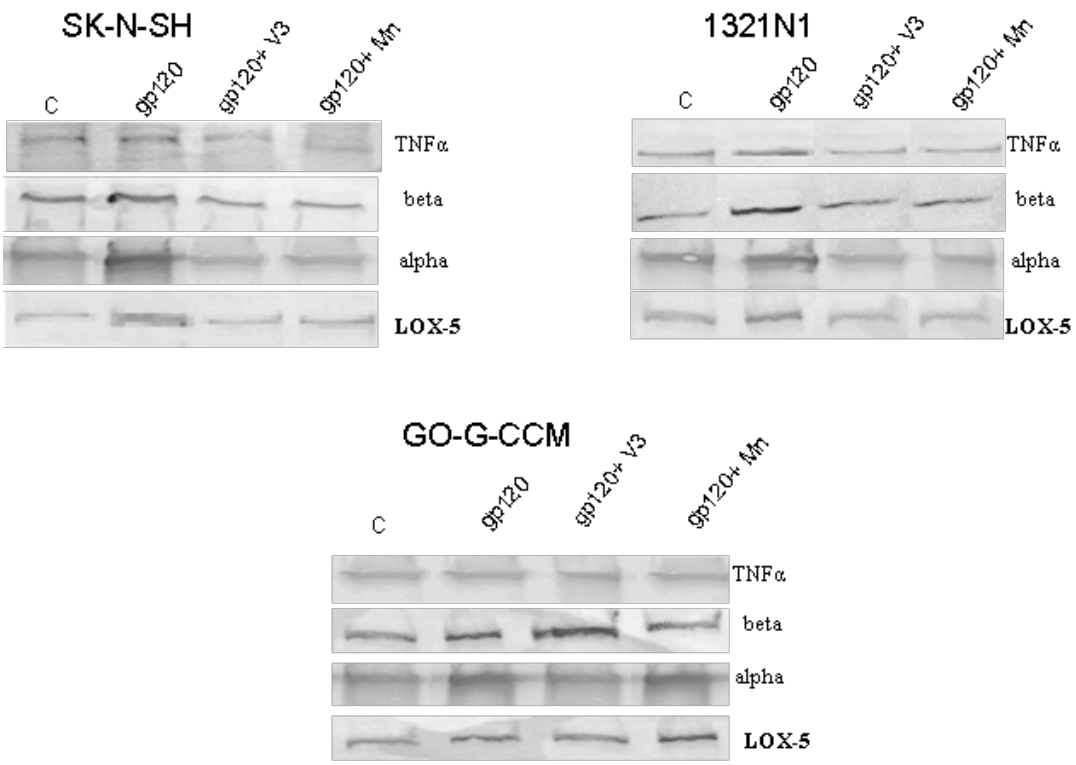
Figure 5.8A



gp120 induced inflammation in SK, N1 and GO cells

SK, N1 and GO cells were challenged with increasing concentrations of (100, 200, and 400 pm) gp120 (JR-FL) for 24 h and harvested. One hundred micrograms total protein was resolved on SDS–PAGE and Western blotted and probed with antigen specific antibodies.

Figure 5.8B



Downregulation of gp120 induced inflammation by V3 and MN peptides

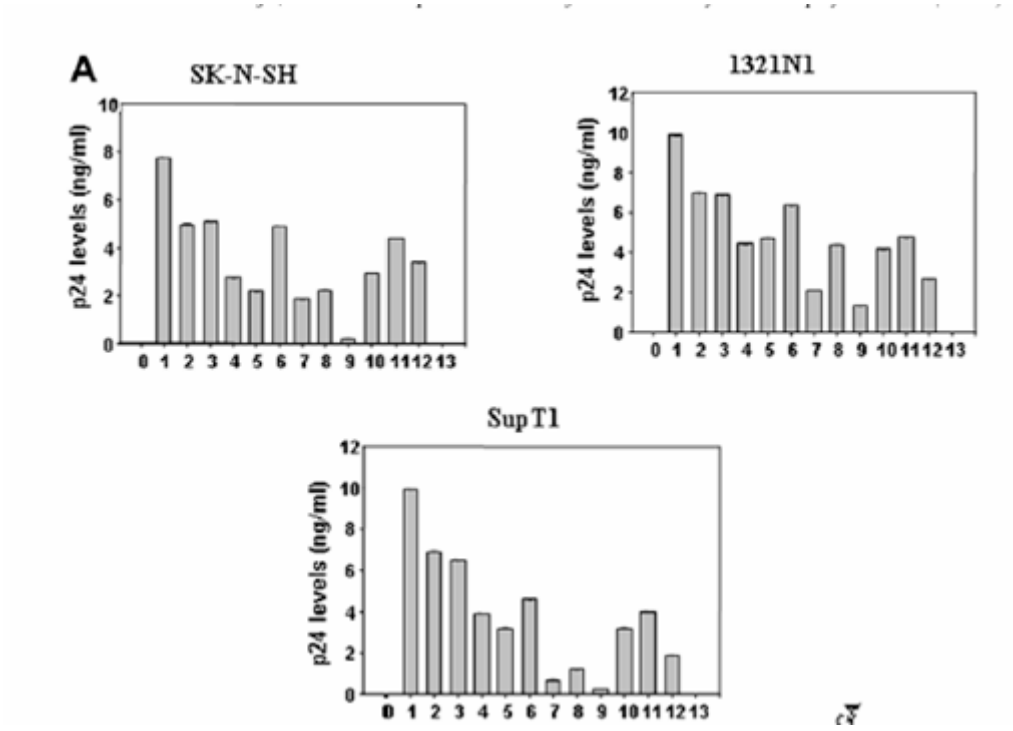
SK, N1 and GO cells were challenged with 200 pm of gp120 in presence of 20 ng of V3 or MN peptide for 24 h and harvested. One hundred micrograms total protein was resolved on SDS–PAGE and Western blotted and probed with antigen specific antibodies

Combination of anti-inflammatory NDGA and gp120-derived peptides can efficiently neutralize HIV-1 virus and decrease virus-induced inflammation

In order to investigate, if peptides and anti-inflammatory drug NDGA can act synergic in terms of virus neutralization, we have conducted infection assay studies in the presence of 25μM of NDGA and 20ng of V3 and MN peptides. The results show that the NDGA and both the peptides in combination can efficiently inhibit virus replication (Fig 5.9A) and proviral DNA synthesis (Fig 5.9B), suggesting that anti-inflammatory condition would help in enhancing the anti-viral activity of gp-120 peptides. To test if such an activity of NDGA is due to the blocking of viral entry, cell fusion assay was conducted in the presence of NDGA. The results show that NDGA cannot block viral entry. It may be inferred that virus associated inflammation may promote post-entry event of replication. To verify if NDGA can exhibit similar activity with HIV-1 drug AZT, we have conducted anti-viral activity experiments with NDGA and AZT in combination. The results (Fig 5.9) show that the NDGA indeed enhances the anti-viral activity of AZT suggesting the importance of controlling inflammation in anti-viral therapy.

To know the time point at which the anti-inflammatory action is important in stimulating anti-retroviral activity of AZT, we have incubated cells with AZT and NDGA is added at different time points during the post infection. The results (Fig 5.10) show that the proviral DNA is completely removed if the NDGA is added along with AZT, while NDGA addition after 30 min would enhance the activity of AZT to a lesser extent. To examine, whether this is due to the down regulation of inflammatory response in the cell, we have analyzed the levels of TNF alpha, Topo II beta, Lox-5 and Topo II alpha in infected cells when NDGA was added during the course of HIV-1 post infection. The results (Fig 5.10) confirm that the priming of cells for 10 minutes can down regulate the viral mediated inflammatory response, enhancing the anti-viral property of AZT.

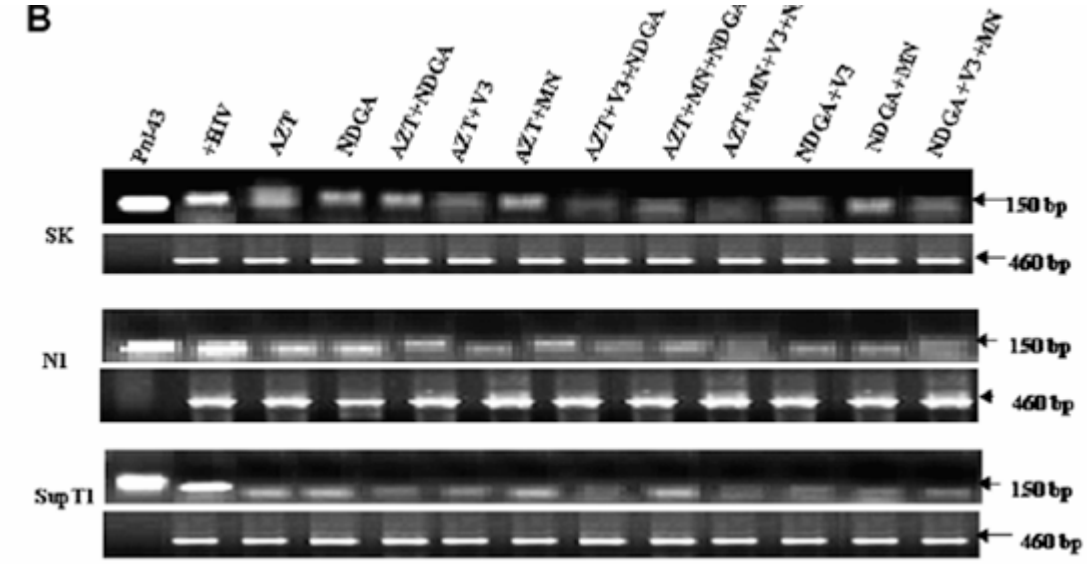
Figure 5.9A



Action of AZT, NDGA and peptides in combination against HIV-1 replication

SK-N-SH, 1321N1 and SupT1 cells were challenged with HIV-1 in presence of 100 nm of AZT, 25 lm of NDGA, 20 ng of V3 and 20 ng of MN peptide in the indicated combination. The amount of virus replicated after day 4 in terms of p24 was quantified, p24 was plotted against samples. #1: HIV alone, #2:AZT, #3:NDGA, #4:AZT+NDGA, #5: AZT+V3, #6: AZT+MN, #7: AZT+V3+NDGA, #8:AZT+MN+NDGA, #9:AZT+V3+MN+NDGA, #10: NDGA+V3,11: NDGA+MN, #12: NDGA+V3+MN

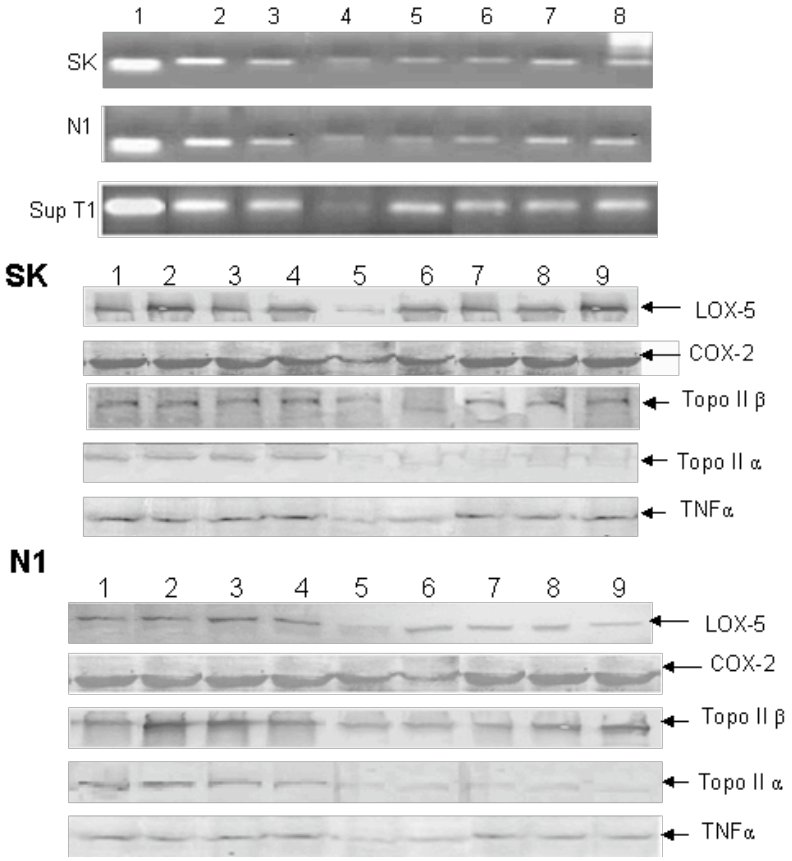
Figure 5.9B



Proviral DNA analysis in NDGA, peptides and NDGA treated cells

SK (a), N1 (b) and SupT1 (c) cells were challenged with HIV-1 in presence of indicated concentrations of drugs and peptides. The infection was stopped at 5 h and the amount of proviral DNA was analyzed using gag specific SK38 and SK39 primers. 150 bp bands is gag specific amplification and 450 bp bands is actin specific amplification. 100 nM of AZT, 25 lm of NDGA, 20 ng of V3 and 20 ng of MN were used for this study

Figure 5.10



Kinetics of anti-inflammatory activity of NDGA during HIV-1 infection conducted in presence of AZT SK, N1 and SupT1 cells (A) cells were incubated with AZT and NDGA was added at 0 (lane 5), 30 min (lane 6), 1 h (lane 7), 2 h (lane 8) and 3 h (lane 9), the amount of proviral DNA was analyzed after 5 h of post infection using gag specific SK38 and SK39 primers.#1: pNL4-3, #2: HIV-1 alone, #3:AZT + HIV, #4: AZT + NDGA, #5: AZT + NDGA at 30 min p.i., #6: AZT + NDGA at 1 h p.i., #7: AZT + NDGA at 2 h p.i., #8: AZT + NDGA at 3 h p.i.; (B,C) cells were challenged with HIV-1 in the presence of AZT and NDGA was added from 0 to 30 min, 1, 2, and 3 h of post-infection, and cells were incubated for 24 h and harvested. One hundred micrograms of total protein was separated on SDS–PAGE and western blotted and probed with antigen specific antibodies. #1: uninfected, #2: uninfected cells with AZT, #3:infected cells alone, #4: infection + AZT, #5: infection + AZT + NDGA #6: infection + AZT + NDGA at 30 min p.i., #7: AZT + NDGA at 1 h p.i., #8: AZT + NDGA at 2 h p.i.,#9: AZT+NDGA at 3 h p.i

Discussion

HIV-1 invades brain, causing impairment of certain cognitive functions at asymptomatic stage of infection (Susana *et al.,* 2005). It replicates in various cell types of brain such as astrocytes, microglia (Kim *et al.,* 2006) and neurons from different regions of brain. The replicated viral antigens can modify various neuronal functions in terms of upregulation/ downregulation of genes. Such a regulation may involve both viral and cellular factors for promoting genetic rearrangements associated with DNA replication (Branzei *et al.,* 2005), transcription, recombination and repair and apoptosis (Xu *et al.,* 2004) in neurons and astrocytes. The synthesized viral proteins are known to interfere with such cellular processes, leading to neurodegeneration and causing HIV associated encephalopathy (Wang *et al.,* 2002) and dementia (McArthur *et al.,* 2004 and Ozdener *et al.,* 2005) during symptomatic stage of infection. The time course of the secondary neurological symptoms may take longer, but emergence of these symptoms would promote rapid progression to AIDS and related mortality. Hence it is important to understand the cellular process associated with invasion of virus in various brain cells, since such information would help in developing therapeutics. In the present investigation, an attempt is made to study the inflammatory responses during viral invasion and replication. The involvement of DNA topoisomerase II enzyme plays an important role in maintenance of DNA topology required for DNA replication, transcription, and recombination of viral and cellular genes. HIV infection in CD4 negative SK-N-SH neuroblastoma, 1321N1 astrocytoma and GO-C-CCM glioblastoma cells are studied. Result show that the expression of inflammatory cytokine TNFα, TopoIIβ and LOX-5 is enhanced immediately after infection. Expressed Topo IIβ is activated through a virus-associated kinase (Kondapi *et al.,* 2005). Topo α and β is required for reverse transcription, DNA rearrangements and proviral DNA synthesis. Anti-inflammatory drug NDGA decreases the levels of inflammatory cytokine TNFα and LOX-5 in both infected and uninfected cells. A low sensitivity of NDGA is exhibited in regulation of TopoIIβ and TNF α uninfected SK and N1 cells, while the regulation of both Topo IIβ and TNFα was marginally decreased in presence of NDGA in infected SK cells, but not in N1 cells.

These results point out

1. TopoIIα and Lox-5 regulated in the same pathways,
2. TopoII beta and TNF α are co-regulated,
3. TopoIIα and Lox-5 show similar regulation in infected and uninfected SK and N1 cells,
4. TopoIIβ and TNFα regulation is same in uninfected SK and N1 cells, but virus infection in SK cells activate an alternative pathway for TopoIIβ and TNF α regulation, that has very low sensitivity to NDGA.

HIV-1 gp120 peptides can down regulate both TNFα and Topo IIβ and inhibit viral replication, suggesting the involvement of gp120 binding in enhancement of the inflammatory response. But the peptides itself could not induce an inflammatory response (Fig. 5, Lanes 2-4), while they could inhibit the HIV-1 virus or gp120 mediated inflammation. Suggesting that these peptides may not be interacting with the co-receptor. They may be interacting with non-CD4 primary receptors present on the cells, thus blocking the virus entry (Fig. 5, lanes 6-9) and envelope induced inflammatory response (Fig.6) Neuroectodermal origin (SK-N-SH) neuroblastoma cells and other neuronal cells has been shown to be suceptable to HIV-1 infection (Li *et al.,* 1990 and Vesanen *et al.,* 1991) through a CD4 independent mechanism. Antibodies against galactosyl ceramide has been shown to inhibit viral entry in those CD4 negative neuronal cells (Harouse *et al.,* 1991) and the interaction of virus with cell surface galactosyl ceramide/3` Sulfo-galactosyl ceramide is dependent on the conformation of gp120 (Harouse *et al.,* 1995), specially V3 domain shown to serve as primary viral determinant for infectivity in CD4 negative cells (Trujillo *et al.,* 1996). Hence, the peptides studied in the present investigation may interact with galactosyl ceramide in inhibition of virus mediated inflammation and infection. Since these neuroblastoma cells are reported to have low expression profile of Galactosyl ceramide receptor (Alvarez *et al.,* 2002) , et is also possible that another non-CD4 receptor may also be involved for peptide interaction in inhibiting HIV-1 induced inflammation and infection.

The binding of virus to cellular receptors may thus enhance inflammatory response leading to the up regulation of TNFα, TopoIIβ, and LOX-5. A combination of AZT (anti-retroviral drug) along with NDGA (anti-inflammatory drug) together can efficiently inhibit viral replication, thus suggesting down regulation of TNFα and TopoIIβ can enhance the anti-retroviral potency of AZT. Hence, TopoIIα and TopoIIβ may show distinct functions of DNA topological maintenance in neurons and astrocytes.

In summary HIV-1 binding to neurons and astrocytes can enhance inflammatory response by up-regulation of TNFα, TopoIIβ, and LOX-5.Anti-inflammatory drug NDGA and gp120 derived peptides can down-regulate TNFα and TopoIIβ and inhibit inflammation and viral replication. NDGA can synergistically act with AZT anti-retroviral activity. Thus a combination of AZT with NDGA can enhance the anti-retroviral potency of AZT..

CHAPTER 6

Conclusions

The work incorporated in the thesis derived the following functional activities of TopoIIα and β.

Topoisomerase IIα

- It is highly expressed during development, while it levels decreased upon birth and become negligible at 4th week old, suggesting association of TopoIIα in replicative functions of cells in the brain.
- TopoIIα is found to be negligible in neurons, while significant levels are expressed in astrocytes.
- TopoIIα accelerates the DNA damage in immortalized proliferation competent cells, while it has no function in DNA repair.
- TopoIIα not expressed during both DNA damage and repair in terminally differentiated neurons.
- TopoIIα and 5-Lox are activated in BDGA sensitive pathway in HIV-1 infected and un-infected neurons.

In summary, TopoIIα isoform is actively associated with replicative state of chromosome during development that exposes the DNA to ROS environment leading to stress related cellular re-organization in DNA damage and HIV-1 infection.

Topoisomerase IIβ

- TopoIIβ isoforms highly expressed throughout the development and growth stages of brain especially localized in three types of neurons viz, Granule neurons, cortical neurons and purkinje neurons, while the levels of TopoIIβ negligible in astrocytes. This suggests that TopoIIβ isoform is significantly associated with non-replicative functions of terminally differentiated cells.
- TopoIIβ isoform is significantly associated with DNA repair functions of both immortalized cell lines as well as differentiated neurons.
- TopoIIβ deficient cells were highly sensitive to DNA damage.
- TopoIIβ promotes NHEJ pathway along with Ku-70, WRN helicase and Pol-β in neurons.

- Another important function of TopoIIβ is that it co-regulates along with TNF-α in HIV-1 infected cells that promote virus infection in susceptible cells.

In summary, TopoIIβ actively participates in non-replicative functions of neurons such as DNA recombination, transcription and repair functions, which are important in development and cellular recovery from stress induced due to DNA damage and viral infection.

