Chapter 2

Review of Literature
2. REVIEW OF LITERATURE

2.1 Brain tumors and Therapeutics

Conventional treatment of brain tumors has advanced only incrementally in the last 30 years and still yields poor outcomes. The current strategy of surgery, radiation, and chemotherapy has increased median survival to approximately 15 months. With the advent of molecular biology and consequent improved understanding of basic tumor biology, targeted therapies have become cornerstones for cancer treatment. Despite the growing understanding of the complex networks regulating CNS tumors, many targeted therapies have fallen short of expectations. This could be partially attributed to blood-brain barrier (BBB), which restricts the entry of most pharmaceuticals into the brain. The developmental process for new drugs for the treatment of CNS disorders has not kept pace with progress in molecular neurosciences because most of the new drugs discovered are unable to cross the BBB (Patel et al., 2011). Moreover, the improvement in the survival and quality of life of cancer patients requires the design of new therapies or therapeutic combinations that are effective and preferably have fewer side effects than those presently available. Differentiation therapy, using agents that modify cancer cell differentiation, has shown promise in the spectrum of agents used against tumors (Leszczyniecka et al., 2001). Wang and Chen (2000) demonstrated the clinical application for differentiation therapy by introducing all-trans-retinoic acid (ATRA).

Current cancer therapies are highly toxic and often nonspecific. A potentially less toxic approach to treating this prevalent disease employs agents that modify cancer cell differentiation, termed 'differentiation therapy'. This approach is based on the tacit assumption that many neoplastic cell types exhibit reversible defects in differentiation, which upon appropriate treatment, results in tumor reprogramming and a concomitant loss in proliferative capacity and induction of terminal differentiation or apoptosis (programmed cell death). Laboratory studies that focus on elucidating mechanisms of action are demonstrating the effectiveness of 'differentiation therapy', which is now beginning to show translational promise in the clinical settings. These compounds include butyroids (e.g. butyric acid), hydroxamic acids (e.g. trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA)),


cyclic peptides (e.g. trapoxin and apicidin) and benzamides. Most of these agents have been found to induce differentiation and/or apoptosis of transformed cells in vitro and some also suppressed tumour growth in vivo (Marks et al., 2000; Leszczyinkeca et al., 2001). Differentiation agents for malignant gliomas and neuroblastomas remain a real challenge.

Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover new therapeutic agents that lack the toxic side effects associated with current chemotherapeutic agents. Epidemiological studies suggest that consumption of diets containing fruits and vegetables, major sources of phytochemicals and micronutrients, may reduce the risk of developing cancer (Reddy et al., 1997). One promising new source of therapeutic agents has been discovered in plant secondary metabolites (Seigler, 1998). Recent interest in these secondary metabolites has been focused upon their medicinal properties (Harborne, 2000). Several natural plant extract and purified components have shown promise for differentiation potential in glioblastoma and neuroblastoma. Some of them include Corosolic acid (Fujiwara et al., 2011), panaxydol (Hai et al., 2008), Tanshione (Wang et al., 2007), Saikosaponins (Tsai et al., 2002), Maharishi Amrit Kalash-Ambrosia (Prasad et al., 1992), Ashwagandha (Kuboyama et al., 2002), Pentacyclic triterpenes (Laszczyk, 2009), green tea (Mandel et al., 2007).

Brain cancer is now the leading cause of death from cancer in children under the age of 15 and the second leading cause of death from cancer from age 15 to 34. In adults, brain cancer is proportionately less common than other cancers, yet it accounts for a disproportionate percentage of deaths from cancer (Legler et al., 2000). Primary brain tumors are classified according to the tissue from which they arise. The most common are gliomas which arise from tissue that supports and nourishes the brain, the glial tissue. They account for about 45-50% of all primary brain tumors and include astrocytomas, oligodendrogliomas, and tumors with mixtures of two or more of these cell types (Penas-Prado et al., 2012). With the advancement in technology, Glioblastoma and Neuroblastoma remains to be leading cause of death in adults and children, respectively. Conventional treatment of brain tumors has advanced only incrementally in the last 30 years and still yields poor outcomes. The current strategy of surgery, radiation, and chemotherapy has increased median survival to approximately 15 months (Gilbert, 2011). With the advent of molecular biology and
consequent improved understanding of basic tumor biology, targeted therapies have become corner stones for cancer treatment.

Gliomas represent a group of low-grade and high-grade brain tumors that originate from glia (from the Greek for “glue”). One major theory postulates that neural stem cells or neural progenitors undergo transformation events when they are in a transit-amplifying phase during development (Hadjipanayis and Van Meir, 2009). The major group of malignant gliomas in the brain are anaplastic astrocytomas and glioblastomas. Anaplastic astrocytomas are diffusely infiltrating neoplasms that demonstrate focal or dispersed anaplasia and an increased proliferation index compared with astrocytomas of a lesser grade. Glioblastoma (GBM) is a grade IV glioma and accounts for approximately 75% of all high-grade gliomas with approximately 9,000 new cases per year diagnosed in the United States alone, making it the most common adult brain tumor. GBM is the most aggressive glial neoplasm, and despite advances in medical management, the outcomes remain quite poor. The current standard of care for high-grade glioma patients is maximum surgical resection combined with radiation and concomitant and adjuvant temozolomide can increase survival further to approximately 15 months (Swartling et al., 2012).

Neuroblastoma is the most common cancer during infancy and the most common solid extracranial cancer of childhood. At the time of diagnosis, >70% of patients present with distant metastases. Neuroblastic tumors are derived from neuroectodermal cells that originate from the neural crest during fetal development. These cells are normally destined to form the adrenal medulla and sympathetic nervous system (Turkel and Itabashi, 1974; Brodeur, 2000; 2003; Maris et al., 2007). Failure of these cells to respond to differentiation signals is the first step towards malignant transformation of these neuroblastic cells. Histological markers of the developmental lineage from which tumor cells originate can still be found in the mature tumor (Hoehner et al., 1998). The neuroblastoma is the most aggressive of this family of tumors and in turn may be classified as differentiating, poorly differentiated and the most aggressive undifferentiated. The undifferentiated neuroblastoma are composed almost entirely of neuroblasts which appear as small round blue cells. The ganglioneuroblastoma contain neuroblasts with a more mature appearance that are clustered in small foci surrounded by schwannian stroma. Ganglioneuroma are predominantly composed of schwann cells with mature ganglion cells (Ambros et al., 2002; Shimada et al., 2009). Standard treatment for
neuroblastoma involves radiation and chemotherapy. Chemotherapeutic agents include cisplatin, doxorubicin, etoposide, and cyclophosphamide. However, the side effects of these treatments in children can be serious due to both acute damage and toxicity and increased occurrence of secondary tumors. In addition, even with aggressive treatment, mortality is still high in more advanced stages of the disease with <50% survival rate (Ebb et al., 2001). Therefore, the search for new nontoxic drugs for single or multidrug therapy is especially important.

### 2.2 Withania somnifera and its potential role in integrative oncology:

*Withania somnifera* (L.) Dunal commonly known as “Ashwagandha”, “Asgandh” and “Winter Cherry” belongs to the family Solanaceae. Ashwagandha is high in medicinal value and extensively used in Ayurvedic formulations. The drug has been positively tested for adaptogenic activity (Singh et al., 2001), anti-tumor and radiosensitizing (Devi et al., 1996; Singh et al., 2001), anti-telomerase (Singh et al., 2001), chemopreventive (Prakash et al., 2002; Ppadmavathi et al., 2005), cardiovascular (Adams et al., 2002), neuroprotective (Jain et al., 2001; Ahmad et al., 2005), anti-inflammatory (Sahni and Srivastava, 1995), anti-convulsant (George and Kulkarni, 1996) and antioxidant and pharmacological (Bhattacharya et al., 1997; Dhuley, 1997, 1998; Singh and Kumar, 1998; Thiagarajan et al., 2003; Govindarajan et al., 2005), nootropic (Dhuley, 2001) properties. Roots of Ashwagandha are however, widely used to source a restorative drug (Asthana and Raina, 1989). Few reports are available on the toxicity of some withanolides (Budhiraja et al., 2000). Similarity between the properties of its roots with those of Ginseng has led it to be called “Indian Ginseng” (Devi, 1996).

Ashwagandha, however, is often underutilized in the oncology arena, despite the fact that it shows direct antitumor and cancer preventive activity. Furthermore, Ashwagandha has the potential to increase tumor sensitization to radiation and chemotherapy while reducing some of the most common side effects of these conventional therapies. Experiments in test tubes and in animal models demonstrated that Ashwagandha plays an anticancer role by inducing apoptosis and cell cycle arrest, enhancing the immune system, and inhibition of angiogenesis and metastasis. Interestingly, as Ashwagandha exhibits both anti-oxidant and pro-oxidant activities, it has been reported to sensitize tumors to radiation while presenting itself a radio/chemo-protector for normal cells (Mishra et al., 2000; Winters, 2006). P. Uma
Devi, a radiation biologist from Jawaharlal Nehru Cancer Hospital and Research Centre (Manipal, India), was one of the early pioneers to research on ASH effects on cancer growth. When crude alcoholic extract from ASH roots was injected (i.p., 200–1000 mg/kg body wt daily for 15 days) in mice, complete regression of injected sarcoma occurred within 100 days (Devi, 1992). Then the usefulness of Ashwagandha as an anti-cancer agent from a radiation oncology perspective was demonstrated in synergism with other treatment modalities, namely radiotherapy and hyperthermia, a phenomenon that was related to depletion of cellular glutathione with Ashwagandha treatment (Devi et al., 1993). It was demonstrated that the cumulative doses of the extract (500–750 mg/kg daily) did not show any toxicity, in contrast to pure withaferin A that was toxic even at a low dose. The results supported the importance of Ashwagandha extract as a novel candidate herbal clinical sensitizer. But before such appreciation of clinical uses of crude extracts came to fore, it is noteworthy that it had taken almost two decades since the first series of reports on the anti-neoplastic potentials of the purified component withaferin A were published (Shohat, 1973). Additionally, it possesses anti-angiogenic activity (Mathur et al., 2006). Orally administered ashwagandha extract significantly inhibited experimentally induced stomach cancer in laboratory animals. Tumor incidence was reduced by 60% and tumor multiplicity (number) by 92%. Similarly, in a rodent model of skin cancer, ashwagandha inhibited tumor incidence and multiplicity by 45% and 71%, respectively (Padmavathi et al., 2005).

Only limited in vitro studies are available on the role and action of Ashwagandha in CNS related tumors. Several withanolides from the leaves of Ashwagandha has been tested on SF-268 CNS cell line along with other cell lines from different origins and was shown that Withanolides inhibit the cell proliferation in dose dependent manner (Jayaprakasam, 2003). Ashwagandha constituent, ashwagandhanolide, from the root extract has displayed growth inhibitory effect on CNS cell line SF-268 along with colon (HCT-116), lung (NCI H460), human gastric (AGS), breast (MCF-7) cell lines, with IC50 values in the range 0.43-1.48 µg/mL. (Subbaraju et al., 2006). Ethanolic extract of root, stem and leaves of Ashwagandha has been shown to possess cytotoxicity activity with maximum in leaf extract against human brain cell line IMR-32 (neuroblastoma) and other cell lines of different tissues (Yadav et al., 2011).
2.3 Glutamate induced excitotoxicity and Neuroprotection