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Distinct roles of Topoisomerase II isoforms: DNA damage accelerating α, double strand break repair promoting β ☆

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Abstract

Topoisomerase II α (TopoIIα) and Topoisomerase II β (TopoIIβ) isoforms are different gene products having conserved catalytic activities. The α isoform is present in proliferating cell, while β isoform is predominantly present in non-proliferating cells namely neurons suggesting its role in non-replicating functions of DNA. The functions of TopoIIα and TopoIIβ isoforms are analyzed in peroxide-mediated DNA damage and double strand breaks (DSBs) repair in neuroblastoma and astrocytoma cells. The results show a strong correlation of TopoIIα level with the progression of DNA damage, while the TopoIIβ expression is correlated with the DNA DSBs repair activity of cells in Ku70, Werner's helicase and pol-β dependent pathways. The functional roles of TopoIIα and TopoIIβ are assessed using siRNA mediated TopoIIα and TopoIIβ knockdown in cells. The results show that TopoIIα^{-}TopoIIβ^{+} cells are resistant to peroxide-mediated DNA damage, while TopoIIα^{+}TopoIIβ^{-} cells are 2-fold more sensitive to peroxide and TopoIIβ deficiency lead to cellular apoptosis. These results are correlated with cell survival from peroxide-mediated insult. The result of this study that TopoIIα accelerates peroxide-mediated DNA damage, while TopoIIβ promotes DNA DSBs repair activity should provide new directions toward understanding of normalytic ageing processes in human brain.

Keywords: DNA repair; Hydrogen peroxide; Topoisomerase II α; Topoisomerase II β; DNA damage

Human Topoisomerase II present in two isoforms, 170 KDa TopoIIα[1] and 180 KDa TopoIIβ, catalytically show similar activity, but their distinct functional activity is not yet understood [1–3]. While the TopoIIα is found to be present in proliferating cells [4], TopoIIβ is present in all cell types, with a predominant localization in brain especially in neurons [5–8]. The decreasing activity of TopoIIβ with aging suggests its possible role in DNA repair activity in neurons during aging [5]. The frequently occurring insults to the DNA in an aging brain are due to enhanced oxidative metabolism and insufficient antioxidant pool that causes reduction in the removal rate of the reactive oxidative intermediates [9,10]. Exposure of chromosomal DNA to reactive oxidative intermediates can cause double stand breaks damage to DNA. While higher repair competent cells can correct such damaged DNA, the repair deficient cells are prone to increased DNA damage leading to a wide spectrum of cellular dysfunctions [11,12]. Hydrogen peroxide is one such frequently released intermediates of oxidative metabolism; it induces double stand breaks (DSBs) in chromosomal DNA [13–16]. It has been reported that several DNA damaging agents and TopoII poisons can enhance the activity of DNA repair enzymes [17]. In absence of isoform-specificity of poison employed, the studies could not address the differential activities of α and β isoforms. Emmons et al. [18] studied activities of

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[1] *Abbreviations used:* TopoIIα, Topoisomerase II α; TopoIIβ, Topoisomerase II β; DSBs, double stand breaks; SDS, sodium dodecyl sulfate; NHEJ, Non-homologous end-joining.

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TopoIIα and β in repair of melphalan-induced cross-links in the cellular DNA of K562 Leukemia and U937 histocytic lymphoma cells, the results could correlate TopoIIβ levels with the repair of cross-links, but could not clearly address the associated repair pathways as well as the activity of α isoform in DNA damage and repair. In the present investigation, using H_2O_2 mediated DNA damage as a model; we studied the DSBs repair efficiency of neuroblastoma and astrocytoma cells in the presence as well as in the absence of Topoisomerase II isoforms. The results clearly bring out the distinctly different functional activity pattern of these two Topoisomerase II isoforms.

Materials and methods

Monoclonal TopoIIα, TopoIIβ, Ku70 and β-actin antibodies (BD Biosciences, Singapore), polyclonal DNA polymerase β (Santa Cruz) and Werner helicase antibodies (BD Biosciences), Goat anti-mouse-ALP conjugated and Goat anti-rabbit-ALP conjugated secondary antibodies (UPSTATE), propidium iodide (Sigma). All the other chemicals and reagents are biochemical grade.

Cell culture

SK-N-SH and 1321N1 cell lines were obtained from National Centre for Cell Sciences. Pune, India. SK-N-SH was cultured in MEM containing 0.5 mM L-glutamine, 0.1 mM sodium pyruvate and 1 mM non-essential amino acids with 10% FBS. 1321N1 cell line was cultured in DMEM containing 10% FBS.

siRNA synthesis

Double strand siRNA oligos were synthesized as described by Donzé and Picard [19]. Briefly, desalted DNA oligonucleotides were ordered from Sigma (India). The oligonucleotide-directed production of small RNA transcripts with T7 RNA polymerase has been described Milligan et al. [20]. For each transcription reaction, 1 nM of each oligonucleotide was annealed in 50 μl of TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA) by heating at 95 °C; after 2 min, the heating block was switched off and allowed to cool down slowly to obtain dsDNA. Transcription was performed in 50 μl of transcription mix: 1× T7 transcription buffer (40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl and 2 mM spermidine) 1 mM NTPs, 0.1 U yeast pyrophosphatase (Sigma), 40 U RnaseOUT (Life Technologies) and 100 U T7 RNA polymerase (Invitrogen) containing 200 pM of the dsDNA as template. After incubation at 37 °C for 2 h, 1 U RNase-free DNase (Genetix) was added at 37 °C for 15 min. Sense and antisense 21-nt RNAs generated in separate reactions were annealed by mixing both crude transcription reactions, heating at 95 °C for 5 min followed by 1 h at 37 °C to obtain 'T7 RNA polymerase synthesized small interfering double-stranded RNA' (T7 siRNA). The mixture (100 μl) was then adjusted to 0.2 M sodium acetate, pH 5.2, and precipitated with 2.5 volume of ethanol. After centrifugation, the pellet was washed once with 70% ethanol, dried and resuspended in 50 μl of water.

siRNA transfection

Cells (1×10^6 million) were (SK-N-SH and 1321N1) transfected by using Lipofectamine-2000 (Invitrogen) with 0.5 μm of non-silencing TopoIIα and β separately, TopoIIα and β siRNA separately and with both TopoIIα and β siRNA. The analysis was performed after 48 and 72 h.

Western blot analysis

Cells were harvested by scraping in 25 mM Tris–HCl, 137 mM NaCl, 3 mM KCl, pH 7.4, and centrifuged at 300*g* for 7 min at 4 °C as previously described by Angley et al. [21]. The cell pellet was homogenized in 0.2 ml homogenization buffer (25 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 lg/ml leupeptin, 0.1% aprotinin, 1 mM iodoacetamide, 200 μg/ml bacitracin and 20 μg/ml soybean trypsin inhibitor) for 10 min on ice followed by sonication for 15–20 s. The protein concentrations in cell lysates were measured using the Bradford method. Twenty micrograms of total protein/lane was separated on 7.5% sodium dodecyl sulfate (SDS) gels and then transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in TBS containing 0.05% Tween 20 for 1 h and then incubated overnight at 4 °C with corresponding protein specific antibodies (see Material and methods). After washing and incubating for 1 h at 22 °C with a secondary antibody conjugated with horseradish peroxidase, the membranes were washed and immunoreactive bands were visualized by chemiluminescence (Pierce Western Blot Chemiluminescence Reagent). Relative levels of protein in the different lanes were compared by analyzing scanned images using the NIH IMAGE program. All studies were performed a minimum of three times using independent cultures.

MTT assay

Reduction of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma) is chosen as a cell viability measurement optimal endpoint [23,24]. Non-silenced and TopoIIα and β silenced SK-N-SH or 1321N1 cells (0.2×10^6 cells per well) in corresponding medium with 10% FBS were seeded in 96-well plates. Increasing concentrations of H_2O_2 were added to the cells and incubated at 37 °C for 24 h in a CO_2 incubator with 5% CO_2. The media were replaced with a fresh growth medium along with 20 μl of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma). After incubation for 4 h in a humidified atmosphere, the media were removed and 200 μl of 0.1 N acidic isopropyl alcohol was added to the wells to dissolve the MTT-formazan crystals. The absorbance was recorded at 570 nm immediately after the development of purple color. Each experiment was conducted in triplicate. Experiments were repeated three times and the data were represented as averages with SE.

Neutral comet assay

Cells were transfected with ds-siRNA oligos, incubated for 48 h and treated with increasing concentrations of H_2O_2 for 24 h followed by 48 h recovery. Subsequently, 5000 cells were placed in a micro-centrifuge tube containing 1 ml cold PBS, and the neutral comet assay was done as described in [25]. Briefly, cells were centrifuged and resuspended in 500 ml cold PBS, and 1.5 ml of 1% agarose was added to each sample. The agarose-cell suspension was gently layered on a frosted-glass microscope slide, allowed to solidify for 5 min, and then placed immediately in ice-cold lysis buffer containing 30 mM disodium ethylenediamine tetraacetic acid (EDTA, pH 8.0), 0.5% sodium dodecyl sulfate (SDS) and 0.25 mg/ml proteinase K (Sigma). The samples were lysed for 1 h at 4 °C and then kept at 37 °C for 12–16 h. After cell lysis and digestion of protein–DNA complexes with proteinase K, the agar slides were re-equilibrated in TBE (90 mM Tris–HCl, 90 mM boric acid and 2 mM EDTA, pH 8.0) for 2 h, with a change of buffer every 15 min. The samples were electrophoresed with TBE buffer for 20 min at 25 V. The DNA was then stained with 20 μg/ml of ethidium bromide (Sigma) for 20 min and slides were washed twice for 5 min in TBE. To ensure random sampling, 50 images/slide were captured and, in some experiments, the observer was blinded to the conditions. The images were captured on a Confocal microscope (Lieca) and quantified by using Comet-IV software (Perceptive Instruments, UK). The comet moment was calculated by using the following equation described by Kent et al., [26]: comet moment Σ_{0-n} ((intensity of DNA at distance X) × (distance))/intensity of total DNA). The mean comet-moment value

obtained from control samples was subtracted from the mean comet-moment value for each H_2O_2 dosage. Data shown are the means and SD values from three independent experiments (50 images for each dose of each independent experiment).

Annexin-V assay

Annexin-V staining of apoptotic cells was used to determine whether reducing the levels of either TopoIIα or β increased the sensitivity of SK-N-SH and 1321N1 cells to H_2O_2 induced cell death. Forty hours after transfection with appropriate siRNA constructs, cells were treated with 1 mM H_2O_2 for 24 h and then placed in drug free media for an additional 48 h then peroxide induced apoptosis was measured by annexin-V staining (Invitrogen kit) and FACS analysis.

Cell cycle analysis

Cells were H_2O_2-treated for 24 h and re-incubated in normal medium. After 48 h of recovery, they were processed using the CycleTest PLUS DNA reagent Kit (Becton–Dickinson). Analysis was carried out in a Facscalibur (Becton–Dickinson), cell-cycle distribution was assessed using the ModFit LT 2.0 software (Verity Software House).

Data analysis

All of the numerical data are presented as means ± SEM. Statistical significance was calculated using Student's *t*-test to determine whether compared groups are distinct. Differences were considered significantly different if $p < 0.05$.

Results

The activity of TopoIIα and β in DNA damage and DSBs repair was studied using Human neuroblastoma cell line, SK-N-SH and astrocytoma cell line, 1321N1. The cells were incubated in the presence of increasing concentrations of H_2O_2 (0.5, 1, 2 mM) for 24 h followed by the re-culturing cells in the fresh medium and the cellular recovery at 72 h of post-exposure was monitored. The DNA damage in the cells was analyzed by single cell neutral comet assay using confocal microscope (Leica). The results show that H_2O_2 can synergistically damage the DNA (Supporting data Fig. 2A and B). The damaged DNA could be repaired when cells were recultured in fresh media for 72 h. Western blot analysis showed (Fig. 1) enhanced levels of TopoIIα with increasing DNA damage, while the TopoIIβ levels remained unaltered (Fig. 1). The levels of TopoIIα were found downregulated to negligible, while the levels of TopoIIβ were enhanced along with Ku70, WRN helicase and pol-β (Fig. 1) during recovery. XRCC2 did not show any expression in this pathway (Fig. 1). The results thus point out to a distinct role of TopoIIα in promoting H_2O_2 mediated DNA damage and to an active involvement of TopoIIβ in DNA DSBs repair activity in Ku70, mediated non-homologous end-joining (NHEJ) repair pathway.

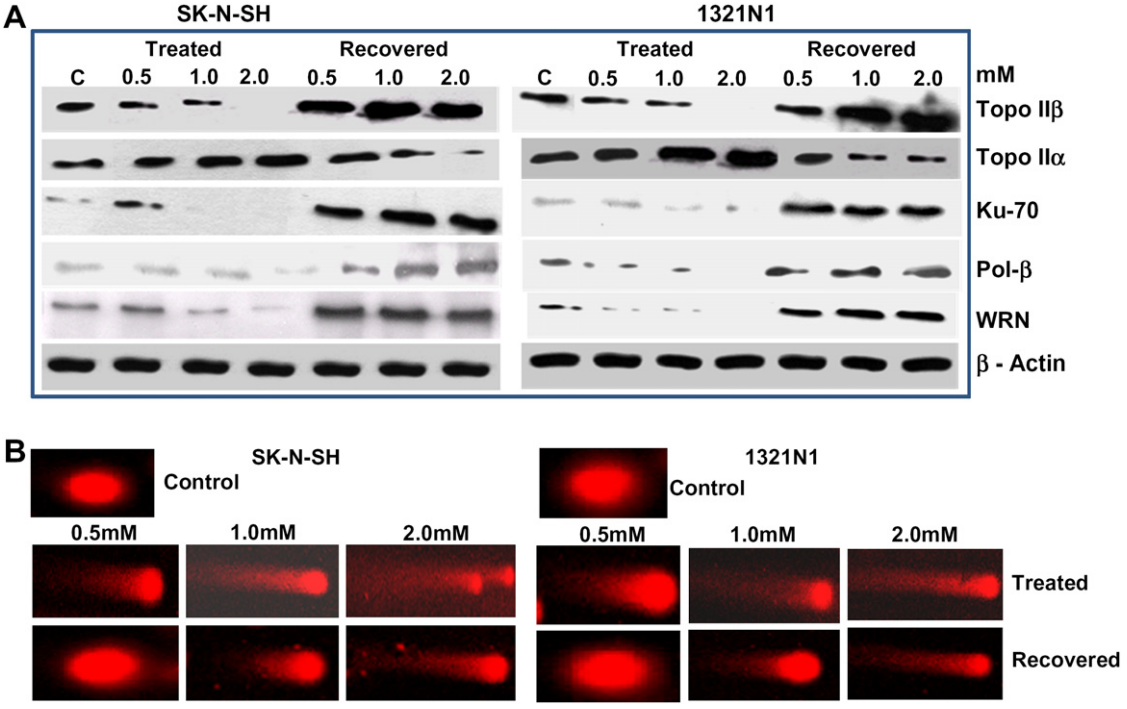

Fig. 1. The expression profile of TopoIIα, TopoIIβ and repair proteins during H_2O_2 mediated DNA damage and repair. (A) The SK-NSH and 1321N1 cells were incubated in the presence of indicated concentrations of H_2O_2 for 24 h (Treatment). After 24 h the cells were washed with fresh medium and recultured in complete medium for 72 h. The proteins in whole cell extract (100 μg) analyzed by Western blot analysis and probed with corresponding antibodies. The control lane (C) is in absence of H_2O_2 treatment ($n = 3$). Densitometry data were presented in Supporting data Fig. 1A and B. (B) Quantification of DNA double strand breaks by neutral comet assay: SK-N-SH and 1321N1 cells were treated with indicated concentrations of peroxide. Double strand breaks were quantified in treated (24 h) and recovered (72 h) cells using neutral comet assay ($n = 3$). Raw data were submitted as Supporting data Fig. 2A and B.

Table 1
SiRNA template sequences for *in vitro* transcription

Name	siRNA sense template sequence	siRNA antisense template sequence
α-Scramble	5′-ACCTCGACTGAGCAATATGTT-3′	5′-CATATTGCTCAGTCGAGGTTT-3′
α-siRNA	5′-ACTGAATAATCAGGCTCGCTT-3′	5′-GCGAGCCTGATTATTCAGTTT-3′
β-Scramble	5′-ACACTCGATCAATCCAGTGTT-3′	5′-CACTGGATTGATCGAGTGTTT-3′
β-siRNA	5′-GCTTAACAATCAAGCCCGTTT–3′	5′-ACGGGCTTGATTGTTAAGCTT-3′
T7 promoter	5′-TAATACGACTCACTATAG-3′	5′-ATTATGCTGAGTGATATC-3′

TopoIIα accelerated the formation of DSBs, while TopoIIβ promotes DSBs repair

To confirm the role of TopoIIα and TopoIIβ, further investigations were carried out through siRNA-mediated knockdown experiments involving downregulation of TopoIIα and TopoIIβ isoforms. The siRNA sequences for TopoIIα and TopoIIβ were designed as per standard protocols. The cells were transfected with TopoIIα and TopoIIβ specific siRNA (Table 1) and the protein levels were analyzed using Western blots. The results showed an efficient downregulation of these isoforms in both SK-N-SH and 1321N1 cell lines. Scrambled sequences were used as negative controls (Fig. 2).

The functional role of TopoIIα and TopoIIβ in H_2O_2 mediated DNA damage and DSBs repair was monitored by challenging TopoIIα and TopoIIβ deficient cells with H_2O_2 and the extent of double strand breaks were quantified by single cell neutral comet assay. The results showed that the TopoIIα$^-$TopoIIβ$^+$ cells (Fig. 3) were resistant to H_2O_2 mediated DNA damage, while TopoIIα$^+$TopoIIβ$^-$ cells were more than 2-fold sensitive

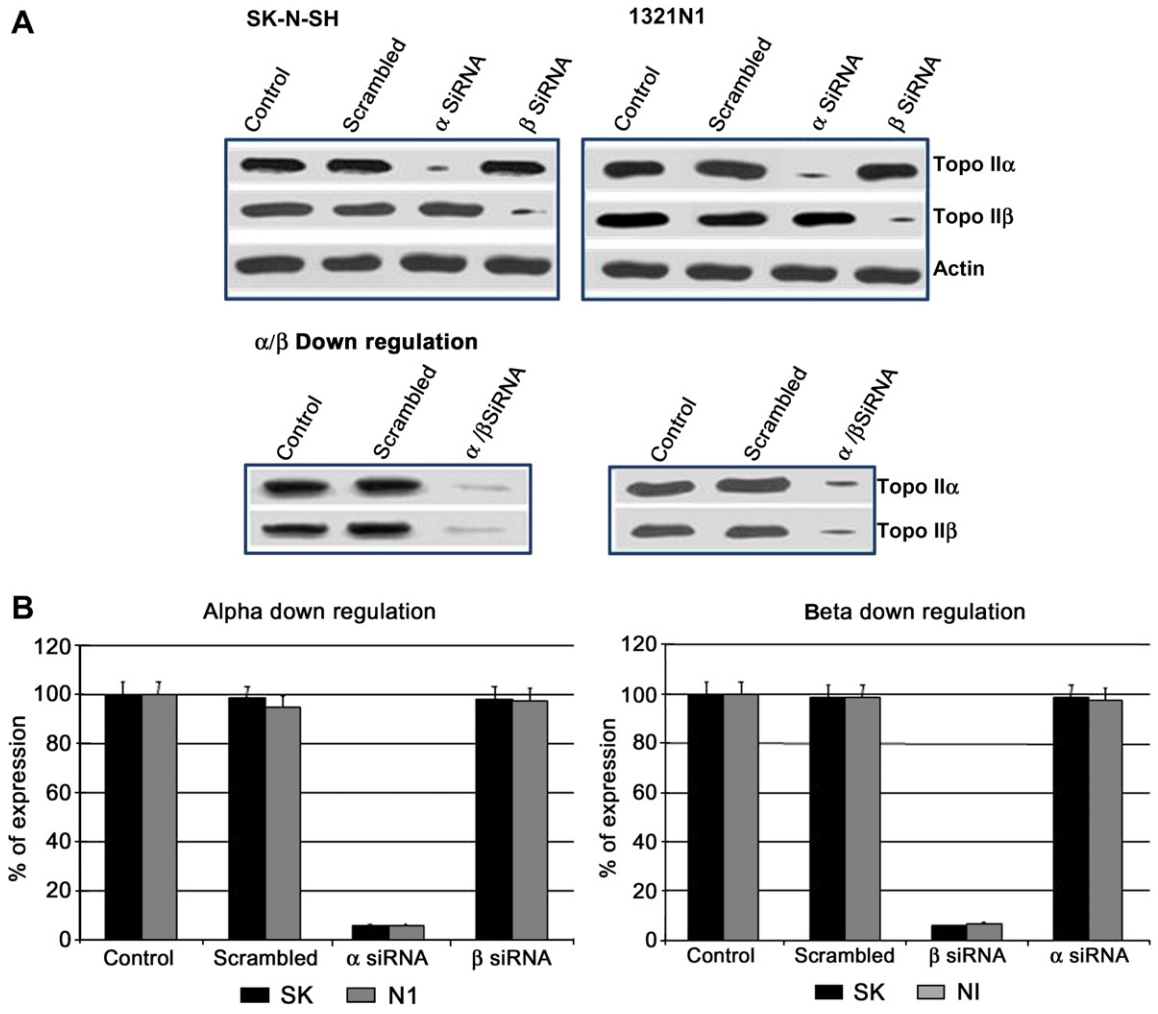

Fig. 2. siRNA mediated downregulation of TopoIIα and β in SK-N-SH and 1321N1 cells. (A) TopoIIα and β specific siRNA (Table 1) was synthesized by *in vitro* transfection and was transfected into the cells at 0.5 μm/1 × 10^6 cells. The cells were incubated for 48 h. The downregulation of TopoIIα and β was analyzed by Western blot analysis. Experiments were repeated three times and corresponding densitometry error bars were showed in (B) ($n = 3$).

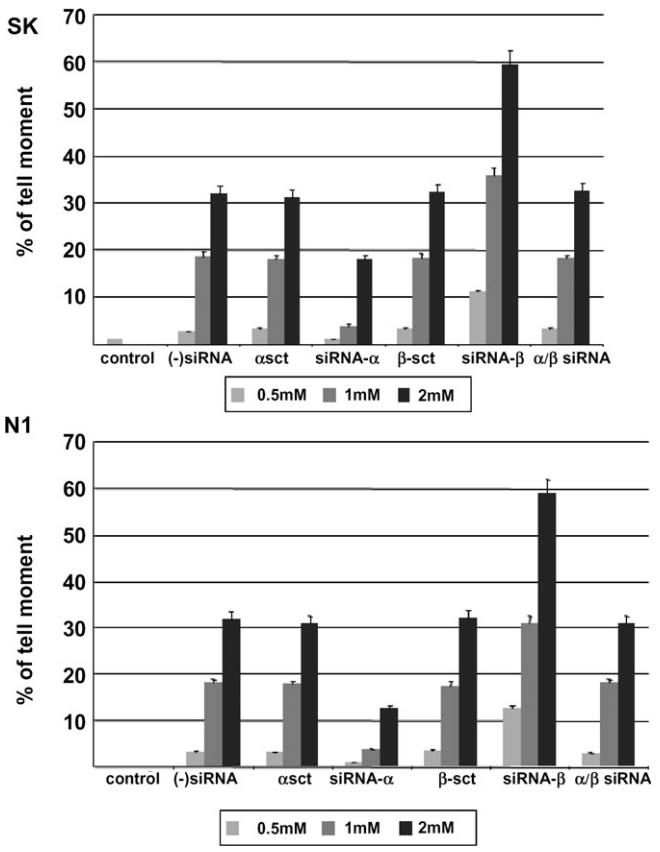

Fig. 3. TopoIIα and β downregulation drastically effect the double strand breaks formation. The TopoIIα and β downregulated SK-N-SH and 1321N1 were incubated with indicated concentrations of H_2O_2 for 24 h and followed 72 h recovery. The extent of DNA damage and recovery was analyzed by single cell neutral comet assay. The percentage of tail length was measured by using Comet-IV software (Perceptive Instruments, UK) and the extent of DNA damage was plotted on *Y*-axis. Experiments were repeated three times and means ± SE was plotted ($p < 0.05$, $n = 3$). Raw data were submitted as Supporting data Fig. 3A and B.

to the H_2O_2 mediated DNA damage suggesting that TopoIIβ present in TopoIIα deficient cells could protect them from H_2O_2 mediated DNA damage. TopoIIα$^+$TopoIIβ$^+$ (normal cells) and TopoIIα$^-$TopoIIβ$^-$ cells (Fig. 3) show similar sensitivity to H_2O_2. Alternatively while α isoform accelerates the damage of DNA, a 2-fold increase in sensitivity of TopoIIα$^+$TopoIIβ$^-$ cells to H_2O_2 indeed demonstrated the important role of TopoIIβ in promoting the DSBs repair of damaged DNA. In the absence of β isoform, H_2O_2 mediated overexpression of TopoIIα accelerates DNA damage. Further, knocking down of both TopoIIα and TopoIIβ did not effect the H_2O_2 mediated DNA damage suggesting that the resistance of TopoIIα deficient cells to H_2O_2 is either due to constitutively expressed TopoIIβ and/or due to repair-signaling associated with the upregulation of TopoIIβ. These results clearly demonstrate the distinct functions of these two isoforms, the TopoIIα being the accelerator of DNA damage and TopoIIβ, the promoter of the repair of damaged DNA.

TopoIIβ deficient cells are very sensitive to peroxide-mediated apoptotic cell death

These results are further confirmed by analysis of survival of TopoIIα$^+$TopoIIβ$^+$, TopoIIα$^-$TopoIIβ$^-$, TopoIIα$^+$TopoIIβ$^-$ and TopoIIα$^-$TopoIIβ$^+$. The results of these experiments clearly demonstrate that the TopoIIα deficient cells can survive from the insults of reactive oxidative intermediates, while TopoIIβ deficient cells show more sensitivity to this damage (Fig. 5A). The results showed that TopoIIα$^-$TopoIIβ$^+$ cells are resistant to H_2O_2 induced oxidative DNA damage compared to TopoIIα$^+$TopoIIβ$^-$ and TopoIIα$^+$TopoIIβ$^+$ cells confirming the repairing potential of TopoIIβ and the damaging function of TopoIIα.

Furthermore, large proportion of TopoIIα$^+$TopoIIβ$^-$ cells were annexin-V positive (Fig. 4) suggesting that they undergo a rapid apoptotic cell death in the presence of H_2O_2, thus pointing out to an important cell survival function of TopoIIβ in promoting DNA repair and promoting cellular recovery form H_2O_2 mediated insult. The absence of TopoIIα coupled with the presence of TopoIIβ can help cells to become repair efficient and hence show resistance to H_2O_2 mediated DNA damage due to the dominance of DNA repair process. Furthermore, the endogenous expression of TopoIIβ is thus essential for cell survival, in promoting DNA repair process against insults from oxidative reactive intermediates.

In addition, there were no significant variations in the cell population in different phases of cell cycle of TopoIIα or β downregulated cells with and without treatment of H_2O_2 (Fig. 5B).

Discussion

Topoisomerase II α known to localize in the nucleus and play important role during cell cycle, while β isoform reported be away from nucleus during cell cycle and show an reticulocyte distribution during cell cycle [6,27]. Various studies showed TopoIIβ uniformly distributed in all cell types, predominantly in neurons [5]. It has been shown to be involved in neuronal differentiation [28,29], its deficiency reported to induce neuromuscular defects in mice [30]. These observations implicate the wide spectrum of housekeeping activities of TopoIIβ in cellular development and survival. Since all types cells are frequently encountered by various toxic agents leading to the damage of DNA followed by cellular dysfunctions. The signaling mechanisms of cells rescue the damaged cells and repair the DNA followed by restoration of cellular activity. The repair and recombination functions are highly conserved among all the cell types and very important for the non-dividing cells like neurons. Since Topoisomerase II β is uniformly distributed in all cell type, shown to be predominantly present in non-dividing neurons indicating possible role of this enzyme in DNA repair and recombination activities. This is further strengthened by our observation that TopoIIβ

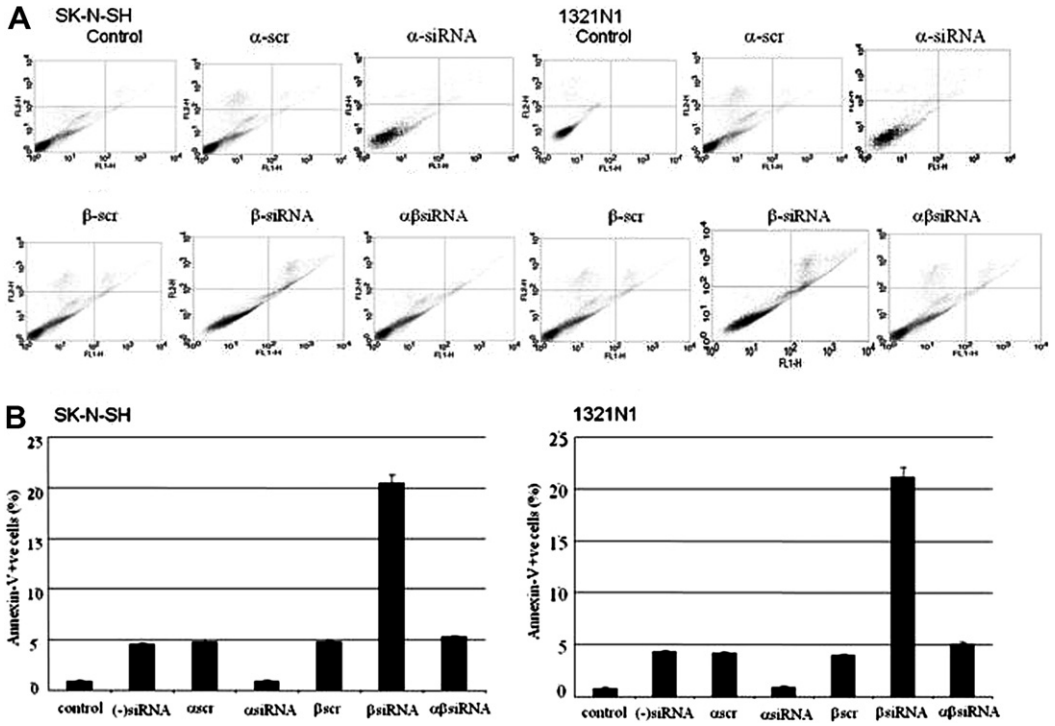

Fig. 4. Apoptosis in TopoIIα and β downregulated cells during H_2O_2 treatment. (A) TopoIIα and β downregulated SK-N-SH and 1321 N1 cells were incubated with H_2O_2 for 24 h and recultured for 72 h in peroxide free medium. The cell death in TopoIIα and β downregulated SK-N-SH and 1321N1 cells was monitored with annexin-V (FITC conjugated, Invitrogen) by Flow cytometry (BD FACS). Percent of annexin-V positive cells were represented on *Y*-axis (B) ($p < 0.01$, $n = 3$).

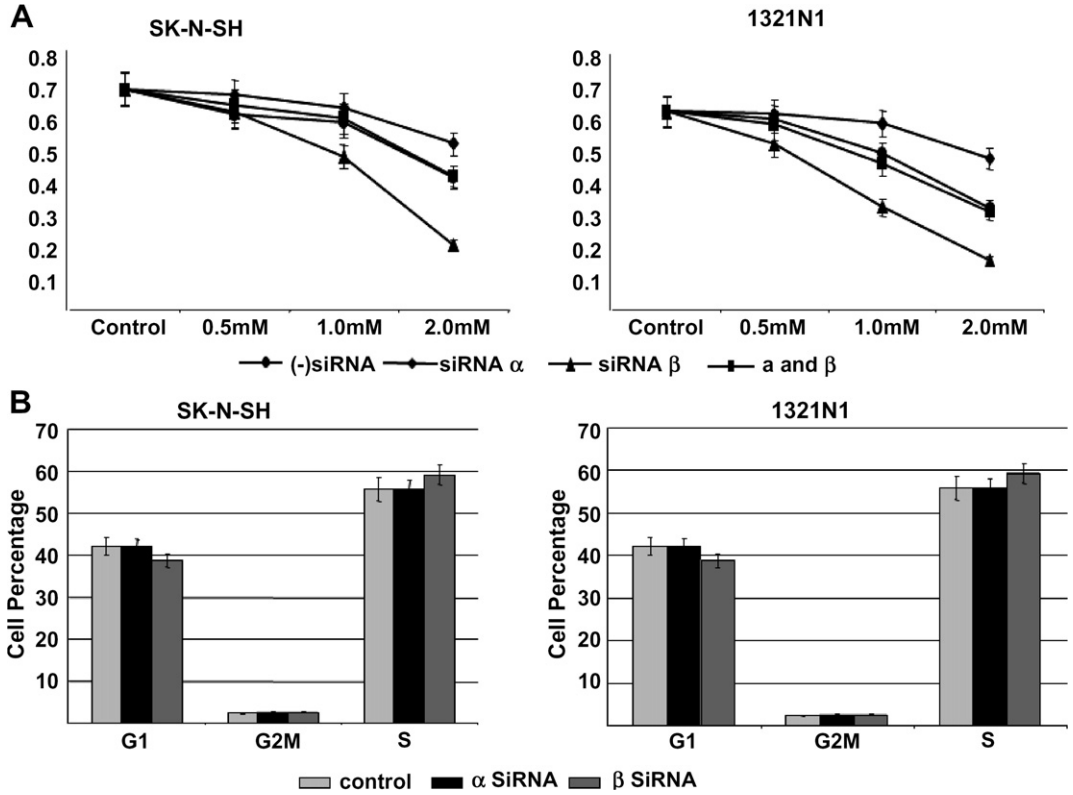

Fig. 5. Analysis of cell survival in TopoIIα and β downregulated cells during H_2O_2 treatment. (A) TopoIIα and β downregulated SK-N-SH and 1321 N1 cells were incubated with H_2O_2 for 24 h and cell survival was analyzed by MTT assay. Viable cells (in OD) were represented on *Y*-axis and the concentration of H_2O_2 on *X*-axis. Experiments were repeated three times and means ± SE was plotted ($p < 0.05$, $n = 3$). (B) TopoIIα and β downregulated SK-N-SH and 1321 N1 cells were incubated with H_2O_2 for 24 h and cell population at different phases of cell cycle was analyzed by Flow cytometry (BD FACS): composite figures of means and SD of three independent experiments.

decrease during ageing [5], the aged cells reported to have deficiencies in DNA repair machinery. Furthermore, Emmons et al. [18] using alkaline Comet assay showed that Topoisomerase II β is required for single stand breaks repair of DNA cross-links. In the present paper, we have addressed the important functions of Topoisomerase II β in double stand breaks repair. Furthermore, our results show that pro-apoptotic activity of TopoIIα through enhancement of DNA double stand breaks in the presence of peroxide. This is further supported by the observations of Liu at al. that H_2O_2 can enhance TopoIIα and DNA cross-links suggesting the enhancement of DNA damage is indeed mediated by TopoIIα. One can argue that the variation in TopoIIα and β levels is due to the presence of such cross-links. Since we have extensively boiled with SDS, such a possibility can be ruled out. If such problem exists, one should not detect Ku70, which part of protein DNA complexes that are known to be formed during DNA repair. These results further demonstrate that the DNA repair activities of TopoIIβ are conserved in both double strand and single breaks repair mechanisms.

TopoII has been shown to mediate DNA clustering and apoptosis in higher order chromatin fragmentation [31]. It has been proposed that TopoIIα binding and cleavage at scaffold associated regions (SARs) or the regions with low energy level are vulnerable to damage due to genotoxic agents and serves as breakage sites [32]. The TopoIIα may be stimulated through a double strand break (DSB)-dependent signaling pathway [33,34], with the activated TopoIIα promoting the unwinding of chromosomal DNA to expose it to reactive oxidative intermediates as well as to free radicals, leading to enhanced DNA damage. In contrast, the TopoIIβ may be activated through signaling proteins expressed during DNA repair. Repair process mediated by TopoIIβ may also promote repair of DSBs formed due to TopoIIα mediated DNA damage, through Non-homologous end-joining (NHEJ) repair pathway [35] it has been shown that TopoIIβ is involved in regulation of transcription of protein involved in DNA repair [36]. It has been reported that TopoIIα and β interact distinctly with various forms of DNA repair activities *in vitro*, these results addresses the importance of these enzymes in cellular damage and recovery against peroxide mediate-insult. Thus, TopoIIβ may be involved in both in transcriptional activation of repair enzymes or in maintaining DNA topological changes during NHEJ repair process. The functional activity of TopoIIβ may occur through an association with DNA rearrangements during branch migration and resolution of crossover intermediates. This facilitates the release of torsional stress in order to maintain the topological integrity in DNA. In the absence of such an activity of TopoIIβ, the migration and forward movement of the repairing DNA ends will be inhibited, thereby blocking the progression of DNA repair. The most important finding of this study is thus to bring out that the two catalytically conserved isoforms of Topoisomerase II, exhibit distinctly different functions *in vivo*, viz the TopoIIα accelerates the DNA damage and TopoIIβ participates in resolution of DNA rearrangements required for the DNA repair. This will be possible when the nature of substrate binding affinity of these enzymes is distinctly different. TopoII shown to bind to specific points of helix–helix juxtra positions on negatively super coiled plasmid containing crossover junctions [37] suggesting possible interaction of TopoII at DNA crossover. Furthermore, the cleavage reaction on double stranded DNA substrates contain nicks and deletions can cause the suicide to the substrates [38], especially apurinic sites are shown to stimulate DNA scission by 10- to 18-fold [39] as well as they are preferred compared to apyrimidine site in substrates that contain multiple lesions [40]. These enzymes can use RNA containing substrates, which stimulates at 8-fold higher activities than deoxyribose [41] suggesting that TopoIIα and β recognize RNA, DNA and RNA/DNA hybrids as substrates [42]. Since TopoIIα displays higher affinity and cooperativity in binding to SARs [43], association of α isoform at SAR may promote unwinding activity in chromosome leading to its exposure to reactive oxygen species that induce nicking and such nicks may stimulate TopoIIα activity and promotes strand cleavage and enhance double strand breaks. Since geometry and conformation of DNA is determining factor in TopoIIβ binding, it is possible that the conformation of DNA occur during the repairing complex formation may promote the TopoIIβ binding at the damaged DNA termini and progress the DNA crossover [37] during repair process. The activity of TopoIIβ at the repair ends may maintain the torsional equilibrium in the chromosome thus prevent the transmission of torsional stress to SARs, thus TopoIIα mediated unwinding process may not be activated.

Assessment of DNA repair activity of TopoII using TopoII poisons etoposide [44] and Novobiocin may activate mixed signaling pathways viz. One due to the drug-mediated DNA damage, and the other due to drug–protein complex mediated signaling processes. In the present case, since such a problem does not arise, one can utilize the role of these enzymes themselves in understanding DNA damage and repair processes without such involvement of any non-specific signaling pathways. The result that TopoIIα accelerates DNA damage and TopoIIβ promotes DNA repair suggesting an important repair function of TopoIIβ in all cell types provides new directions toward understanding the important functional role of this isoform in aging brain.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2007.10.017.

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Regulation of topoisomerase II α and β in HIV-1 infected and uninfected neuroblastoma and astrocytoma cells: Involvement of distinct nordihydroguaretic acid sensitive inflammatory pathways ☆

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Abstract

The activity of Topoisomerase II α and β isoforms is tightly regulated during different phases of cell cycle. In the present study, the action of anti-inflammatory agents, nordihydroguaretic acid (NDGA) is analyzed in HIV-1 infected $CXCR4^+$, $CCR5^+$ and $CD4^-$ SK-N-SH neuroblastoma, $CXCR4^+$, $CCR5^+$ and $CD4^-$ 1321N1 astrocytoma and $CXCR4^+$, $CCR5^{+/-}$ and $CD4^-$ GO-G-CCM glioblastoma cell lines. In SK-N-SH and 1321N1 the expression of Topoisomerase II α is concomitant with that of LOX-5 and is highly sensitive to NDGA, while the Topoisomerase II β is expressed along with TNFα and exhibits low sensitivity to NDGA, suggesting distinct pathways of regulation for the two isoforms. HIV-1 infection in these cells enhanced the expression of Topo II α and β. Further, the regulation of Topo II β and TNFα in infected and uninfected SK cells is distinctly different. HIV-1 gp120 derived peptides could block HIV-1 mediated inflammation and Topoisomerase II α and β expression, suggesting the viral mediated response. A combination of NDGA, gp-120 derived peptides and AZT has completely blocked the viral replication, suggesting the enhancement of potency of AZT under the suppression of inflammatory response. In contrast, the expression of Topo II α and β was stimulated by NDGA in GO-G-CCM cells showing distinct regulatory pathway in these cells that was resistant to HIV-1 infection. This suggests the requirement of inflammatory response for productive viral infection. In summary, an induction of co-receptor mediated inflammatory response can distinctly enhance regulated expression of the cellular Topo II α and β and promote productive infection in neurons and astrocytes.

Keywords: Neurons; Astroytes; Inflammation; Actin cleavage; Topoisomerase II; TNFα; gp120 peptides; NDGA; HIV-1

Neurological disorders are the first manifestation of symptomatic HIV-1[1] infection in 10–20% of population, while about 60% of seropositive persons with advanced HIV disease will have clinically detectable neurological dysfunction [1–3]. The incidence of sub clinical neurological disease is even higher along with peripheral neuropathy and HIV associated cognitive dysfunction leading to HIV associated dementia [4,5] in early infection. HIV crosses the blood brain barrier and enters the neuronal system with concomitant internal systemic infection [6]. HIV virus has been cultured from brain, nerve and cerebrospinal fluid (CSF) from patients during HIV infection [7,8]. 50% of patients with AIDS eventually show neurological complications directly attributable to the infection of the brain by the HIV-1. While HIV-1 viral turnover in circulation is primarily due to infection of $CD4^+$ cells, specifically T-lymphocytes and monocytes/macrophages, however, experimental evidence suggests that expression of the CD4 molecule is not the sole factor determining viral entry

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[1] *Abbreviations used:* HIV, human immunodeficiency virus; LOX-5, lipoxygense-5; TNFα, tumor necrosis factor α; IFNγ, interferon γ; HAD, HIV-associated dementia; NDGA, nordihydroguaiaretic acid; AZT, 3′-azido-3′-deoxythymidine; Topo II, topoisomerase II.