Neurodegeneration results from the cumulative loss in structure or function of neurons. Neuronal death in these cases can be caused by triggers for programs that lead to cellular demise or programmed cell death, such as apoptosis, autophagy. Processes triggering cell death include defects in protein degradation, reactive oxygen species, calcium dysregulation, mitochondrial dysfunction and excitotoxicity (Clarke, 1990). Cross talk between pathways has been reported (Gonzalez-Polo et al., 2005; Hsieh et al., 2009) and neurodegenerative disease samples can show morphological signs of necrosis (Artal-Sanz and Tavernarakis, 2005). Thus, the mechanisms responsible for neuronal death may represent more of a continuum rather than a process that can simply be defined categorically. Despite more than 30 years of extensive research neuroprotection remains an unmet need in the treatment of stroke and chronic neurodegenerative disorders such as Parkinson’s disease (PD), Alzheimer’s disease (AD), and Huntington’s disease (HD). All these disorders share an excitotoxic component that is largely, albeit not exclusively, mediated by an overactivation of N-methyl-D-aspartate (NMDA) receptors (Olney et al., 1997; Zipfel et al., 1999). Drugs that selective block NR2B-containing extrasynaptic NMDA receptors are more promising as neuroprotective agents (Hardingham and Bading, 2010). This highlights the need for targeting receptors that “modulate” and not “mediate” excitatory synaptic transmission in an attempt to obtain neuroprotective drugs with a good profile of safety and tolerability.

2.3.1 Glutamate as a Neurotransmitter

Glutamate is one of twenty essential amino acids and is the main excitatory neurotransmitter in the mammalian CNS. Glutamate has been reported to regulate neurogenesis, neurite outgrowth, synaptogenesis and neuron survival (reviewed in Mattson, 2008). The concentration of glutamate is strictly maintained in the CNS, and glutamate released into the synaptic gap is promptly recovered by neurons and/or glial cells (especially astrocytes) via the glutamate transporter 1. Glutamate receptors are categorized into two groups, ionotropic and metabotropic. Metabotropic glutamate receptors (mGluRs) mediate slow response by activating different downstream second messenger molecules via heterotrimeric G-protein. The ionotrophic glutamate receptors (iGluRs) are ligand-gated ion channels, which permit the flow of $\text{Na}^+$ and/or $\text{Ca}^{2+}$ in response to activation. The major function of iGluRs is
mediating fast excitatory synaptic transmission (Rang et al., 1995). Glutamate activates ionotropic receptors such as alpha-amino-5-methyl-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-Methyl-D-aspartic acid (NMDA) receptors, which regulate neuronal membrane depolarization and \( \text{Ca}^{2+} \) influx and are integral in the functional responses of neurons in processes such as learning and memory (Dingledine et al., 1999).

2.3.2 Mechanism of glutamate excitotoxicity

Excitotoxicity is the paradoxical property exhibited by excitatory amino acids such as glutamate, of causing acute neuronal degeneration by excessive stimulation of postsynaptic EAA ionotropic receptors, i.e. receptors through which glutamate functions as a transmitter (Olney, 1990). Overstimulation of the glutamatergic system, as observed when glutamate concentration in the synaptic cleft increases, may be neurotoxic (Izquierdo & Medina, 1997; Ozawa et al., 1998). This is primarily mediated via an influx of calcium due to NMDA receptor activation (Manev et al., 1989), which causes calcium, sodium, chloride and zinc influx, and efflux of potassium. This results in depolarisation and increased calcium and water content, which in turn disturbs metabolic function and produces neuronal swelling, damaging the cell membrane (Sapolsky, 2001, Hou and MacManus, 2002). A further effect of glutamate excitotoxicity is the development of dendritic beading, an effect induced by a collapse in mitochondrial ATP production associated with ion (sodium or calcium) influx, which leads to influx of extracellular water and resultant formation of beading on dendrites (Greenwood et al., 2007).

Glutamate-induced excitotoxicity depends on the presence of extracellular calcium (Choi and Rothman, 1990). Activation of NMDA receptors causes calcium influx, which initially accumulates in mitochondria prior to a subsequent efflux of calcium into the cytoplasm (Budd and Nicholls, 1996). This lead to an increase in mitochondrial respiration resulting in elevated levels of superoxide and other genotoxic free radicals (Sengpiel et al., 1998; Chinopoulos et al., 2000). It does not appear however, that this calcium is emitted from the mitochondria (Ward et al., 2005). Glutamate-induced excitotoxicity leads to MPT pore opening, leading to leakage of calcium, ROS and cytochrome c from the mitochondria (Chalmers and Nicholls, 2003). Further damage to the cell membrane results from glutamate-induced ROS production, which damages cell membrane lipids (Sapolsky, 2001; Hou and
MacManus, 2002). The reactive oxygen species (ROS), together with rapid mitochondrial membrane permeability changes, trigger cell death in a process termed excitotoxicity (Reynolds, 1999; Mattson, 2003). High concentrations of glutamate can cause neuronal death, which typically involves DNA damage and induction of apoptosis (Kruman et al., 2000; Culmsee et al., 2001).

![Figure 1: Mechanism of glutamate excitotoxicity](image)

Figure 1: Mechanism of glutamate excitotoxicity: Glutamate accumulation as a result of decreased functional EAAT2 activity triggers ionotropic glutamate receptor activation, which causes an increase in the \([\text{Ca}^{2+}]_i\). At the same time, elevated levels of extracellular glutamate inhibit system \(x_c\) activity. This inhibition leads to decreased intracellular cysteine and subsequent impairment of glutathione production, culminating in the inability to neutralize reactive oxygen species. Reactive oxygen species cause membrane oxidation and ATP-dependent \(\text{Ca}^{2+}\) release from the endoplasmic reticulum (ER), leading to mitochondrial damage and further ATP depletion. With severe reduction in ATP levels, \(\text{Na}^+\) and water enter the cell and precipitate a massive cell volume increase. \(\text{K}^+\) efflux fails to maintain ionic homeostasis, which results in further increases in \(\text{Na}^+\) and water influx. As a result, the cell swells, and its plasma membrane ruptures, leading to cellular collapse. In the final stage, the leakage of intracellular contents results in the activation of extracellular proteases, which induces inflammation and eventual cell death (Adapted from Noch and Khalili, 2009).

Destruction of the plasma membrane calcium pump by calcium-activated calpains is another feature of excitotoxicity (Bano et al., 2005). This may explain the link between excitotoxicity and necrosis, as plasma membrane calcium pump
cleavage leads to excessive intracellular calcium, despite initial activation of apoptosis mechanisms (Schwab et al., 2002). The results of glutamate excitotoxicity can themselves limit the damage produced, specifically the increases in calcium and ROS: NMDA receptors can be damaged by the action of calpain, and calcium currents inhibited by the calcium-dependant activation of calcineurin and calmodulin, which are all activated by increasing levels of intracellular calcium (Sapolsky, 2001). Furthermore, damage to intracellular proteins initiates production of 'heat shock proteins', which protect against calcium and ROS-induced protein damage, and also antioxidant enzymes production rises to compensate for increased ROS levels (Sapolsky, 2001).

**2.3.3 Role of glial cells in glutamate transport**

Astrocytes constitute the majority of glial cells within the brain and may account for up to 50% of the brain’s volume (Tower and Young, 1973). Numerous discoveries continue to uncover a plethora of astrocytic functions. The astrocyte is a versatile cell tactically located between blood vessels and neurons (Kettenmann and Ransom, 2005). Among their important functions, astrocytes take up glucose, influence cerebral blood flow (Mulligan and MacVicar, 2004; Takano et al., 2006; Gordon et al., 2008), maintain extracellular potassium (Karwoski et al., 1989) and neurotransmitter levels (Danbolt, 2001), including glutamate. The role of astrocytes in neuronal degeneration has been extensively studied. The astrocyte selective glutamate transporter EAAT2 has been shown to be paramount in keeping extracellular glutamate below excitotoxic levels (Rothstein et al., 1996). Astrocytes in close proximity to the neural synapse permits detection and reaction to increased levels of extracellular neurotransmitters (Volterra and Meldolesi, 2005). When they are damaged in a way that affects their ability to sense or respond to increases in glutamate levels, the microenvironment for nearby neurons is disrupted. This may lead to an acceleration of the neurodegenerative process (Rossi and Volterra, 2009). In addition to neurodegenerative disease, excitotoxicity may be a factor in ischaemic models of neuronal damage (Bruijn et al., 2004). Channel dysregulation leading to $\text{Ca}^{2+}$ influx during ischaemia can overload neurons in a manner similar to excitotoxic conditions (Xiong et al., 2004). An important $\text{Na}^+ / \text{Ca}^{2+}$ exchanger, NCX, is cleaved both under ischaemic conditions and when exposed to glutamate, leading to neuronal cell death (Philipson and Nicoll, 2000).
2.3.4 Role of Glutamate in neurodegenerative disorders

Many studies have reported that glutamate excitotoxicity increases oxidative stress in both in vitro and in vivo models, and accumulating evidence indicates that glutamate-induced oxidative stress contributes to neuronal death in neurodegenerative diseases (Mattson, 2003). There is considerable evidence that this capacity declines with age, raising the notion that glutamate-induced oxidative stress could pose a much greater burden in aging neurons (Rao et al., 2001; Raji et al., 2002; Intano et al., 2003). Further, if the glutamate system is dysregulated during aging, or if additional oxidative stress is placed on neurons, then the neurodegenerative process gets accelerated.

Alzheimer’s disease (AD) is the most common neurodegenerative disorder in the elderly population. Glutamate excitotoxicity undoubtedly has a role in AD, and likely accelerates disease progression (Choi, 1988; Facheris et al., 2004; Dong et al., 2009; Lau and Tymianski, 2010). Amyloid b-peptide (Ab) tends to aggregate with both itself and other proteins, forming inter-cellular senile plaques potentially increasing the vulnerability of neurons. Studies of mutations in the b-amyloid precursor protein (APP) and presenilin 1 (PS1) that cause inherited early-onset forms of AD have provided considerable evidence for a pivotal role for Ab in the disease process (for review see Mattson, 2004). Ab induces membrane-associated oxidative stress which can render neurons vulnerable to excitotoxicity (Mattson et al., 1992). By increasing ROS production, Ab can cause damage to both nuclear (Kruman et al., 2002) and mitochondrial (Bozner et al., 1997) DNA in neurons.

Parkinson’s disease (PD) is another neurodegenerative disease commonly afflicting the elderly. There are a number of genetic mutations known to cause PD including mutations in genes encoding a-synuclein, parkin, DJ1 and LRRK2 (Hardy et al., 2006). The parkin and DJ1 proteins play roles in the regulation and stability of the excitatory glutamate synapses, enhancing the plasticity of glutamate synapses and may protect neurons against glutamate excitotoxicity (Wang et al., 2008; Dong et al., 2009). Huntington’s disease (HD) is a genetically inherited autosomal dominant neurodegenerative disorder caused by trinucleotide (CAG) repeat expansions in the huntingtin gene. The expression of mutant huntingtin enhances NMDA activity and sensitizes type 1 inositol 1,4,5-trisphosphate receptors, causing a disturbance in calcium homeostasis (Bezprozvanny and Hayden, 2004; Zhang et al., 2008). The
disrupted calcium homeostasis induces mitochondrial dysfunction resulting in reduction of ATP concentration and impairment of mitochondrial energy metabolism associated with generation of ROS (Brouillet et al., 1999). Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder linked to progressive motor neuron loss. The hallmark of ALS is motor neuron degeneration, which may result, in part, from over activation of glutamate receptors in motor neurons (Corona et al., 2007). Motor neurons from Cu/Zn-SOD mutant mice (ALS model) are sensitive to glutamate toxicity associated with ROS production and display elevated intracellular calcium levels and mitochondrial dysfunction (Kruzan et al., 1999). Collectively, glutamate-induced oxidative stress and DNA damage has important pathogenic roles in many neurodegenerative disorders (Rog and McKinnon, 2000; Facheris et al., 2004; Dong et al., 2009).