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[9]. Additionally, CD4 independent HIV-1 infection has been demonstrated in-vitro in several human cell lines and in primary human cell cultures [10]. These observations indicate that other cell surface molecules function as co-receptors in the presence of CD4 molecule or as alternative receptor in the absence of CD4. Recent findings *in vivo* indicated that $CD4^-$ cells in the brain, including astrocytes, endothelial cells and neurons harbor HIV-1 infection [11]. But some neurological cell types are sensitive to virus, while others are resistant. Several theories have been proposed regarding HIV-1 mediated neuropathogenesis, such as the aberrant cytokine production by HIV infected microglial and glial cells, neurotoxicity of gene products of virus (gp120 and tat) and alteration of function of astrocytes by cytokines and toxins produced by HIV gene products [12,13].

The cytokines TNFα and IFNγ have been implicated in the development and progression of multiple sclerosis (MS) and AIDS associated dementia complex. TNFα is a 17 KDa peptide produced by a wide range of cells [14]. TNFα plays an important role in (1) inflammation, (2) the modulation immunoresponse by affecting the expression of class I and class II MHC molecules and adhesion molecules, (3) stimulation of cytokines such as IL-1, IL-6, IL-8 and IFNγ [15]. TNFα participates in the inflammatory reaction within the CNS. TNFα positive macrophages and astrocytes have been identified in the brain of MS patients, particularly in the plaque region [16]. TNFα induction of IL-6 expression in astrocytes occurs by protein kinase C dependent pathway. Inflammatory markers shown to be enhanced in HIV-1 associated dementia, HIV-1 gp120 is shown to be involved in such response in neuroblastoma cells [17].

Topoisomerase II α (Topo II α) and Topoisomerase II β (Topo II β) are 170 and 180 KDa proteins; they promote the replication of viral DNA and chromosomal DNA [18]. Topo II α, is highly regulated during cell division, Topo II β is associated with non-proliferating function. The objective of the present investigation is to examine if systemic inflammation can regulate Topo II α and β in neuronal and astrocytes. The regulation of Topo II α and β were analyzed based on the sensitivity of their expression to NDGA. The results showed that inflammatory response in neurons and astrocytes regulate the expression of Topo II α and β in different pathways. The systemic inflammation in neurons and astrocytes was also induced as a stress response related to the HIV-1 infection and the results showed the regulation of induction of inflammatory response for productive HIV-1 replication mediated by cellular receptor CCR5 in neurons, astrocytes and glial cells.

Materials and methods

The following reagents were obtained from AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA. The reagent contributor name is given in parenthesis. SupT1 cell line (Dr. J. Hoxie). HIV-1 virus subtype C, HIV-$1_{93IN101}$ (Dr. R. Bollinger), pNL4-3 (Dr. Malcolm Martin), SK-N-SH (Neuroblastoma), GO-G -CCM (Glioblastoma) and 1321N1 (astrocytoma) cell line were obtained from NCCS, Pune, INDIA.

V3-peptide (TRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAH-NH2., (5), MN-peptide (306-327 YNKRKRIHIQRGPGRAFYTTKNII (C),. [19,20] from NIH USA.

Mouse anti human Topoisomerase II α and β were from BD biosciences. Monoclonal anti-human CD4, CXCR4 and CCR5 from NIH USA. Monoclonal anti-human TNFα, Rabbit anti-human COX-2 and LOX-5 from US biological. The secondary antibodies were from UPSTATE USA. NDGA and AZT from Sigma–Aldrich. Each experiments was repeated three times. ELISA assays were carried out in triplicates. Data was plotted as an average of triplicates with standard deviation.

Cell culture

SK-N-SH neuroblastoma cell line was maintained in EMEM with 0.1 mM non-essential amino acids, 0.01 mM sodium pyruvate and 10% fetal bovine serum, GO-C-CCM and 1321N1 cell line were maintained in DMEM with 10% FBS. SupT1 cell line were maintained in RPMI with 10% FBS.

FACS analysis

The FACS analysis was done according to Bruce et al. (1990) [21] Briefly to prepare cells for fluorescence-activated cell sorter (FACS) analysis, adherent cells were removed from plastic tissue culture flasks by trypsinization for 8 min at 37 °C. Fetal calf serum was added to 25% to block trypsin activity. The cells were then filtered through nylon mesh to remove clumps, counted, and distributed to a 96-well tray (5×10^5 cells per well) in 50 μl of RPMI 1640 medium containing 0.01 M NaN_3, washed twice with RPMI-azide, and then incubated with mouse anti-human monoclonal antibodies (1:50 dilution) CD4, CXCR4 and CCR5 in 50 μl of RPMI-azide for 1 h at 4 °C. After two washes with RPMI-azide, cells were incubated in 150 μl of 1:180 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin in RPMI-azide for 1 h at 4 °C. after two washes with RPMI-azide, the cells were suspended in 1 ml of PBS containing 1% formaldehyde. The stained and fixed cells were analyzed on a FACSTAR cell sorter (Becton Dickinson, Mountain View, Calif.) with an argon ion laser emitting 488-nm light at 200 mW.

Infection assay

5×10^6 cells were seeded into the 60 mm dishes one day before the experiment, the cells were challenged with HIV-1 (20 ng/ml) in a medium containing 2% serum, and after 2 h post-infection, serum was increased to 10%. The infection was stopped at 4 and 24 h the time at which Topo II α and β and TNFα show significant expression.

For the p24 quantification the medium is aspirated out completely 48 h post-infection and cells were washed twice with the medium and supplemented with complete medium containing 10% FBS. HIV-1 was quantified in terms of p24 after 96 hours of post-infection.

HIV-1 infection in presence of NDGA and peptides

One day before the experiment, 5×10^6 cells were cultured in the 60 mm dishes. Drug and peptides were added to the cells at indicated concentrations and incubated for 10 min. These cells were challenged with HIV-1 as explained in infection assay.

Proviral DNA isolation

Cells (0.5×10^6) were challenged with HIV-$1_{93IN101}$ (200pg of p24 viral core protein) in the presence of peptides and NDGA at 5% CO_2 and 37 °C. The cells were harvested at 5 h p.i and washed with phosphate-buffered

saline. They were then lysed with 50 µl lysis buffer containing 10× Solution A (1 M KCl, 100 mM Tris–HCl (pH 8.3), 2.5 mM $MgCl_2$), 10× Solution B (100 mM Tris–HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.45% Tween 20, 0.45% Nonidet P 40, 50 mM NaCl). The cell lysates were treated with RNaseI (10 µg/ml) and incubated at 37 °C for 30 min. Proteinase K (60 µg/ml) was added to the lysates and incubated at 56 °C for 2 h followed by the inactivation of Proteinase K at 95 °C for 10 min. The lysates were then stored at −20 °C until they were used for PCR.

PCR analysis of proviral DNA

The Cell lysates were added to the 50 µl of reaction mixture comprising of 10x PCR buffer, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 2.5 mM $MgCl_2$, 0.40 µM SK38, SK39 primers (5′-ATAA TCCACCTATCCCAGTAGGAGAAAT-3′), SK39 (5′-TTTGGTCCT TGTCTTATGTCCAGAATGC-3′) [7] (Synthesized by Integrated DNA Technologies (IDT), USA) and 0.5 U of *Taq* DNA Polymerase (Biogene, USA) [22,23]. The mixtures were heated to 94 °C for denaturation for 2 min and then subjected to amplification for 30 cycles of PCR (1 min 94 °C, 1.30 min 60 °C and 2 min 72°), and a final step for extension at 72°C for 5 min. Control amplification was done using β-actin specific primers Froward:5′-GGCCCAGAGCAAGAGAGGTATCC-3′, Reverse-5′-CGCACGATTTCCCTCTCAGC-3′ [24]. The products were resolved on 2% agarose gel electrophoresis, ethidium bromide stained and photographed.

Preparation of cell extracts

HIV-1 challenged cells were pelleted down at 300*g* for 10 min and washed twice with PBS (Phosphate-buffered saline). SK-N-SH and GO-C-CCM cells were harvested by trypsinization with 0.25% of trypsin and 1 mm EDTA The trypsin was inactivated by 20% serum., the cells were pelleted at 300*g* for 10 min and washed twice with PBS. Cells were lysed in RIPA (50 mM Tris–HCl (pH 7.5), 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 150 mM NaCl, 1 mM PMSF, 1% Aprotenine, 10 µg/ml pepstatin and leupeptin) buffer incubated in ice for 1hour and centrifuged at 3000*g* for 30 min. Supernatant was used for the protein analysis.

Western blotting

Western blots were performed as described in Toubin et al. 1979 [25]. 100 µg of protein was isolated from infected cell. Lysate boiled with buffer containing SDS and resolved on 7.5%, 12% SDS–PAGE. The proteins on the gel were transferred to nitrocellulose membrane, then immunoblotted [26] with a 1 ng dilution of mouse anti-human TNFα, Topoisomerase II α and β monoclonal antibodies and incubated with alkaline phosphatase (ALP) conjugated goat anti-mouse IgG antibody (1:2000 dilutions in TBS) for 60 minutes at room temperature and washed with TBS. The blots were developed with NBT-BCIP substrate in ALP buffer and documented.

Results

The present study aims at understanding the HIV-1 induced inflammatory response mediated signaling pathway and the regulation of the expression of DNA Topoisomerase II (Topo II) isoforms in CD4 negative cells. Further, the significance of anti-inflammatory compound NDGA and gp120 derived peptides in progression of viral replication in neurons and astrocytes is investigated. The neuroblastoma SK-N-SH (SK) was used as representative cell line for neurons, 1321N1 (N1) for astrocytes and GO-C-CCM (GO) for glial cells. Indian isolate HIV-$1_{93IN101}$ was used in all experiments.

The sensitivity of SK, GO and N1 cell lines to HIV-1 infection

Neuroblastoma, astrocytoma, and glioblastoma, were challenged with HIV-$1_{93IN101}$, the amount of virus replicated at day 4 were analysed, the results presented in Fig. 1A show that SK, and N1 were sensitive to HIV-1 infection, while GO cell line is resistant to HIV-1 infection. These results are confirmed by the analysis of proviral DNA after 5 h of post-infection (Fig. 1B). SupT1 cells were used as a control cell line.

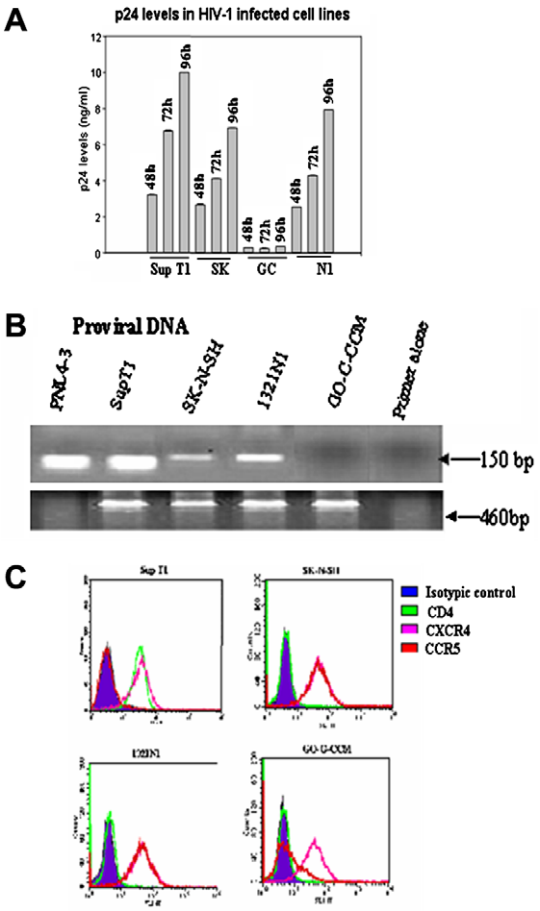

Fig. 1. Sensitivity of neurons and astrocytes to HIV-1 infection. SK-N-SH (SK), 1321N1(N1), SupT1 and GO-G-CCM (GO) cell lines (5×10^6) were infected with 10 ng/ml of HIV-$_{93IN101}$. Cell supernatant was collected at 48, 72 and 96 h of post-infection (p.i). HIV was estimated in terms of p24. SupT1 is used as positive control. (A) HIV-1 replication in terms of p24 on *Y*-axis and cell lines on the *X*-axis. Each experiment was repeated three times and data was plotted as an average of triplicates and standard deviation. (B) Proviral DNA in infected SK, N1, SupT1 and GO cell lines. The cells were harvested after 5 h of post-infection and proviral DNA was analyzed by amplification with gag specific SK38 and SK39 primers. Top panel is 150 bp gag specific product and the bottom panel is 450 bp of actin. (C) Expression of receptors in neurons and astrocytes SupT1, SK, N1 and GO were probed with mouse anti-human CD4, CXCR4 and CCR5 antibodies, the bound antibody was probed by Goat anti-mouse IgG secondary antibody conjugated with FITC was sorted using FACS. The result was overlaid (Blue: isotypic control, green: CD4, pink: CXCR4 and red: CCR5).

Expression of CD4, CXCR4 and CCR5 receptors in SK, N1 and GO cell lines

Since SK and N1 cells are sensitive to HIV-1 infection, while GO cells are resistant, these cells are analyzed for the presence of primary receptor CD4, and co-receptors CXCR4 and CCR5 using Flow cytometry. The results show (Fig. 1C) that the SK, N1 and GO cell lines are CD4 negative. Further, SK and N1 cell lines express both CXCR4 and CCR5 receptors, in contrast GO expressed only CXCR4, with very low expression of CCR5.

Expression of Topo II α and β and inflammatory cytokines in SK-N-SH, 1321N1 and GO-G-CCM cells in presence and absence of HIV-1

The cells were incubated with HIV-1 for 24 h and the whole cell extract was analyzed for TNFα, LOX-5 and Topo II α and β by western blot. The results show that Topo II β expression is enhanced along with TNFα, while Topo II α expression increases concomitant with LOX-5 expression (Fig. 2). This Suggests that Topo II β may follow TNFα mediated inflammatory pathway, while Topo II α may be up regulated during stress response in the cells due to induction of activity of LOX-5, thus suggesting distinct modes of Topo II α and β regulation.

Action of anti-inflammatory molecule NDGA during HIV-1 induced inflammation and viral replication

The cells were challenged in presence and absence of HIV-1 with increasing concentrations of NDGA. The results in uninfected cells show (Fig. 3) that the Topo II α and LOX-5 expressions are diminished at 10μm in SK (Fig. 3) and N1 (Fig. 3) cells, and there is no effect on the TopoII α expression in GO cell line (Fig. 3). The analysis of expression of TopoII β and TNFα shows that 50 μm of NDGA is required to inhibit these proteins expression in SK, N1 cells, in contrast, the expression of TNFα and TopoII β are enhanced with increasing concentration of NDGA in GO cell line. Where as in infected cells (Fig. 4) the effect of NDGA on Topo II α and Lox-5 remains the same as that of uninfected cells (Fig. 3) suggesting the regulation of Topo II α is NDGA sensitive and follow similar pathways in both infected and uninfected SK and N1 cells. Where as Topo II β and TNFα expression shows low sensitivity to NDGA in HIV infected SK cells (Fig. 4) compared to uninfected SK cells suggesting HIV-1 infection induces an alternative NDGA resistance pathway for Topo II β and TNFα regulation in SK cells, while Topo II β and TNFα regulation remain same in both infected and uninfected N1 cells suggesting that the HIV-1 infection follow similar Topo II β and TNFα regulatory pathways in presence and absence of HIV infection.. Hence, the expression of Topo II β may be tightly linked with TNFα or it is possible that TNFα co-regulates Topo II β. These results suggest that under stress (virus infection) condition, the expression of Topo II α is regulated in LOX-5 dependent pathway, while the expression of Topo II β is regulated in TNFα dependent path way. In HIV-1 resistant cells the regulation of Topo II α and β may be distinctly different: For example in GO cell line, the levels of Topo II α remain unaltered, while Topo II β and TNFα are enhanced by blocking an NDGA sensitive pathway.

Action of gp-120 derived peptides on replication

We have chosen two gp120 peptides namely V3 and MN peptides. These peptides inhibit viral entry in-vitro (data not shown). To monitor the involvement of gp-120 epitopes in virus induced inflammation, we have performed competitive experiments of HIV-1 infection with two gp-120 derived peptides. The peptides did not show any cytotoxicity to the cells (data not shown). The cells were incubated in the presence of increasing concentrations of peptides and were challenged with the virus for 5 h. The virus was washed and the cells were then recultured in fresh media and the amount of virus replicated after 4 days was estimated in SK and N1 (Fig. 7). The results show both the peptides can compete with the virus interaction to the

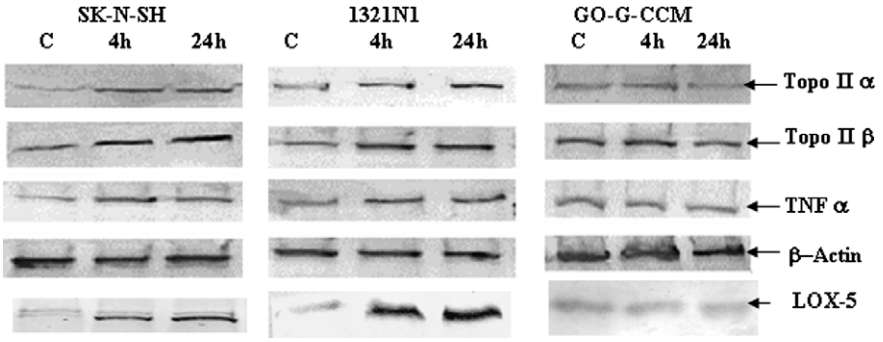

Fig. 2. Cellular expression profile in neurons and astrocytes during HIV-1 infection. SK-N-SH, 1321N1 and GO-G-CCM cell lines were infected with 10 ng/ml of HIV-$1_{93IN101}$. The cells were harvested at indicated time points of post-infection, and lysed. Total protein was estimated and 100 μg of total protein was loaded onto each well and separated on SDS–PAGE and western blotted. Then probed with indicated antigen specific antibodies and detected with ALP conjugated secondary antibodies.

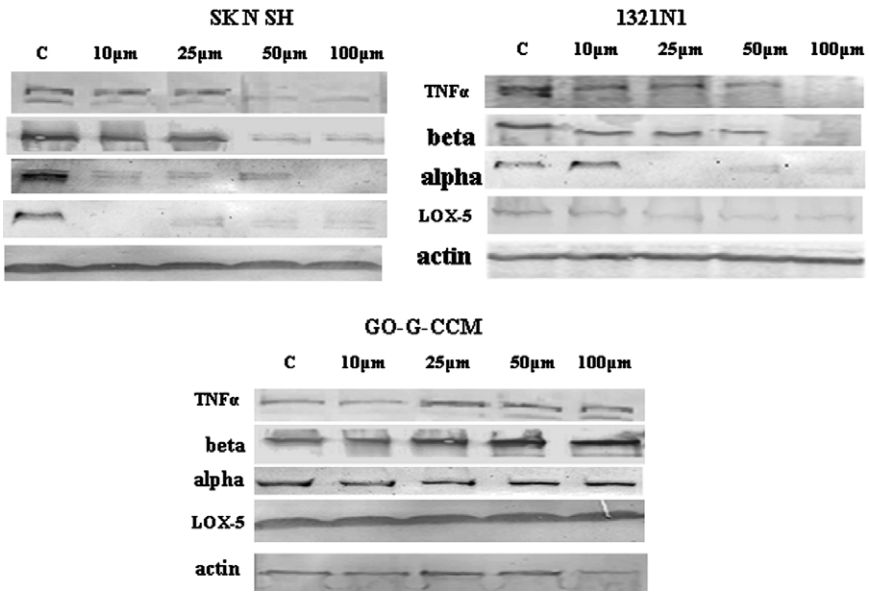

Fig. 3. NDGA sensitivity of Topo II α and β in SK, and N1 cells. SK-N-SH, 1321N1 and GO-G-CCM cells were incubated with increasing indicated concentration of NDGA in complete media and incubated for 24 h at 37 °C. Total protein (100 μg) was separated on SDS–PAGE and Western blotted and probed with antigen specific antibodies.

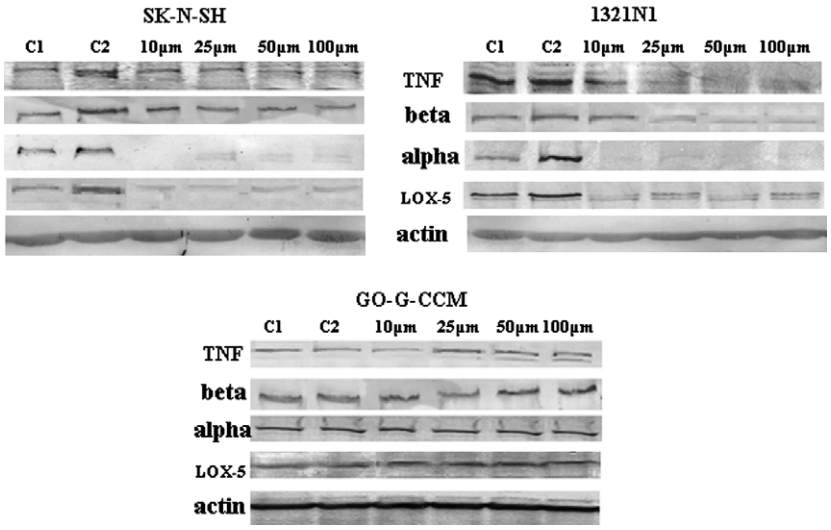

Fig. 4. NDGA sensitivity of expression of Topo II α and β in HIV-1 infected SK, and N1 cells. SK-N-SH, 1321N1 and GO-G-CCM cells were incubated for 24 h in the presence of both HIV-1 and indicated concentration of NDGA at 37 °C. Total protein (100 μg) was separated on SDS–PAGE and western blotted and probed with antigen specific antibodies. C1: Uninfected cells without NDGA and C2: Infected cells without NDGA.

receptors in cell lines, suggesting that the interaction of virus in these cell types can be competed by the peptides. The analysis of TNFα, Topo II α and TopoII β in the infection, conducted in the presence of peptides alone, show that these peptides itself cannot induce any inflammatory response in uninfected cells (Fig. 5, lanes 2–4), while these peptides can produce a 50% decrease in HIV-1 mediated up-regulation of TNFα and Topo II β levels in SK and N1 (Fig. 5, lanes 6–9) cells. While the levels remain unchanged in GO (Fig. 5) cells. While the Topo II α is significantly decreased in SK and N1 cells and no change was observed in Topo II α in GO cells. The results suggest that the blockage of gp-120 mediated virus binding by the neutralizing peptides can inhibit the up-regulation of TNFα, Topo II β and Topo II α. Hence gp120 mediated virus binding may be involved in the induction of inflammatory response and Topo II α and β in neurons and astrocytes.