2.3.5 Neuroprotection- mechanism and strategies

The CNS is particularly vulnerable to injury and to a range of late onset degenerative conditions. Coupled with the limited ability of the neuronal population in the adult CNS to regenerate or to be replenished, this means that these conditions are particularly damaging to individuals and to society as a whole. It is salient to note that there are no effective treatments or cures neither for traumatic CNS injury nor for any of the major neurodegenerative diseases. It is perplexing that in some cases, the nature of the presumptive initiating agent is well known, for example, the aberrant proteins which appear to lie at the heart of familial versions of Alzheimer’s disease, Parkinson’s disease, and motor neuron disease (Brundin, 2010). All these disorders share an excitotoxic component that is largely, albeit not exclusively, mediated by an overactivation of N-methyl-D-aspartate (NMDA) receptors (Olney et al., 1997; Zipfel et al., 1999). The lack of success of NMDA receptor antagonists in the treatment of stroke is one of the major pitfalls in the history of neuroscience. A non-selective block of NMDA receptors, regardless of their subunit composition, severely impairs excitatory synaptic transmission and plasticity causing sedation, ataxia, and memory loss (Lipton, 2006; Koller and Urwyler, 2010), and restrains the trophic effect of NR2A-containing synaptic NMDA receptors on neurons (reviewed by Hardinghaim and Bading, 2010). Drugs that selectively block NR2B-containing extrasynaptic NMDA receptors are more promising as neuroprotective agents (Hardingham and Bading, 2010). MK-801 (dizocilpine), Memantine, Cerestat, dextromethorphan and
its metabolite dextrophan are all drugs that block the NMDAR at the level of the channel pore, thereby reducing calcium entry. Each of these drugs has been shown in animal models to provide histological and behavioural neuroprotection following focal ischaemia (Steinberg et al., 1988, Ozyurt et al., 1988, Seif el Nasar et al., 1990, Block and Schwarz, 1996).

2.4 Ayurveda and role of Ashwagandha in the Central Nervous System

Ashwagandha - the *Queen of Ayurveda* is considered as “Rasayana” (CSIR, 1976). Its roots and leaves are used in a number of preparations for their anti-inflammatory, anticonvulsive, antitumor, immunosuppressive and antioxidant properties besides for promoting vigor and stamina (Al-Hindawi et al., 1992; Devi et al., 1996; Bhattacharya et al., 1997; Kulkarni et al., 1998). Therapeutic value of its roots is considered comparable to that of Panax ginseng and it is often referred to as ‘Indian ginseng” (CSIR, 1976). Pharmacological investigation suggests its safe and better utility than P. ginseng (Korean drug Ginseng) notably in view of “Ginseng abuse syndrome” of the latter (Grandhi et al., 1994). Moreover, Ashwagandha has a shorter life cycle; it takes only 8 months to reach maturity while Ginseng requires 7 years to develop fully. Ashwagandha is increasingly becoming a popular adaptogenic herb and is available throughout the Western world as a dietary supplement.

The medicinal properties of Ashwagandha are attributed to the group of compounds called withanolides. A large number of withanolides have been identified in its roots and leaves. Withanolides are C–28 steroidal lactones. Withaferin A represents the first natural lactone of the withanolide series isolated from *W. somnifera* shoots (Nigam and Kandalkar, 1995). Till date, about forty steroidal lactones structurally related to Withaferin A, have been reported (Ray and Gupta, 1994).

2.4.1. Neuroprotective role of Ashwagandha in Neurodegenerative disorders

Ashwagandha has general stimulating and regenerative qualities and is used among others for the treatment of nervous exhaustion, memory related conditions, insomnia etc. Clinical trials and animal research support the use of Ashwagandha for treatment of neurological disorders. Research studies have proven that Ashwagandha preparations have potential therapeutic role in almost every CNS related disorders. It modulates GABAergic, cholinergic and oxidative systems and the phytochemicals
present in it have been proven to be responsible for overcoming the excitotoxicity and oxidative damage (Parihar and Hemnani, 2003; Russo et al., 2001) in various in vitro and animal models.

The potential of Ashwagandha for regeneration has been explored in some of the in vitro as well as in vivo studies. It is well known that the extension of dendrites and axons in neurons may compensate for and repair damaged neuronal circuits in the dementia brain. Ashwagandha root extract significantly increased the percentage of cells with neurites in human neuroblastoma SK-N-SH cells in dose and time dependent manner which was associated with the increase in expression of dendritic markers MAP2 and PSD-95 (Tohda et al., 2000). The Methnaolic extract of Ashwagandha has been characterized to contain withanolides such as withanolide A, withanoside IV and withanoside VI, which induce neurite outgrowth in human neuroblastoma SHSY5Y. Withanolide A, withanoside IV, withanoside VI and coagulin Q has been shown to possess significant neurite outgrowth activity on a human neuroblastoma SH-SY5Y cell line (Zhao et al., 2002). In another study using methanolic extract of Ashwagandha, withanolide A, withanoside IV and withanoside VI showed neuritic regeneration and synaptic reconstruction in Aβ(25-35)-induced damaged cortical neurons (Tohda et al., 2005). Ashwagandha extract was tested for its potent neuroprotective properties in H2O2 and Aβ (1–42) induced cytotoxicity for novel approaches to treat dementia, especially dementia of the Alzheimer’s type (AD). Aqueous root extract significantly protected the differentiated PC12 cells against both H2O2 and Aβ (1–42) induced cytotoxicity, in a dose dependent manner demonstrating the neuroprotective properties of Ashwagandha (Kumar et al., 2010).

Parkinson's disease (PD) is a neurodegenerative disease which belongs to a group of conditions called motor system disorders due to loss of dopamine (DA) neurons in substantia nigra. The exact mechanism of the cell death of DA neurons is still unknown. In a clinical study of 18 clinically diagnosed parkinsonian patients, Ashwagandha treatment (a concoction in cow's milk of powdered Withania somnifera, Mucuna pruriens and Hyoscyamus reticulatus seeds and Sida cordifolia roots) showed significant improvement in activities of daily living and on motor examination following Ayurveda medication (Nagashayana et al., 2000). One of the acceptable models used for screening drugs for Parkinsonism is haloperidol or reserpine-induced catalepsy in mice. Administration of BR-16A (Mentat®), a polyherbal formulation of Ashwagandha (50 and 100 mg/kg, po), significantly
reversed the haloperidol or reserpine-induced catalepsy suggesting that Ashwagandha has protective effect against Neuroleptic induced catalepsy (Kumar and Kulkarni, 2006).

Table I: Therapeutic use and proposed mechanism of action of *Withania somnifera* in various CNS related disorders

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<td>Antioxidant mechanism</td>
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<td>Cerebral ischemia</td>
<td>Antioxidant mechanism</td>
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<td>Anxiety</td>
<td>Increasing GABA levels in the brain</td>
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<td>Drug addiction</td>
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6-Hydroxydopamine (6-OHDA) elicits its toxic manifestations through oxidant stress and is one of the most widely used rat models for Parkinson's disease. Ahmad *et al.*, (2005) in their study pretreated animals with 100, 200 and 300 mg/kg b.w. of the Ashwagandha extract orally for 3 weeks before 6-OHDA infusion into the right striatum. Ashwagandha extract was able to revert all the physiological and biochemical parameters of oxidative stress in dose dependent manner as tested by neurobehavioral activity and oxidative enzymes, catecholamine content, dopaminergic D2 receptor binding and tyrosine hydroxylase expression as compared to 6-OHDA treated animals (Ahmad *et al.*, 2005). In another study on Parkinson’s model, parkinsonism was induced by 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP). Ashwagandha root extract (100 mg/kg body weight) oral treatment resulted
in a significant improvement in the mice's behavior and antioxidant status, along with a significant reduction in the level of lipid peroxidation (Sankar et al., 2007). Ashwagandha leaf extract (100mg/kg bw) has been shown to normalize the levels of GSH, GPx and TBARs in the MPTP induced PD animals. There was improvement in the motor function of the Ashwagandha treated animals as evident by rota rod and hang test proving it to be strong contender in treating catecholamines, oxidative damage and physiological abnormalities seen in the PD disease (Rajasankar et al., 2009a; Rajasankar et al., 2009b).

The amnesia induced by scopolamine is also associated with main symptom of Alzheimer’s Disease. In a recent study by Konar et al., (2011) Ashwagandha was shown to ameliorate scopolamine induced downregulation of expression of BDNF and GFAP in dose and time dependent manner in animal model. Ashwagandha leaf extract and its bioactive component, Withanone was able to revert the scopolamine induced cytotoxicity in the brain cell IMR32 neuronal and C6 glial cells which was associated with downregulation of neuronal proteins (NF-H, MAP2, PSD-95, GAP-43 and GFAP). It was also able to augment the scopolamine induced upregulation of DNA damage- γH2AX and oxidative stress- ROS markers in these cell lines (Konar et al., 2011).

Another landmark study showed that Withanolide A, isolated from the Ashwagandha root extract could regenerate neurites and reconstruct synapses in severely damaged neurons both in vitro and in vivo systems (Kuboyama et al., 2005). Withanoside IV also induced neurite outgrowth in cultured rat cortical neurons. Oral administration of withanoside IV (10 µM/kg) significantly improved memory deficits in Aβ (25-35)-injected mice and prevented loss of axons, dendrites, and synapses. Sominone, an aglycone of withanoside IV, was identified as the main metabolite after oral administration of withanoside IV. Sominone induced significant axonal and dendritic regeneration and synaptic reconstruction in cultured rat cortical neurons damaged by Aβ (25-35). Ashwagandha constituent withanoside IV has been reported to ameliorate neuronal dysfunction in Alzheimer's disease model (Kuboyama et al., 2006; Kuboyama et al., 2002). Sominone was further found to reinforce the morphological plasticity of neurons by activation of the RET pathway and thus enhance memory. It has been proposed to be a GDNF-independent stimulator of the RET pathway and/or a novel modulator of RET signalling (Tohda and Joyashiki, 2009). In another study related to AD, Withanamides from Ashwagandha fruit were
tested for their ability to protect the PC-12 rat neuronal cells, from beta-amyloid induced cell damage. Withanamides negated the amyloid induced cell death and it was shown that withanamides uniquely bind to the active motif of beta-amyloid (25-35) and thus prevent the fibril formation (Jayaprakasam et al., 2010). Similarly, aqueous Ashwagandha extract was able to inhibit fibril formation by the amyloid-β peptide in vitro as evident by transmission electron microscopy and ThT fluorescence assay in a concentration-dependent manner as compared with control samples, thus proving to be an important candidate in AD therapeutics (Kumar et al., 2011).

Tardive dyskinesia (TD) is a serious motor side effect of chronic neuroleptic therapy. The protective effects were observed in haloperidol-induced vacuous chewing in animal model, when Ashwagandha (100 and 200 mg, p.o.), was administered concomitantly with haloperidol for 28 days (Bhattacharya et al., 2002). Vacuous chewing movements in rats are widely accepted as an animal model of tardive dyskinesia. Chronic treatment with Ashwagandha root extract for a period of 4 weeks to reserpine treated animals (TD induced animals) significantly and dose dependently (50 and 100 mg/kg) reduced the reserpine-induced vacuous chewing movements and tongue protrusions and reversed reserpine-induced retention deficits. It also significantly reversed the reserpine-induced decrease in brain SOD and catalase levels in rats. Thus Ashwagandha root extract could be a potential drug for the treatment of drug-induced dyskinesia (Naidu et al., 2006). 3-Nitropropionic acid (3-NP) induced HD animal model has also been used to investigate the effects of Ashwagandha root extract. Ashwagandha extracts (100 and 200 mg/kg) for a period of 2 weeks dose-dependently improved 3-NP-induced behavioral, biochemical, and enzymatic changes via its antioxidant activity (Kumar and Kumar, 2009).