Both the Peptides inhibited gp120$_{JR\text{-}FL}$ mediated inflammation in SK-N-SH and 132N1 cells

SKN-SH neuroblastoma and 132N1 astrocytoma cells were incubated with increasing concentrations of gp120$_{JR\text{-}FL}$, and the levels of TNFα, Topo II β, Topo II

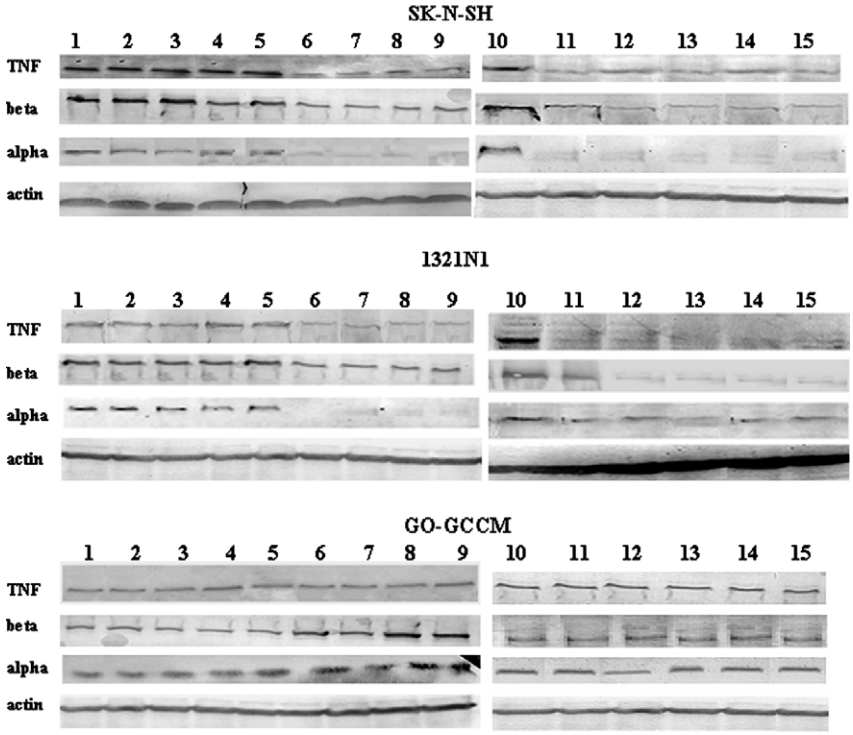

Fig. 5. The sensitivity of cells to peptides in presence and absence of HIV-1. SK-N-SH, 1321N1 and GO-G-CCM cells were treated with peptides in presence and absence of HIV-1, cells were harvested after 24 h. One hundred micrograms of total protein was resolved on SDS–PAGE and Western blotted and probed with antigen specific antibodies. (#1: No peptide, # 2: 20 ng V3 and #3: 40 ng V3; # 4: 20 ng Mn #5: 40 ng Mn, # 2: 20 ng V3 and #3: 40 ng V3; # 4: 20 ng Mn #5: 40 ng Mn; # 6: HIV+ 20 ng V3 and #7: HIV +40 ng V3; #8: HIV + 20 ng Mn #9: HIV+ 40 ng Mn, # 10: HIV alone, #11: HIV+ 25 μM NDGA; # 12: HIV+ 25 μM NDGA+ 20 ng V3; # 13: HIV+ 25 μM NDGA+ 40 ng V3; # 14: HIV+ 25 μM NDGA+ 20 ng Mn; # 15: HIV+ 25 μM NDGA+ 40 ng Mn.

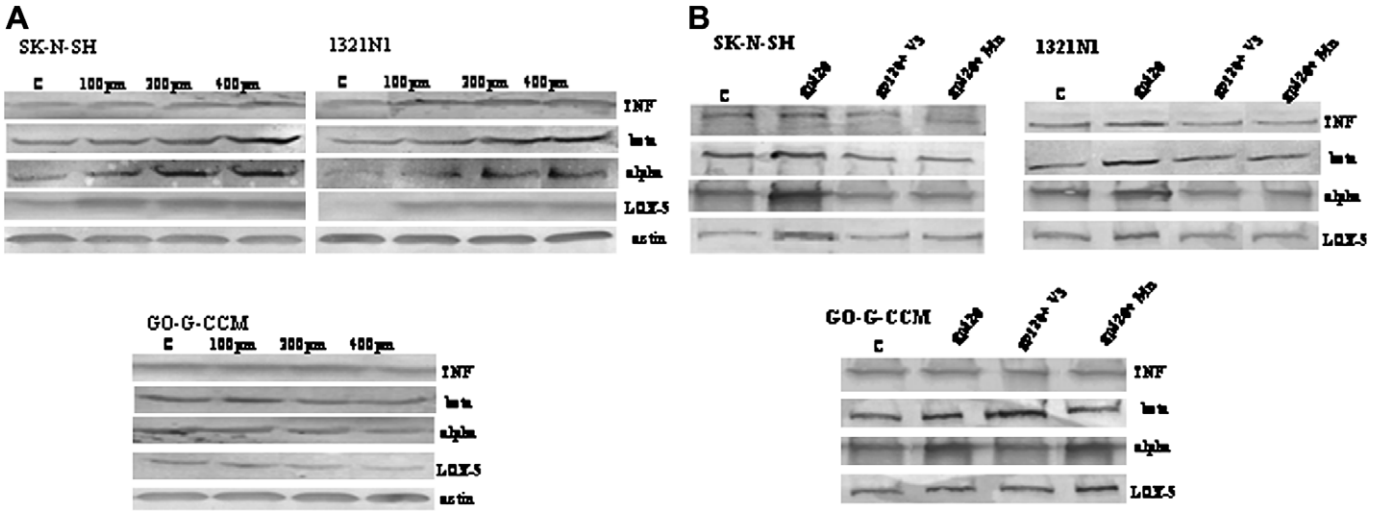

Fig. 6. (A) gp120 induced inflammation in SK, N1 and GO cells. SK, N1 and GO cells were challenged with increasing concentrations of (100, 200, and 400 pm) gp120 (JR-FL) for 24 h and harvested. One hundred micrograms total protein was resolved on SDS–PAGE and Western blotted and probed with antigen specific antibodies. Blots were quantified using Image J (NIH) software and density of bands was represented on *y*-axis (Supporting A). (B) Down-regulation of gp120 induced inflammation by V3 and MN peptides: SK, N1 and GO cells were challenged with 200 pm of gp120 in presence of 20 ng of V3 or MN peptide for 24 h and harvested. One hundred micrograms total protein was resolved on SDS–PAGE and Western blotted and probed with antigen specific antibodies. Blots were quantified using Image J (NIH) software and density of bands was represented on *y*-axis (Supporting B).

α and Cox 5 were monitored by Western blot analysis. The results in Fig. 6A show that the levels of Topo II α and β increased significantly along with TNFα and Cox 5 suggesting that gp120 induces inflammation of SN-N-SH and N1 cells, GO-G-CCM Gliobalstoma cells show an increase of Topo II α and β at 100 pM of gp120, while their

levels decreased from 200 pM of gp120. While Cox-5 levels decreased with increasing concentrations of gp120. No change in the levels of TNFα has been observed thus suggesting that these cells are resistance to gp120-mediated inflammation.

Analysis of activity of peptides on the gp120 mediated inflammatory activity show that both peptides inhibits $gp120_{JR\text{-}FL}$ mediated inflammation in SK-N-SH and 12N1 cells (Fig. 6B), where as the peptides did not show any effect on TNF and Cox 5 levels in GO cells (Fig. 6B). In addition, the results indicate that the peptides have distinct action on gp120-mediated Topo II α and β regulation in GO cells.

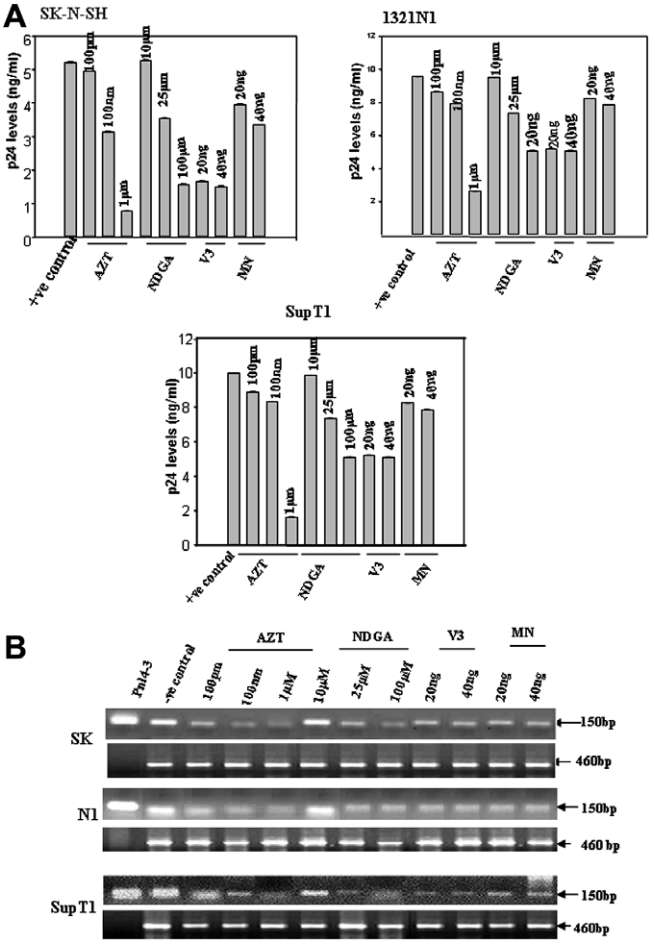

Fig. 7. Action of AZT, NDGA, and peptides on HIV-1 replication: (A) SK, N1 and SupT1 cells were challenged with virus in presence of AZT (100 pm, 100 nm and 1 μm), NDGA (10, 25 and 100 μm), V3 (20 and 40 ng) and MN (20 and 40 ng), the replicated virus was quantified in terms of p24 at day 4 of post-infection. p24 levels were plotted on *Y*-axis. The positive control was HIV-1 infected SupT1 cells in absence of agents. (B) Proviral DNA synthesis in AZT, NDGA and peptides treated cells: SK, N1 and SupT1 cells were challenged with HIV-1 in presence of increased concentration of AZT, NDGA and peptides as indicated and cells were lysed and proviral DNA was analysed using gag specific SK38 and SK39 primers. The 150 bp band is gag specific amplification and 450 bp band is actin specific amplification.

Combination of anti-inflammatory NDGA and gp120-derived peptides can efficiently neutralize HIV-1 virus and decrease virus-induced inflammation

In order to investigate, if peptides and anti-inflammatory drug NDGA can act synergic in terms of virus neutralization, we have conducted infection assay studies in the presence of 25 μM of NDGA and 20 ng of V3 and MN peptides. The results show that the NDGA and both the peptides in combination can efficiently inhibit virus replication (Fig. 8A) and proviral DNA synthesis (Fig. 8B), suggesting that anti-inflammatory condition would help in enhancing the anti-viral activity of gp-120 peptides. To test if such an activity of NDGA is due to the blocking of viral entry, cell fusion assay was conducted in the presence of NDGA. The results show that NDGA cannot block viral entry (data not shown). It may be inferred that virus associated inflammation may promote post-entry event of replication. To verify if NDGA can exhibit similar activity with HIV-1 drug AZT, we have conducted anti-viral activity experiments with NDGA and AZT in combination. The results (Fig. 8A and B) show that the NDGA indeed enhances the anti-viral activity of AZT suggesting the importance of controlling inflammation in anti-viral therapy.

To know the time point at which the anti-inflammatory action is important in stimulating anti-retroviral activity of AZT, we have incubated cells with AZT and NDGA is added at different time points during the post infection. The results (Fig. 9) show that the proviral DNA is completely removed if the NDGA is added along with AZT, while NDGA addition after 30 min would enhance the activity of AZT to a lesser extent. To examine, whether this is due to the down regulation of inflammatory response in the cell, we have analyzed the levels of TNFα, Topo II β, LOX-5 and Topo II α in infected cells when NDGA was added during the course of HIV-1 post infection. The results (Fig. 9) confirm that the priming of cells for 10 min can down regulate the viral mediated inflammatory response, enhancing the anti-viral property of AZT.

Discussion

HIV-1 invades brain-causing impairment of certain cognitive functions at asymptomatic stage of infection [27]. It replicates in various cell types of brain such as astrocytes, microglia [28] and neurons from different regions of brain. The replicated viral antigens can modify various neuronal functions in terms of up-regulation/down-regulation of genes. Such a regulation may involve both viral and cellular factors for promoting genetic rearrangements associated with DNA replication [29], transcription, recombination and repair and apoptosis [9] in neurons and astrocytes. The synthesized viral proteins are known to interfere with such cellular processes, leading to neurodegeneration and causing HIV associated encephalopathy [18] and dementia [30,31] during symptomatic stage of

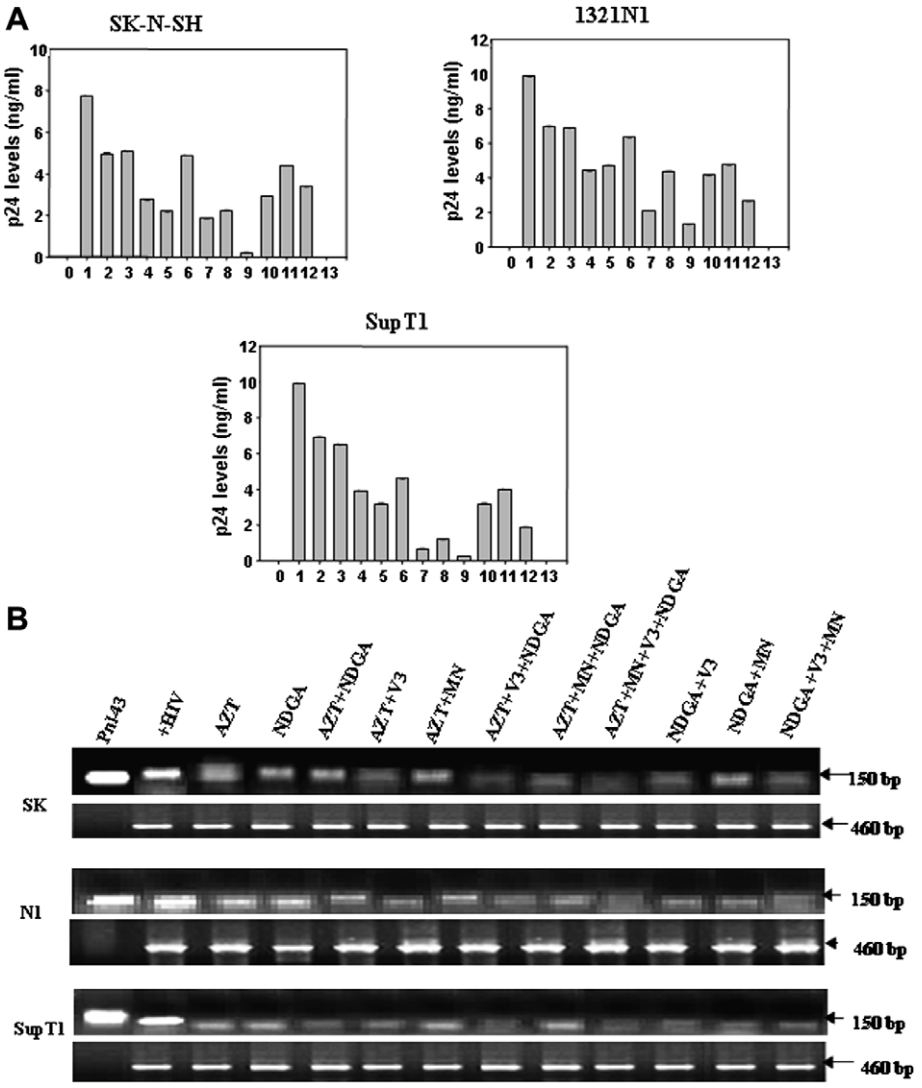

Fig. 8. Action of AZT, NDGA and peptides in combination against HIV-1 replication. Panel A: SK-N-SH, 1321N1 and SupT1 cells were challenged with HIV-1 in presence of 100 nm of AZT, 25 μm of NDGA, 20 ng of V3 and 20 ng of MN peptide in the indicated combination. The amount of virus replicated after day 4 in terms of p24 was quantified, p24 was plotted against samples. #1: No addition, #2: AZT+NDGA, #3: AZT+V3, #4: AZT+MN, #5: AZT+V3+NDGA, #6: AZT+MN+NDGA, #7: AZT+V3+MN+NDGA, #8: NDGA+V3, #9: NDGA+MN and #10: NDGA+V3+MN. Panel B: Proviral DNA analysis in NDGA, peptides and NDGA treated cells: SK (a), N1 (b) and SupT1 (c) cells were challenged with HIV-1 in presence of indicated concentrations of drugs and peptides. The infection was stopped at 5 h and the amount of proviral DNA was analyzed using gag specific SK38 and SK39 primers. 150 bp bands is gag specific amplification and 450 bp bands is actin specific amplification. 100 nM of AZT, 25 μm of NDGA, 20 ng of V3 and 20 ng of MN were used for this study.

infection. The time course of the secondary neurological symptoms may take longer, but emergence of these symptoms would promote rapid progression to AIDS and related mortality. Hence it is important to understand the cellular process associated with invasion of virus in various brain cells, since such information would help in developing therapeutics. In the present investigation, an attempt is made to study the inflammatory responses during viral invasion and replication.