Total alkaloid extract from the roots of Ashwagandha has been studied for its effects on the CNS (Malhotra et al., 1965). Ashwagandha exerted a mild depressant tranquilizer effect on the CNS in monkeys, cats, dogs, albino rats, and mice. Effects of sitoindosides VII-X and withaferin isolated from aqueous methanol extract of roots of Ashwagandha enhanced acetylcholinesterase (AChE) activity in the lateral septum and globus pallidus, and decreased AChE activity in the vertical diagonal band in male Wistar rats. There was increase in cortical muscarinic acetylcholine receptor capacity which might partly explain the cognition-enhancing and memory-improving effects of Ashwagandha extracts in animal models and human studies (Schliebs et al., 1997). The anxiolytic and antidepressant actions of the bioactive glycowithanolides
from Ashwagandha roots have been studied by Bhattacharya et al., (2000). There was a dose-related reversal of the stress effects as evident by augmentation of SOD and LPO activities and enhanced the activities of CAT and GPX, lending support to the clinical use of Ashwagandha as an antistress adaptogen. (Bhattacharya et al., 2001). The neuroprotective effects of Ashwagandha were studied on stressed adult female Swiss albino rats. Treatment with root extract of Ashwagandha (Stresscom capsules, Dabur India Ltd) significantly reduced the number of degenerating cells in the brain (Jain et al., 2001). In another study the Ashwagandha extract was examined to retard or reverse excitotoxic neuronal injury induced by kainic acid (KA) in female Swiss albino mice. Ethanolic extract of Ashwagandha mitigated the effects of excitotoxicity and oxidative damage in hippocampus possibly due to its antioxidative properties (Parihar and Hemnani, 2003). Ashwagandha extract has been shown reducing oxidative damage in cortex and hippocampus induced by streptozotocin (STZ) in diabetic mice model, possibly via antioxidative mechanisms (Parihar et al., 2004).

In a study, to explore the underlying molecular mechanism of neuroprotective action of the root extract of Ashwagandha, restrain induced stress model was used (Bhatnagar et al., 2009). Activity of NADPH diaphorase (NADPH-d) and factors (Acetylcholine, serotonin and corticosterone), which regulate NADPH-d activity were studied. Treatment with Ashwagandha extract significantly reversed the stress induced NADPH-d activation. It was proposed that inhibition of NADPH-d by Ashwagandha was not a direct effect of extract on NADPH-d, instead it was inhibited via suppressing corticosterone release and activating cholineacetyltransferase, which in turn increase serotonin level in hippocampus to inhibit NADPH-d. Thus, the main mechanism underlying the neuroprotective effects of Ashwanagadha could be attributed to its role in the down regulation of nNOS and neurochemical alterations of specific neurotransmitter systems. Ashwagandha extract could also suppress glucocorticoid release in chronic stress which could be exploited for treatment of neurodegenerative disease like Alzheimer’s as well as in oxidative stress (Bhattacharya et al., 2001; Bhatnagar et al., 2009). Therefore, Ashwagandha constituents Withanone, Sominone, Withanolide A, withanoside IV, and withanoside VI are important candidates for the therapeutic treatment of neurodegenerative diseases such as PD, AD and HD (Tohda et al., 2008; Konar et al., 2011).
2.5 Molecular markers for neural stress, adhesion and plasticity

2.5.1 Glial intermediate filament protein and its physiological role

A key component of the astrocyte’s cytoskeleton, that warrants cell integrity and resilience, is the intermediate filament (IF) network. Besides the pivotal role in the cell’s structural properties, novel IF network functions associated with transduction of biomechanical and molecular signals have emerged. GFAP is the main IF protein in astrocytes, in addition to vimentin, nestin and synemin. A noteworthy asset of GFAP is that about eight different isoforms of this IF proteins have been identified. Recent data show that these isoforms are expressed in specific subsets of astrocytes and that they can change the properties of the IF network of a cell. Classically GFAP is a marker for astrocytes, known to be induced upon brain damage or during CNS degeneration, and to be more highly expressed in the aged brain. GFAP is a highly regulated protein, whose expression is induced by multiple factors such as brain injury and disease (Eng et al., 2000), and it was shown to fluctuate under the circadian light-dark cycle (Hajos, 2008).

Although the cell volume of astrocytes increases during aging, the number of astrocytes expressing GFAP shows much more modest changes. The increased GFAP expression during aging is due to an increased transcription of GFAP, as shown by in situ hybridization at a cellular level with intronic cRNA probes (Morgan et al., 1997). It has been suggested that increased GFAP transcription during aging is caused by the increased load of oxidatively damaged proteins, which appear in tissues throughout the body during aging, including the brain (Morgan et al., 1997; Sohal and Weindruch, 1996). The enlargement of astrocytes with enhanced expression of GFAP is an indication of reactive gliosis, a process which has shown to be highly related to brain damage and aging (Nichols et al., 1993). Diseases which show increased GFAP mRNA and protein expression include Alzheimer’s disease, scrapies and Creutzfeldt-Jacob disease. Other types of injuries in the CNS which show increased GFAP are for instance cerebrovascular accidents, stab wounds and other lesions and experimental allergic encephalomyelitis, an animal model for multiple sclerosis (Eng and Ghirnikar, 1994). In addition to these neurodegenerative diseases and others like Pick’s and Huntington’s disease, GFAP expression has also been reported to be altered in different neurological conditions including developmental, infectious and inflammatory, vascular, and mood disorders. For example a decrease in GFAP
expression in different brain areas has been correlated to depression (Johnston-Wilson et al., 2000; Miguel-Hidalgo et al., 2000; Muller et al., 2001; Si et al., 2004).