The involvement of DNA topoisomerase II enzyme plays an important role in maintenance of DNA topology required for DNA replication, transcription, and recombination of viral and cellular genes. HIV infection in CD4 negative SK-N-SH neuroblastoma, 1321N1 astrocytoma and GO-C-CCM glioblastoma cells are studied. Result show that the expression of inflammatory cytokine TNFα, Topo II β and LOX-5 is enhanced immediately after infection. Expressed Topo II β is activated through a virus-associated kinase [32]. Topo α and β is required for reverse transcription, DNA rearrangements and proviral DNA synthesis. Anti-inflammatory drug NDGA decreases the levels of inflammatory cytokine TNFα and LOX-5 in both infected and uninfected cells. A low sensitivity of NDGA is exhibited in regulation of Topo II β and TNFα uninfected SK and N1 cells, while the regulation of both Topo II β and TNFα was marginally decreased in presence of NDGA in infected SK cells, but not in N1 cells. These results point

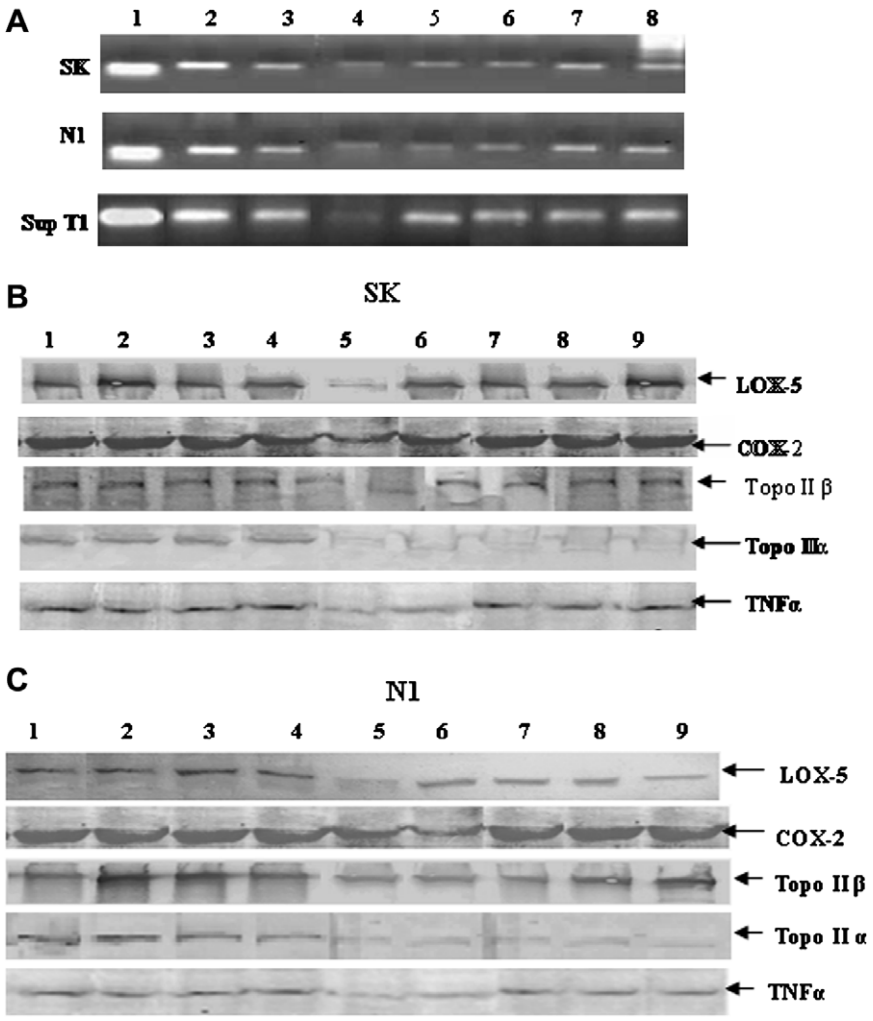

Fig. 9. Kinetics of anti-inflammatory activity of NDGA during HIV-1 infection conducted in presence of AZT SK, N1 and SupT1 cells (A) cells were incubated with AZT and NDGA was added at 0 (lane 5), 30 min (lane 6), 1 h (lane 7), 2 h (lane 8) and 3 h (lane 9), the amount of proviral DNA was analyzed after 5 h of post infection using gag specific SK38 and SK39 primers.#1: pNL4-3, #2: HIV-1 alone, #3:AZT + HIV, #4: AZT + NDGA, #5: AZT + NDGA at 30 min p.i., #6: AZT + NDGA at 1 h p.i., #7: AZT + NDGA at 2 h p.i., #8: AZT + NDGA at 3 h p.i.; (B,C) cells were challenged with HIV-1 in the presence of AZT and NDGA was added from 0 to 30 min, 1, 2, and 3 h of post-infection, and cells were incubated for 24 h and harvested. One hundred micrograms of total protein was separated on SDS–PAGE and western blotted and probed with antigen specific antibodies. #1: uninfected, #2: uninfected cells with AZT, #3:infected cells alone, #4: infection + AZT, #5: infection + AZT + NDGA #6: infection + AZT + NDGA at 30 min p.i., #7: AZT + NDGA at 1 h p.i., #8: AZT + NDGA at 2 h p.i.,#9: AZT+NDGA at 3 h p.i.

out (1) Topo II α and LOX-5 regulated in the same path ways, (2) Topo II β and TNFα are co-regulated, (3) Topo II α and Lox-5 show similar regulation in infected and uninfected SK and N1 cells, (4) Topo II β and TNFα regulation is same in uninfected SK and N1 cells, but virus infection in SK cells activate an alternative pathway for Topo II β and TNFα regulation, that has very low sensitivity to NDGA.

HIV-1 gp120 peptides can down regulate both TNFα and Topo II β and inhibit viral replication, suggesting the involvement of gp120 binding in enhancement of the inflammatory response. But the peptides itself could not induce an inflammatory response (Fig. 5, lanes 2–4), while they could inhibit the HIV-1 virus or gp120 mediated inflammation, suggesting that these peptide may not be interacting with the co-receptor. They may be interacting with non-CD4 primary receptors present on these cells, thus blocking the virus (Fig. 5, lane 6–9) and envelope induced inflammatory responses (Fig. 6).

Neuroectodermal origin (SK-N-MC) neuroblastoma cells and other neuronal cells has been shown to be susceptible to HIV-1 infection [33,34] through a CD4 independent mechanism. Antibodies against galactosyl ceramide has been shown to inhibit viral entry in these CD4 negative neuronal cells [35] and the interaction of virus with cell surface Galactosyl ceramide/3′ Sulfo-Galactosyl ceramide is dependent on the conformation of gp120 [36], specifically V3 domain shown to serve as primary viral determinant for infectivity in CD4 negative cells [37]. Hence, the peptides studied in the present investigation may interact with galactosyl ceramide in inhibition of virus mediated inflammation and infection. Since these neruoblastoma cells are

reported to have low expression profile of Galactosyl ceramide receptor [38], it is also possible that another non-CD4 receptor may also be involved for peptide interaction in inhibiting HIV-1 induced inflammation and infection.

The binding of virus to cellular receptors may thus enhance inflammatory response leading to the up regulation of TNFα, TopoII β, and LOX-5. A combination of AZT (anti-retroviral drug) along with NDGA (anti-inflammatory drug) together can efficiently inhibit viral replication, thus suggesting down regulation of TNFα and Topo II β can enhance the anti-retroviral potency of AZT. Hence Topo II α and Topo II β may show distinct functions of DNA topological maintenance in neurons and astrocytes.

In summary, HIV-1 binding to neurons and astrocytes can enhance inflammatory response by up-regulation of TNFα, Topo II β, and LOX-5. Anti-inflammatory drug NDGA and gp120 derived peptides can down-regulate TNFα and Topo II β and inhibit inflammation and viral replication. NDGA can synergistically act with AZT anti-retroviral activity. Thus a combination of AZT with NDGA can enhance the anti-retroviral potency of AZT.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2007.01.026.

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Analysis of age dependent changes of Topoisomerase II α and β in rat brain

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Abstract

Eukaryotic Topoisomerase II (Topo II) is present in two isoforms α and β. The α isoform is predominantly localized in proliferative tissue, while β isoform is present in all tissues. In the present study we report the activity and protein levels of Topoisomerase II α and β in rat brains of different age groups viz.: E11 (Embryo day 11), E18 (Embryo day 18), post-natal day 1, young (<10 days), adult (<6 months) and old (>2 years). Topoisomerase II β isoform is found to be the predominant form in brain tissue but Topoisomerase II α is found in embryos up to post-natal day 1. The studies to examine the regional distribution of Topoisomerase II β in brain showed highest activity in cerebellar region and that too only neuronal cell fraction. There was a significant age-dependent decline in this activity. Hence, Topoisomerase II β may have some unknown function in cerebellum and the low levels of Topoisomerase II β activity in ageing cerebellum may contribute to the genomic instability in cerebellar region of ageing brain.

Keywords: Brain; Cerebellum; Neurons; Ageing; Topoisomerase II β

1. Introduction

Topoisomerase II (Topo II) is a nuclear enzyme playing a key role in DNA replication, transcription, chromosome condensation, genetic recombination and repair (Wang, 1996). In mammals, Topo II is found to be present as 170 kDa, α and 180 kDa, β isoforms (Drake and Hofmann, 1989). Both the isoforms show structural similarity but are genetically, immunologically and biochemically distinct. They show distinct cellular localization and cell cycle expression profiles. Topo II α activity is shown to be highest during the G_2/M phase of the cell cycle (Woessner et al., 1991). On the other hand, Topo II β activity is constant throughout the cell cycle. Topo II α is distributed in the nucleoplasm, whereas Topo II β is localized in the nucleolus during interphase, and in the cytoplasm during mitosis (Chaly et al., 1996). In the embryonic stage, Topo II β in the brain is a nucleoplasmic enzyme showing higher levels of expression in the differentiating neurons (Tsutsui et al., 2001). Both the isoforms show different patterns of tissue distribution. Topo II α is shown to be higher in testes, spleen, bone marrow and liver. Topo II β is detected in high levels in differentiated tissue like brain (Jurenke and Holden, 1993; Capranico et al., 1992). However, a systematic study to examine the levels of the isoform of Topoisomerase II, α and β in different regions and cell types of brain is lacking.

We have been interested in the DNA repair mechanisms in ageing brain (Rao, 2002; Rao et al., 2000) and also Topo II as possible drug target (Gopal et al., 1999, 2002). Therefore a systematic study of the levels of Topo II α and β in different regions and in two cell types (neurons and astrocytes) in rat brain of different ages has been taken-up. We report the age-associated changes in the protein and activity levels of the Topo II β isoform in different regions of brain. The changes in the levels of Topo II β at the cellular level were also studied, viz. neurons and astroglia from whole brain, cerebellum, and cerebral cortex. The results of our experiments show that Topo II β is the only isoform that is significantly present in rat brain and that too predominantly in cerebellar neurons.

2. Experimental procedures

2.1. Materials

Topo II α and β monoclonal antibodies were obtained from Pharmingen. Goat–anti mouse IgG and FITC

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conjugated Goat–anti mouse IgG was obtained from Bangalore Genei, India. Nonidet, ATP were from Boehringer Manhheim. PMSF, BSA, protein A agarose, Triton X-100, ficoll, DTT, trypsin and trypsin inhibitor were from Sigma, USA, PVDF membrane was obtained from PALL Life Sciences, USA.

2.2. Preparation of tissue extracts from embryos

Tissue extracts were prepared from cerebellum of E11 (Embryo day 11), E18 (Embryo day 18) and post-natal day 1 pups by sacrificing them through decapitation and collecting the cerebellum tissue. At E11 stage there is no organ differentiation so we have taken cephalic region of the embryos as brain source. The tissue was homogenized in extraction buffer (20 mM Tris–HCl pH 7.5, 0.1 mM β-mercaptoethanol, 1 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol, 0.1% Triton X-100, 0.5 mM KCl, 0.5 mM PMSF and 1 μg/μl pepstatin and leupeptin.). The homogenate was kept at 4 °C for 1 h and centrifuged at 100,000 × *g* for an hour in an ultracentrifuge. The supernatant containing the cytosolic and nuclear proteins was used as a source for Topo II.

2.3. Preparation of tissue extracts from whole brain

Whole tissue extracts were prepared from young (<10 days), adult (~6 months), old (>2 years) rats. The brain, liver and testes tissue were collected separately. The tissue was homogenized in extraction buffer (20 mM Tris–HCl pH 7.5, 0.1 mM β-mercaptoethanol, 1 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol, 0.1% Triton X-100, 0.5 mM KCl, 0.5 mM PMSF and 1 μg/μl pepstatin and leupeptin.). The homogenate was kept at 4 °C for 1 h and centrifuged at 100,000 × *g* for an hour in an ultracentrifuge. The supernatant containing the cytosolic and nuclear proteins was used as a source for Topo II.

2.4. Isolation of neurons and astroglia

Neurons and astrocytes were isolated from brain tissues of rats that are collected after decapitation essentially as per the

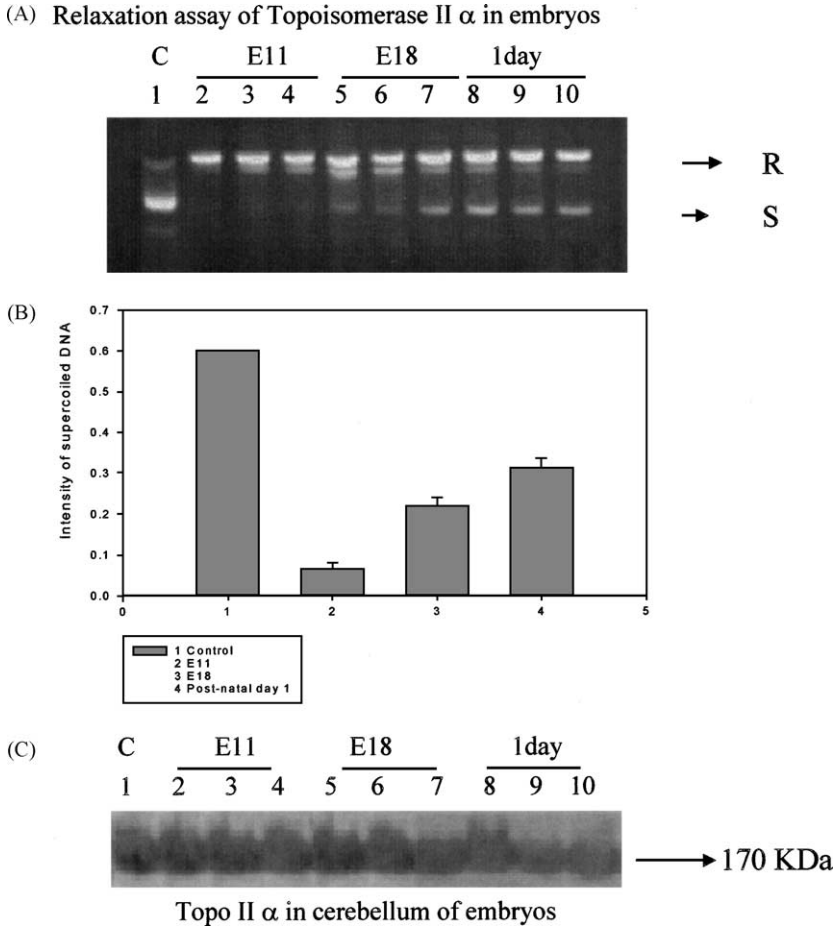

Fig. 1. Enzymatic activity and protein levels of Topo II α in cerebellum of rat embryos: activity: Topo II α activity was measured in 100 μg protein of cerebellum extracts of E11 (lanes 2–4), E18 (lanes 5–7) and post-natal day 1 (lanes 8–10) rat pups, lane 1 shows ~0.6 μg of PRYG plasmid DNA. Activity of Topo II α in cerebellum of E11, E18 and post-natal day 1 shown as gel in panel A, corresponding quantified supercoiled DNA is shown in panel B. Protein: 75 μg of protein of extract was separated on 7.5% SDS PAGE and Western transferred and probed with monoclonal Abs against Topo II α, immunoblot of Topo II α is shown in panel C, lane 1 shows 25 μg of Topo II α positive control.

procedure of Usha Rani et al. (1983). The brain tissue was placed in isolation medium (10 mM KH_2PO_4, 8% glucose, 5% fructose PH 6.0) and cleared of blood vessels and minced well. Young rat brains were incubated with medium; adult and old brains were incubated with medium containing 0.1% trypsin for 30 min. After incubation the trypsin medium was decanted and 0.1% trypsin inhibitor was added and left on ice for 10 min. Tissue was passed through 103, 80 and 48 μm nylon meshes and rinsed with isolation medium thrice. Before washing the tissue was minced well on the mesh using a flat glass rod. Filtrate was centrifuged at 800 × *g* for 15 min the supernatant is decanted and the pellet was resuspended in 7% ficoll medium and centrifuged at 300 × *g* for 10 min and pellet containing the neurons was suspended in 2% ficoll medium. The supernatant containing the astrocytes was diluted with 2% ficoll medium and centrifuged at 1100 × *g* for 10 min. Astrocyte pellet with was suspended in 2% ficoll isolation medium. Both the suspensions were centrifuged at 1500 × *g* for 10 min and the pellets are further washed with medium with out ficoll followed by washing with PBS. The final pellet containing the neurons and the astrocytes was suspended in required amounts of extraction buffer then vortexed and was kept at −20 °C overnight. These suspensions were sonicated and spun down at 100,000 × *g* for 1 h.

2.5. Immunoprecipitation of Topoisomerase II isoforms

Brain extracts (100 μg total protein) prepared from cerebellum of embryos (E11, E18 and 1 day old) and whole brain, cerebellum, cerebral cortex and midbrain regions of the young, adult and old age groups were taken in Eppendorf tubes for immunoprecipitation and Topo II α or β antibody (1:1000 dilution in Immunoprecipitation buffer containing 100 mM Tris–HCl pH 8, 750 mM NaCl, 2 mM EDTA, 1 mM PMSF, 0.75% Nonidet) was added to each sample. The antigen–antibody mixture was incubated at room temperature for 1 h and 25 μl of 6% protein A agarose beads were added. The beads were incubated at 4 °C for 15 min, spun down and the supernatant was removed. The protein A agarose beads were washed twice with 0.5% Triton X-100 in PBS. The beads were directly used for monitoring the relaxation activity of Topo II. The total amount of protein was constant as assessed from the Western blot analysis of β-actin.

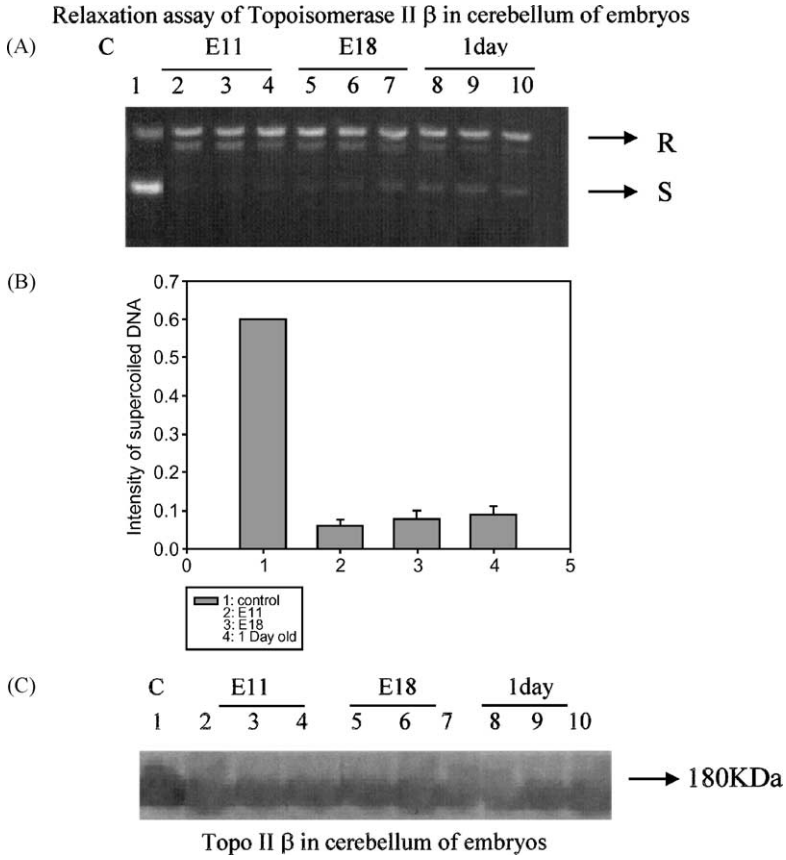

Fig. 2. Enzymatic activity and immunoblotting analysis of Topo II β in cerebellum of rat embryos: activity: Topo II β activity was measured in 100 μg protein of extract of E11 (lanes 2–4), E18 (lanes 5–7) and post-natal day 1 (lanes 8–10) rat pups, lane 1 shows ∼0.6 μg of pRYG plasmid DNA Activity of Topo II β in cerebellum of E11, E18 and post-natal day 1 shown as gel in panel A, corresponding quantified supercoiled DNA is shown in panel B. Protein: immunoblot of Topo II β was shown in panel C.

2.6. DNA relaxation assay

DNA relaxation by Topo II involves the change in the linking number of DNA by 2. During relaxation the super coiled DNA band (Form I) disappears and completely relaxed plasmid DNA (form II) appears. About 0.6 μg of super coiled plasmid DNA is incubated with the immunoprecipitated Topo II captured on to the Protein A agarose beads in relaxation buffer (50 mM Tris–HCl pH 8.0, 120 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 30 μg/ml BSA, 1 mM ATP) for 30 min at 37 °C. The beads were spun down at 300 × *g* for 5 min and the supernatants were collected separately. The reaction was stopped by addition of 10% SDS and the DNA products were resolved on 1% agarose gel and stained with ethidium bromide and photographed.

2.7. Immunoblotting analysis

Seventy-five micrograms of total protein of brain tissue extracts were electrophoresed on a 7.5% SDS polyacrylamide gel and transferred on to polyvinyl diphenyl fluoride (PVDF) membrane for immunoblotting analysis. Blot was incubated with 5% non-fat dry milk powder solution in Tris-buffered saline (TBS: 10 mM Tris and 150 mM NaCl) to block the non-specific binding. The membrane was

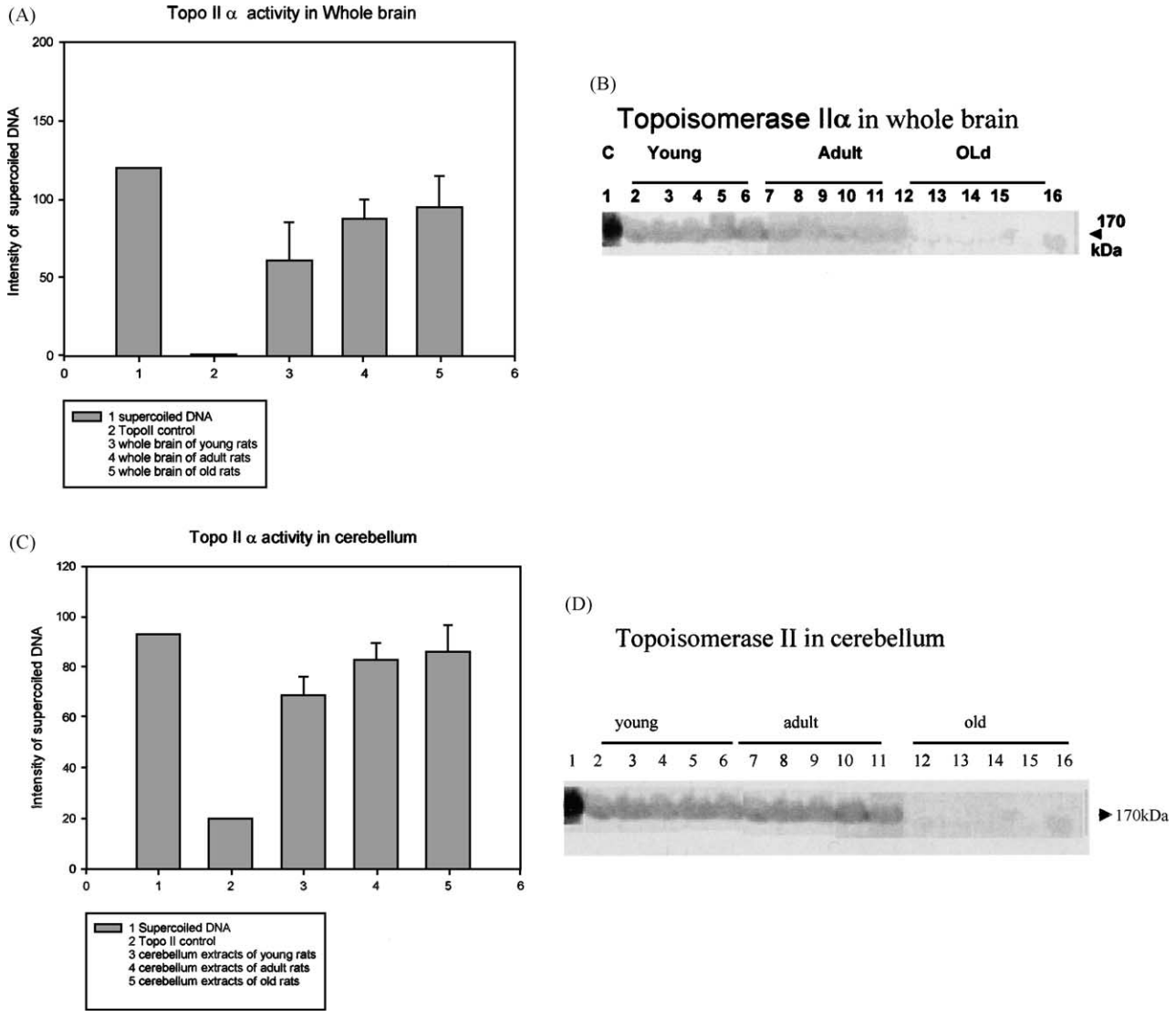

Fig. 3. Enzymatic activity and immunoblotting analysis of Topo II α in whole brain: activity: 100 μg of protein of extract was assayed for Topo II α catalyzed relaxation activity, the activity is measured in terms of amount of supercoiled DNA present in the gel. Quantified supercoiled DNA was plotted on *Y*-axis and sample names were given on *X*-axis. Panels A, C, E and G are the results of activity of Topo II α in extracts of whole brain, cerebellum, cerebral cortex and mid brain respectively. Protein: 75 μg of protein of extract was separated on 7.5% SDS PAGE and Western transferred and probed with monoclonal Abs against Topo II α, lane 1 shows Topo II α control with 25 μg. Panels B, D, F and H show immunoblots of Topo II α in extracts of whole brain, cerebellum, and cerebral cortex and mid brain respectively.

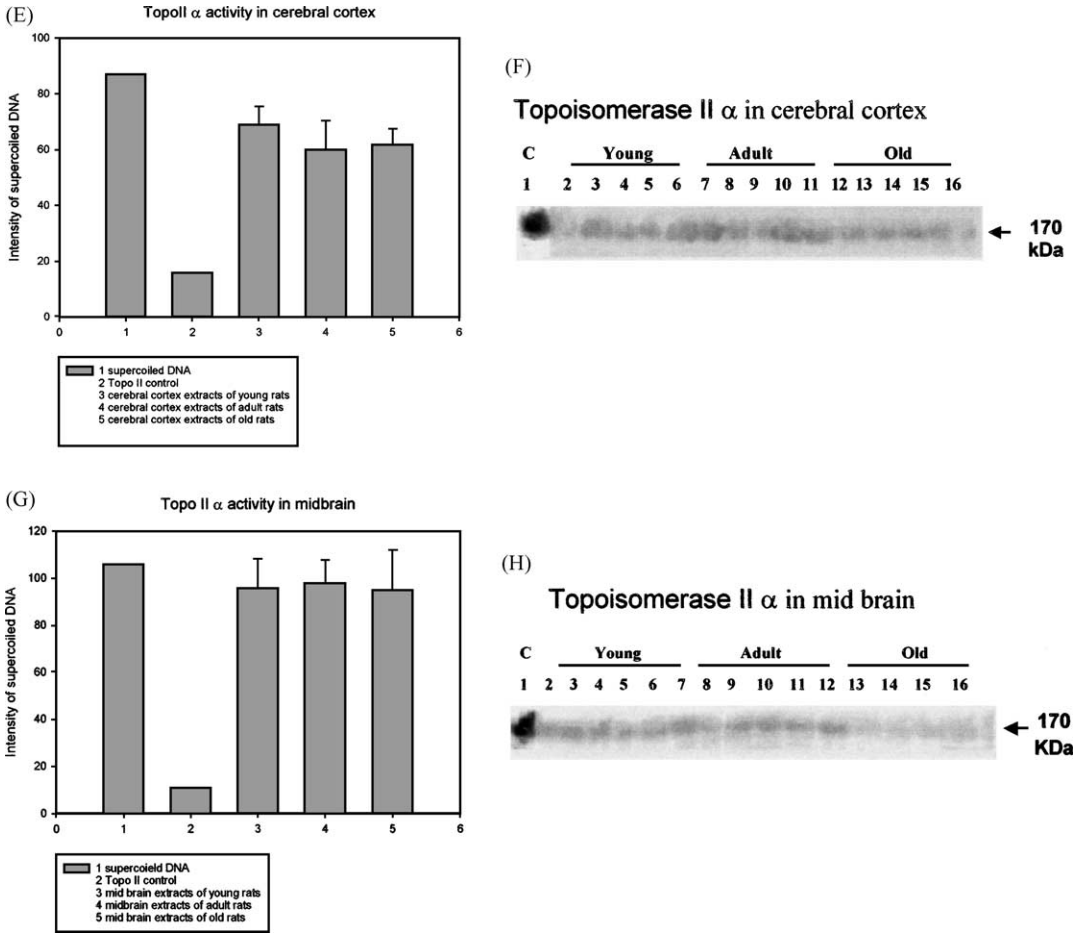

Fig. 3. (*Contineud*).

thoroughly washed using TBS containing 0.15% Tween 20 and incubated with Topo II α or β antibodies (1:1000 dilution in TBS) for 30 min at room temperature. The membrane was washed thrice and incubated with the alkaline phosphatase conjugated anti mouse IgG antibody (1:2000 dilution in TBS) for 60 min at room temperature and washed with TBS containing 0.15% Tween. The blots were developed using NBT-BCIP substrate in TBS.

2.8. Phosphorylation of Topoisomerase II

Fifty micrograms of protein from the extracts of whole brain or cerebellum was phosphorylated with 5 μCi of γ^{32}P-ATP in kinase buffer (10% glycerol 0.1 M EDTA and 50 mM $MgCl_2$). The mixture was incubated at 37 °C for 1 h. Topo II β antibody was added to the above mixture. After incubation at 37 °C for 30 min, 20 μl of protein A agarose was added. The tissue lysates were incubated at 4 °C for 15 min with intermittent shaking and centrifuged at 300 × ***g***. The sediment was washed twice with PBS and eluted with 20 μl of 5% trichloroacetic acid (TCA). Ten microliters of elute was spotted on Whatman no. 1 filter paper discs and ^{32}P was measured with a Wallac 1400 DSA liquid scintillation counter. Each experiment was carried out in triplicate and all data points represent an average of results from three experiments.

2.9. Immunofluorescence

The cerebellar regions from brains of young, adult and old rats were dissected out and placed in medium containing 10 mM potassium phosphate, 8% glucose and 5% fructose. Frozen sections ∼4 microns/μm of the cerebellum from the three different age groups were taken in a cryostat and mounted on glass slides. The sections were treated with ethanol for 10 min followed by washes with double distilled water and PBS. Then the sections were treated with 0.3% Triton X-100 in PBS for 3 min. The sections were blocked with 1% BSA in PBS and incubated for 1 h at room temperature. After washing with PBS twice, the sections were then incubated with Topo II antibodies diluted in PBS containing 1% BSA for 1 h and then washed with PBS twice. The sections were then incubated in FITC conjugated IgG secondary antibody for 1 h and washed with PBS twice. The slides were then viewed using a fluorescence microscope and photographed.

3. Results

The levels of the two isoforms of Topo II were analyzed in whole brain, and also in the three regions of brain viz. cerebellum, cerebral cortex and midbrain. The levels of this enzyme were also examined in the different cell types, viz. neurons and astrocytes, that are widely used for various studies for understanding mechanisms of DNA damage and repair.

Topo II α and β present in the corresponding extract was immunoprecipitated using isoform-specific monoclonal antibody. The activity of the Topo II α and β present in immunoprecipitate was analyzed by monitoring the extent of Topo II catalyzed relaxation of supercoiled plasmid pRYG DNA. PRYG DNA plasmid contains Topo II binding and cleavage sites. The products were analyzed on 1% agarose gels and stained with ethidium bromide. Agarose gels along with quantified supercoiled DNA were shown as bar graphs in results wherever we have observed significant Topo II activity. In the results where we have observed low levels of Topo II activity, we have given data only in bar graph for quantified supercoiled DNA bands that is inversely proportional to the relaxation activity of the enzyme. Topo II α and β proteins were monitored through Western blot analysis of the Protein using isoforms-specific monoclonal antibodies.

3.1. Topoisomerase II α in cerebellum of rat embryos

Analysis of Topo II α in cerebellum extracts of embryos shows high activity in E11 and E18 embryos but in post-natal day 1 pups shows moderate activity (Fig. 1A). This observation was correlated well with corresponding protein levels on the immunoblot analysis (Fig. 1C). This analysis indicates that Topo II α level is high in proliferating tissue like developing rat embryos brain.

3.2. Topoisomerase II β in cerebellum of rat embryos

Our analysis on Topo II β in cerebellum extracts of embryos shows same activity in all three age groups (Fig. 2A). There are no detectable changes in Topo II β activity

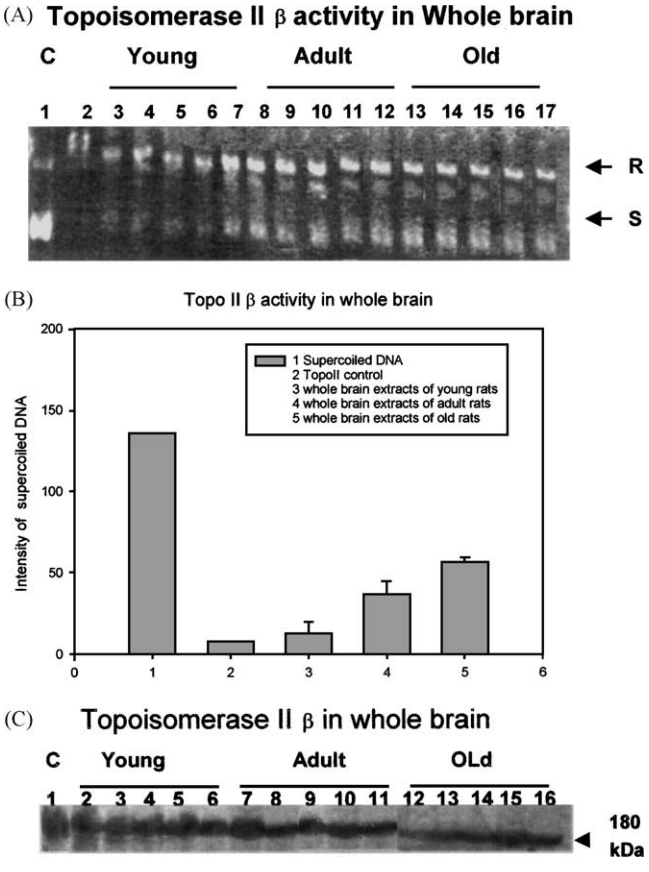

Fig. 4. Enzymatic activity and protein levels of Topo II β in whole brain: ACTIVITY: Topo II β activity is measured in 100 μg protein of extract of young (lanes 3–7), adult (lanes 8–12) and old (lanes 13–17) rats. Lane 1 shows ∼0.6 μg of pRYG plasmid DNA and lane 2 shows DNA with 2 units of Topo II from whole brain and three regions of young, adult and old rats. Activity of Topo II β in whole brain was shown as gel in panel A, corresponding quantified supercoiled DNA is shown in panel B. Protien: immunoblots of Topo II β in extracts of whole brain was shown in panel C.

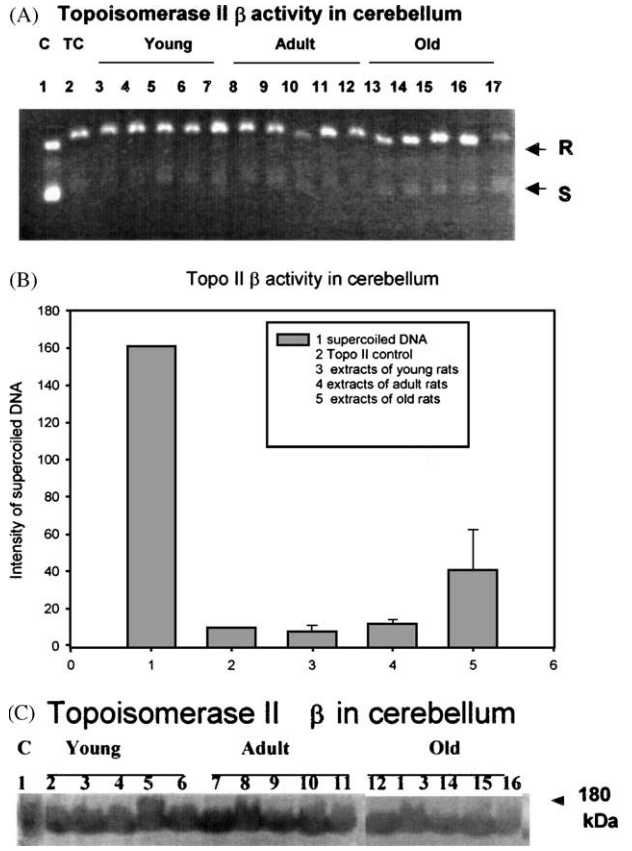

Fig. 5. Enzymatic activity and protein levels of Topo II β in cerebellum: activity: Topo II β Activity is measured in 100 μg protein of extract of young (lanes 3–7), adult (lanes 8–12) and old (lanes 13–17) rats. Lane 1 shows ∼0.6 μg of pRYG plasmid DNA and lane 2 shows DNA with 2 units of Topo II from cerebellum. Activity of Topo II β in cerebellum was shown as gel in panel A, corresponding quantified supercoiled DNA is shown in panel B. Protien: immunoblots of Topo II β in extracts of cerebellum was shown in panel C.

between three age groups. This result closely supported by immunoblot analysis of Topo II β level in embryos (Fig. 2C).

3.3. Topoisomerase II α in whole brain and three regions of rat brain

Analysis of Topo II α in whole brain extracts showed very low activity of this enzyme in young rat brain and negligible activity in adult and old rat brain (Fig. 3A), this observation is correlated well with corresponding protein levels on the immunoblot analysis in Fig. 3B.

Topo II α activity was also examined in the three regions of brain viz. cerebellum (Fig. 3C), cerebral cortex (Fig. 3E) and mid brain (Fig. 3G), the results showed negligible activity of Topo II α in these three regions and at all the ages studied. To know protein levels of Topo II α, we have carried out immunoblot analysis of Topo II α in extracts of cerebellum (Fig. 3D), cerebral cortex (Fig. 3F) and midbrain (Fig. 3H), the results of the these experiments confirm that Topo II α activity as well as protein level remains low in these three regions at all ages.

3.4. Topoisomerase II β in rat whole brain and three regions of brain

Since the above studies indicated that Topo II α activity remained low or even undetectable in brain at all the ages studied, we have taken-up to study the activity and protein levels of Topo II β in whole brain and three regions of brain in young, adult and old rats.

The results are shown in Fig. 4, the results show a significant activity of Topo II β in whole brain extract (Fig. 4A and B). The "Young" rat brain showed high activity of this isoform, while moderate activity is seen in "Adult" brain

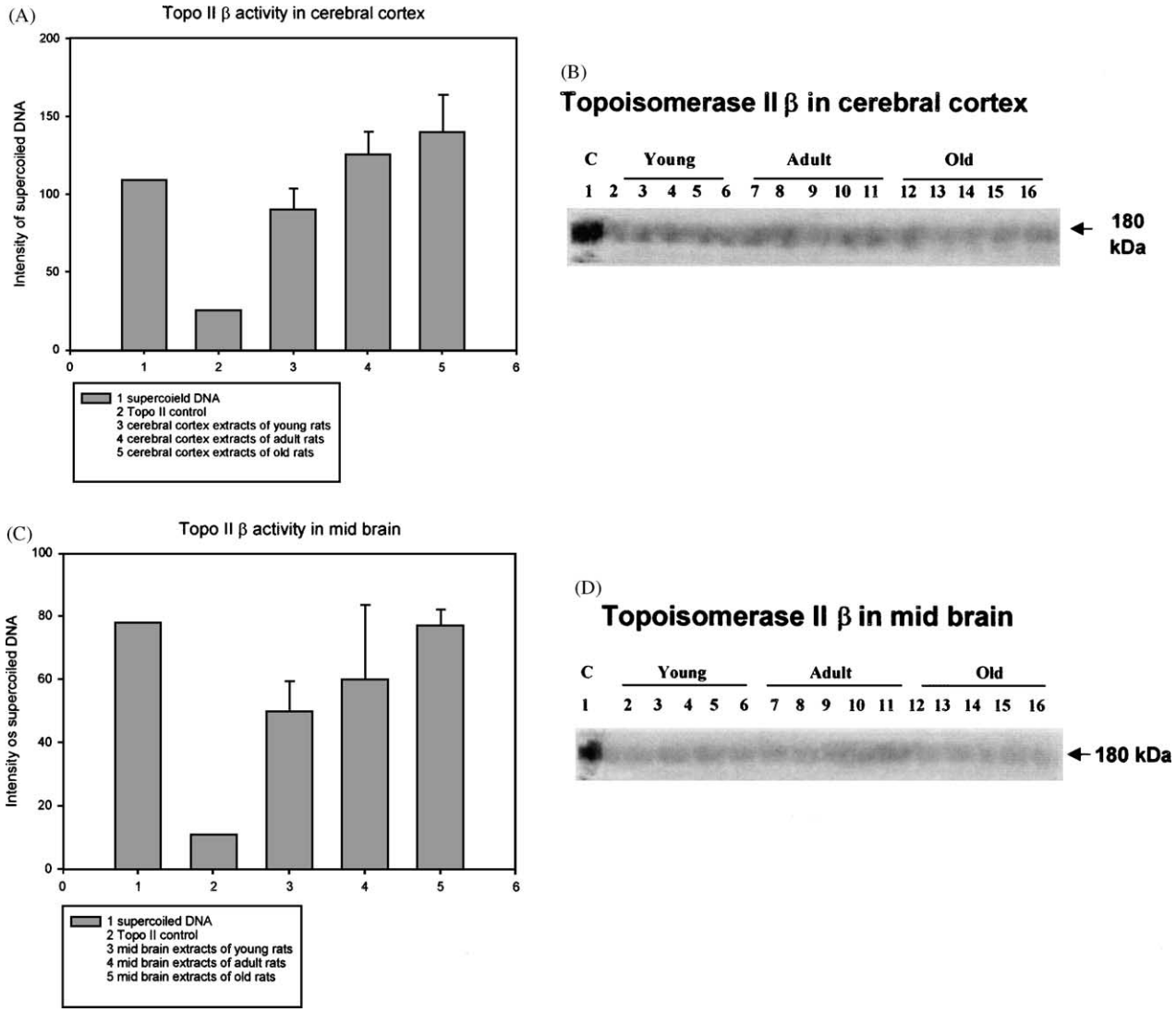

Fig. 6. Panels A and C show the activity of Topo II β in extracts of cerebral cortex and midbrain in bar diagram. Protein: immunoblots of Topo II β in extracts of cerebral cortex and midbrain were shown in panels B and D, the blots labeled appropriately, lane 1 shows Topo II β control with 25 μg.

and least activity was detected in 'Old' brain (Fig. 4A and B). The analysis of protein levels of Topo II β as shown in Fig. 4C, correlate well with the observations seen in the enzymatic activity suggesting that high enzymatic activity and protein of Topo II β is observed in young brain and these levels deceased with increasing age. Further, we notice that the migration of Topo II β protein in "Old" rat brain is slightly ahead of "Young" and "Adult" suggesting the possibility of slight variation in molecular form of Topo II β in ageing brain.

The results concerning the Topo II β activity in cerebellum of brain and at three different ages are shown in Fig. 5A and B and the protein levels are shown in Fig. 5C. The Topo II β activity was highest in "Young" as well as "Adult", while the activity is decreased in "Old" rat cerebellum. Western blot analysis of protein levels of Topo II β in rat cerebellum as shown in Fig. 3C suggest that Topo II β protein remains high in "Young" and in "Adult" rat cerebellar extracts, while protein levels decrease in "Old" rats. These observations closely correlated the results of enzymatic activity (Fig. 5A and B) Further, we notice the migration of Topo II β in "Old" rat cerebellum is slightly faster than that of Topo II β in "Young" and "Adult" rat cerebellum (similar to the observation in whole brain) suggesting the molecular form of Topo II β in "Old" rat cerebellum may a slightly altered one.

On the other hand, activity and protein of Topo II β are negligible in cerebral cortex and mid brain (Fig. 6A–D) at all the ages studied. Similar analysis of Topo II β specifically in hippocampus region also showed negative results (data not shown). These results thus point out the interesting aspect of Topo II β being found only in cerebellar region.

3.5. *Phosphorylation of Topoisomerase II β in whole brain and cerebellum*

Cellular Topo II β activity is known to be regulated by its phosphorylation and the phosphorylated form being the active form (Isaacs et al., 1998). To understand whether the Topo II β activity in brain also maintained through its phosphorylation, Topo II β phosphorylation was analyzed in whole brain and cerebellum extracts. The results in Fig. 7 show that whole brain and cerebellum region have the maximum level of phosphorylated Topo II β and there was a decrease in this level in an age-dependent manner (Fig. 7).

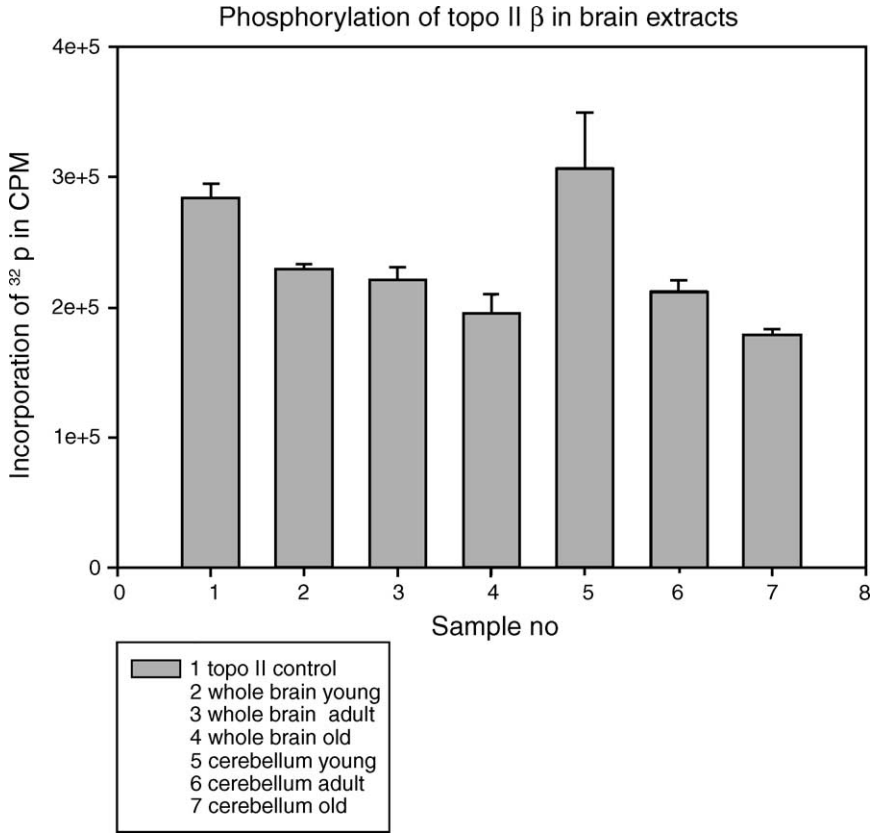

Fig. 7. Phosphorylation of Topo II β in brain extracts: Topo II β from 50 μg total protein of whole brain (lane 2–4), and cerebellum of young, adult and old rats was phosphorylated with 32 p ATP in presence of kinase buffer. Twenty-five micrograms of Topo II (sample no. 1) was used as a control. The phosphorylated enzyme captured on to the protein A agarose beads was TCA eluted and spotted on to Whatman filter papers and the radioactivity was counted using a Wallac scintillation counter. sample nos. 2–4 are Topo II β of whole brain extract of young, adult and old rats respectively, sample nos. 5–7 are of cerebellum extract of young, adult and old rats respectively.

3.6. Levels of Topoisomerase II β in different cell types of brain

Since Topo II β is found only in the cerebellar region of brain, we have examined whether Topo II β has any exclusive localization in a particular cell type. Neuronal and astroglial cell fraction were prepared from whole brain, cerebellum and cerebral cortex and topo II β activity in the extracts of these cell fractions were examined. The results are shown in Fig. 8. Only neuronal extracts from whole brain and cerebellum showed activity of Topo II β. The activity of the cerebellar neuronal extracts (Fig. 8A and B) was higher when compared to whole brain (Fig. 8A and B) and in contrast Topo II β activity was negligible in cerebral cortex neurons (Fig. 8C and D). Analysis of age-dependent changes in Topo II β activity in whole brain and cerebellar neurons showed that the enzymatic activity decreased with increasing age. Similar analysis of astrocytes showed negligible amounts of Topo II β (Fig. 8E).

3.7. Immunohistochemical analysis of Topoisomerase II α and β

To confirm the above findings we have performed the immunohistochemical analysis of Topo II β in brain sections and neurons. The analysis of Topo II β was carried out in the sections obtained from cerebellum (young, adult and old) (Fig. 9, panel A) cerebral cortex (young), midbrain (young) (data not shown). Immunofluorescence of Topo II β was high in cerebellum sections, while Topo II β was negligible in sections of cerebral cortex and mid brain. Further, immunofluorescence of Topo II β showed a decrease with increasing age, highest amount of fluorescence is seen in the young rat cerebellar section (Fig. 9, panel A), in contrast the Topo II α analyzed in the cerebellum sections of the three

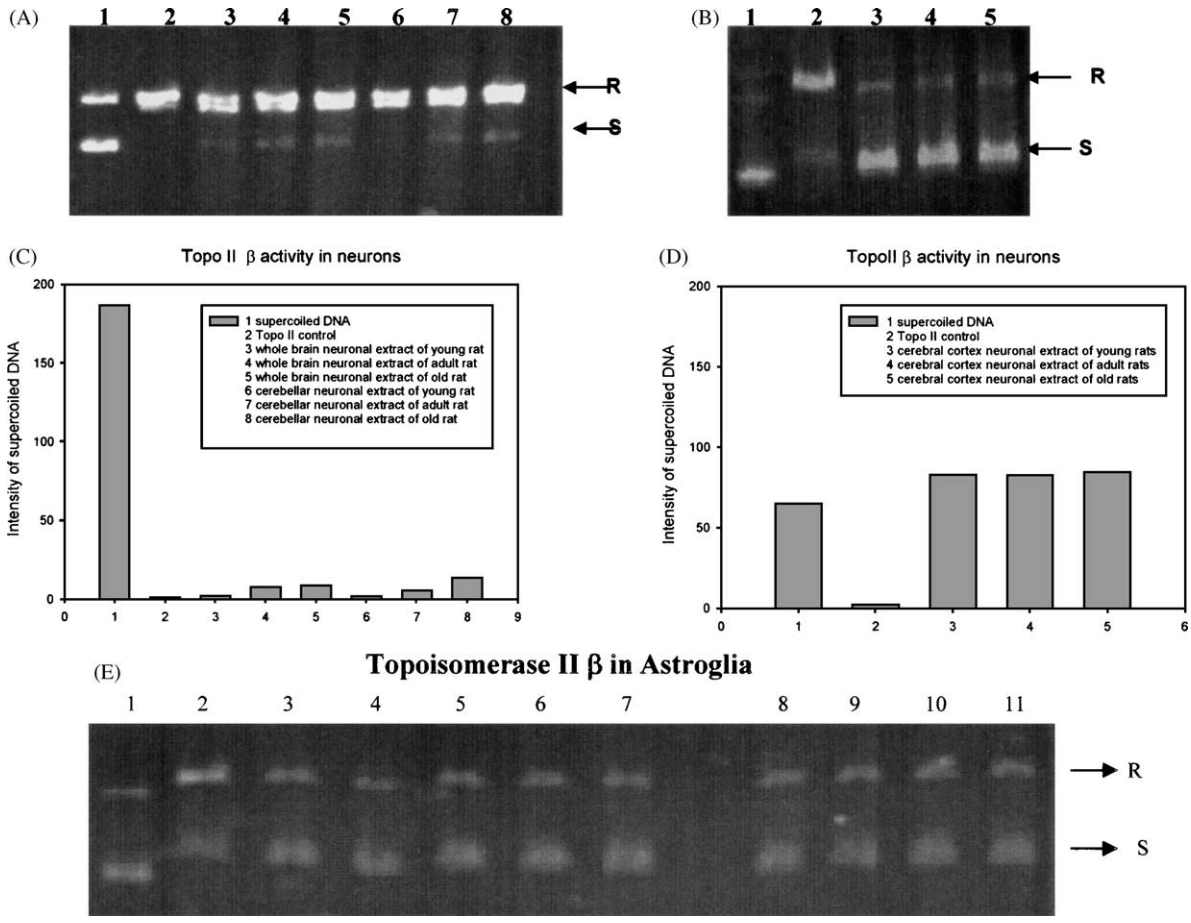

Fig. 8. Relaxation activity of Topo II β from neuronal cells: 100 μg protein of neuronal cell extracts was assays for Topo II β activity and data presented in form of relaxation of supercoiled DNA as analyzed by 1% agarose gel electrophoresis followed by ethidium bromide straining and photography, in addition a bar diagram is appended to show the quantified supercoiled DNA. Panel A, lanes 3–5 show activity of Topo II β in neuronal extracts of whole brain of young, adult and old respectively, while lanes 6–8 show the activity of Topo II β in neuronal extracts of cerebellum of young, adult and old respectively, quantified supercoiled DNA in gel is given in panel B and panel C, lanes 3–5 show activity in neuronal extract of cerebral cortex of young, adult and old rats, quantified supercoiled DNA in gel is given in panel D. Panel E shows activity in astroglial extracts of whole brain (3–5), cerebellum (6–8), and cerebral cortex (9–11) of young, adult and old rats. In all gels, lanes 1 show the DNA alone, lane 2 show DNA with purified Topo II β.

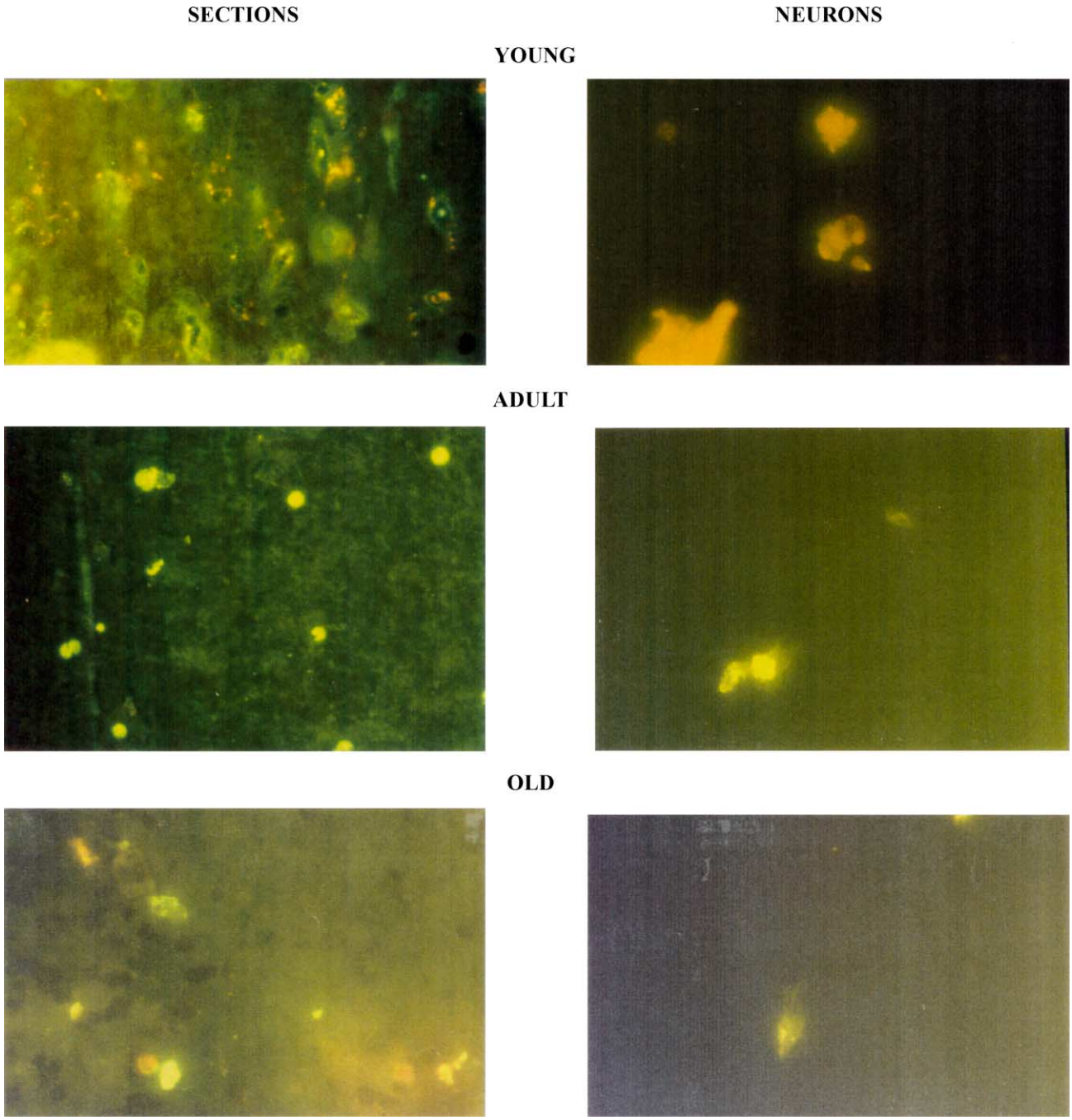

Fig. 9. Immunoflourescence of cerebellar section and cerebellar neurons: cerebellar sections of young (A), adult (B) and old (C) rats were dissected out and frozen in liquid nitrogen. Sections were taken by cryostat, they were fixed with acetone and probed with Topoisomerase II β monoclonal and stained with FITC conjugated anti mouse IgG and fluorescence images were seen in fluorescence microscope and photographed. The cerebral neurons from young (D), adult (E) and old (F) were isolated and cytospinned. The monolayer was fixed with ethanol and probed and stained as explained above.

age groups showed very low amounts of immunofluorescence (data not shown). Analysis of Topo II β immunofluorescence in cerebellar neurons of young, adult and old rats showed that the young rat cerebellar neurons possess highest amount of Topo II β immunofluorescence, while lowest being in the old rats. These observation correlate well with the above studies on the activity and protein levels of Topo II isoforms in three regions of brain.

4. Discussion

The above studies indicated that Topo II β is predominant in brain, while Topo II α is predominant in embryos (up to post-natal day 1) and remains low in aged groups. Further higher activity and protein levels of the topo II β isoform was seen only in cerebellar region, that is having laminar arrangements of cells. The studies of Tsutsui et al. (1993,

2001) on localization of Topo II isoforms in developing rat brain also showed that developing rat cerebellar region possess high levels of Topo II β during the early post-natal period suggesting the importance of Topo II β in rat cerebellum in its development and growth.

The results of the age-dependent changes of Topo II α and β suggested that α isoform remains low and unaltered with age, while the activity of β isoform is higher in young, moderate in adult and low in old rat cerebellar region suggesting an age-dependent decline in Topo II β protein and activity. The enzymatic activity may be regulated by phosphorylation as phosphorylated form of Topo II decreases in an age-dependent manner. Furthermore, we have noticed in Western blot data of Topo II β in whole brain and cerebellum (Fig. 2C and F) that Topo II β migration in whole brain and cerebellum of old rat is slightly faster than that in young ones indicating that the change in migration could be due to different phosphorylation status in addition to protein levels. The above results suggest that Topo II β activity in rat brain may be regulated through its phosphorylation.

The increased topo II β mRNA levels seen by Tsutsui et al. (2001) in the cerebellar granule cells that are differentiating to granular layer during the first two post-natal weeks of the rat cerebellar development suggest that the topo II β is essential for some functions in cerebellum during its development and growth. The present results show that this essential function of Topo II β is depleting during ageing. Higher excision nuclease activity, which is required in excision repair, is detected in cerebellum (Brook, 1998) and it is to be seen whether Topo II β is associated with a specific DNA repair activity in cerebellum.

The present results also suggest that only neurons possess significant Topo II β activity. Further, Topo II β activity in neurons decreased in an age-dependent manner. Studies of Woessner et al. (1991) showed that in developing rat neurons Topo II β is present is Purkinjee cells and granule cells during development suggesting that Topo II β is expressed in cerebellar neurons during cerebellar development and growth. These studies indicate that Topo II β may be required for cerebellar neurons for certain recombination

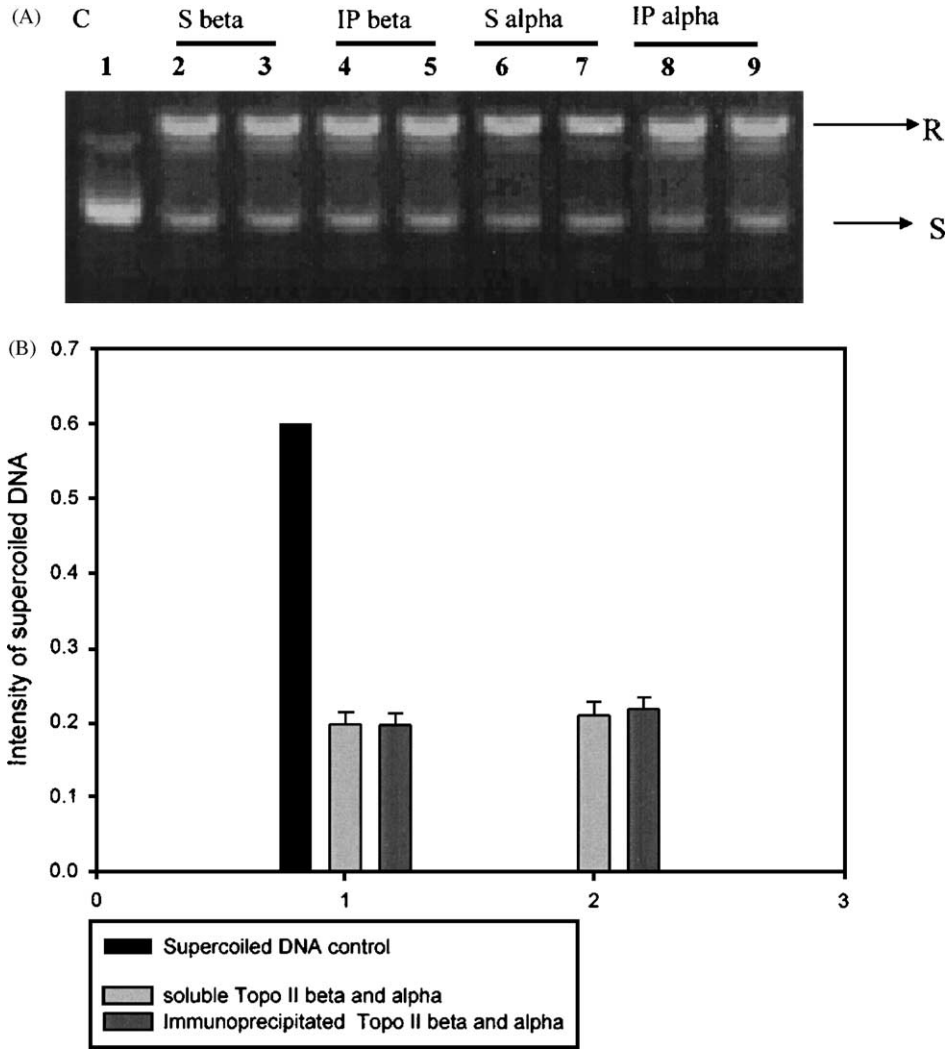

Fig. 10. Comparison of enzymatic activity of soluble and immunoprecipitated Topo II α and β.

and repair activity of differentiated cerebellar cells during their development and growth. Since Topoisomerases are reported to be involved in promiscuous recombination, and cellular response to radiation damage of DNA (Asami et al., 2002; Kohji et al., 1998; Ishii and Ikushima, 2002; Pastor and Cortes, 2002; Franchitto et al., 2000; Wu et al., 1999), the above results on the decreased activity of Topo II β in cerebellum of aged rat may be correlated as one of the factors contributing towards decrease in repair activity in aged brain tissue. Topo II β catalytic activity may be required for resolving certain topological restrictions formed during DNA rearrangements that take place in DNA repair and recombination in cerebellum.

5. Supporting data

Comparison of enzymatic activity of soluble and immunoprecipitated Topo II α and β are shown in Fig. 10. Tissue extracts were prepared from cerebellum of young (1 day old) rat pups. For soluble Topo II, tissue extracts were precipitated with 60% ammonium sulphate and dialyzed then treated with 0.2% heparin (nuclease inhibitor). Panel A, lanes 2 and 3 show soluble Topo II β, lanes 4 and 5 show immunoprecipitated Topo II β, lanes 6 and 7 show soluble Topo II α and lanes 8 and 9 show immunoprecipitated Topo II α. Lane 1 shows ∼0.6 μg of pRYG plasmid DNA and quantified supercoiled DNA are shown in panel B.

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