2.5.2 Neurofilaments

The neuronal cytoskeleton consists of actin microfilaments, microtubules, and a network of 10-nm filaments, intermediate in size between the microfilaments and microtubules, termed intermediate filaments (IFs). In the nervous system, at least seven different proteins contribute to the IF system (Liem, 1993; Xu et al., 1994). Three of these proteins, termed the neurofilament (NF) triplet, assemble into heteropolymeric NFs, which are the most prominent cytoskeletal components in large myelinated axons. Indeed, the NF triplet are the most abundantly expressed IF proteins in neurons. In mammals the triplet proteins, i.e., the light (NF-L), mid-sized (NF-M), and heavy (NF-H) NF subunits, have apparent molecular weights in SDS-PAGE gels of 68,000, 150,000, and 200,000 kD, respectively. Each subunit protein is encoded by a separate gene (reviewed in Perrot et al., 2008). Based on sequence homology and intron placement, the NF genes have been classified as type IV IFs along with a -internexin, which is also expressed in the nervous system (Kaplan et al., 1990). The correlation between NF number in cross sections of mature axons and axonal caliber has long suggested a role for NFs as a major determinant of axonal diameter (Roder et al., 1995). This correlation persists during axonal degeneration and regeneration and changes in NF transport correlate temporally with alterations in the caliber of axons in regenerating nerves (Gold et al., 1991). Additionally, fewer NFs are found at nodes of Ranviers where axonal diameter is reduced (Elder et al., 1998). NFP phosphorylation is topographically regulated within neurons. Recent studies show that phosphorylation of the NF subunits plays a critical role in regulation of filament translocation, formation and function. It is also involved in the pathogenesis of some related neurodegenerative diseases. NF have long been assigned a role in the pathogenesis of several types of neurodegenerative disease (Lee and Cleveland, 1996; Julien and Mushynski, 1998). It is well known that accumulation of NFs is a general hallmark for several neurodegenerative diseases. These include amyotrophic lateral sclerosis (ALS), AD, Lewy bodies in PD, progressive supranuclear palsy, Charcot-Marie-Tooth disease, diabetic neuropathy and giant axonal neuropathy (Liu et al., 2004, Teunissen and Khalil, 2012). It is
widely believed that NF abnormalities in neurodegenerative disorders are the hallmark of neuronal dysfunction.

2.5.3 Neural Cell Adhesion Molecule (NCAM) and its polysialylated form (PSA-NCAM)

NCAM is very conservatively encoded in different genomes, ranging from 70% to 98% residue identities from human to frog. This protein has been found in almost all tissues with the highest expression in the central and peripheral nervous systems. NCAM is encoded by a single gene consisting of 26 exons, however via alternative splicing mechanism, has three major isoforms which slightly differ in their structure. NCAM-180 and NCAM-140 are transmembrane isoforms, able to interact with cytoskeleton and thus be more rigidly stationed in the membrane; whereas NCAM-120 is a nearly free-floating protein since it is attached to the lipid bilayer via a glycosylphosphatidylinositol (GPI) anchor. The numbers following in their names represent its approximate molecular weights. In addition, several forms of soluble NCAM exist generated by truncation or proteolysis (Olsen et al., 1993).

![Diagram of NCAM isoforms](image)

**Figure II:** Structure of three main isoforms of NCAM named according to their molecular weights (from review Kleene and Schachner, 2004).
NCAM has long been proved to interact in homophilic trans-fashion mode (NCAM-NCAM located on opposite cells (Rutishauser et al., 1982). NCAM can bind to other CAMs, for example, it has been shown that NCAM may interact with L1 molecule in cis-fashion, which triggers phosphorylation of tyrosine and serine residues in L1 (Heiland et al., 1998). Another study showed that NCAM-180 and NCAM-140, both can be associated with α- and β-tubulin and α-actinin, major components of the cytoskeleton (Büttner et al., 2003).

Activation of NCAM-mediated intracellular signaling via tyrosine kinase receptors may lead to a wide diversity of cellular events. For instance, neuronal differentiation and axonal growth can be induced by NCAM via interaction with FGFRs, which triggers dimerization of FGFRs and its subsequent autophosphorylation (Doherty and Walsh, 1996). NCAM-induced neuritogenesis is also mediated via another signaling pathway – cAMP/PKA, leading to activation of two transcription factors, CREB and c-Foc, which are downstream of PKA, since cAMP and PKA inhibitors can selectively abolish NCAM-mediated axonal outgrowth (Jessen et al., 2001).

NCAM can be post-translationally modified via polysialylation that is a highly spatially and temporally regulated process in neuronal and glial cells. The polysialylation of NCAM occurs in all vertebrates but appears to be absent in invertebrates (Rutishauser and Landmesser, 1991, 1996; Schachner, 1994). Polysialic acid can be attached to all three NCAM isoforms (including soluble ones), to their 5th Ig-domain, associating with three amino acids Asn-430, Asn-459 and Asn-404 (Finne, 1983, Nelson, et al., 1995). PSA is a highly negatively charged sugar with an unusual α-2,8 linkage in its chains which can be up to 200 residues long. Attachment of PSA to NCAM results in a large hydration cloud appearing around the core protein, which sterically inhibits homophilic binding of NCAM-NCAM on other cells.

PSA-NCAM has been implicated in several morphogenetic processes; these include axonal growth (Doherty et al., 1990), sprouting (Zhang et al., 1992; Muller et al, 1994) and cell migration (Ono et al., 1994; Wang et al., 1994). PSA decreases neurite fasciculation of neuronal fibers promoting an opposite process – defasciculation by affecting the avidity of NCAM and other recognition molecules (Hoffman et al., 1983; Rutishauser and Landmesser, 1991). There have been
discovered two enzymes which catalyze attachment of PSA to NCAM protein backbone – ST8SiaII/STX is involved in polysialylation of NCAM during development, and in adults, in stem cell-derived immature granule cell neurons; and ST8SiaIV/PST is involved in polysialylation of NCAM in mature neurons. Both PST and STX can synthesize PSA on α-2,3- or α-2,6-linked sialic acid on NCAM without an initiator (Muhlenhoff et al., 1996; Kojima et al., 1996) and both are potentially involved in the biosynthesis of PSA associated with NCAM in mammalian tissues.

2.5.3.1 Role of NCAM and PSA-NCAM in pathological conditions

Owing to physiological functions of NCAM and PSA-NCAM, the role of the molecules in different pathological conditions has been investigated. The changes in neuronal structure and connectivity, with atleast partly underlying alterations in PSA-NCAM and NCAM expression or functioning, have been suggested in molecular pathology underlying depression and also as possible target of anti-depressants (Varea et al., 2007), as well as in the development of mood and anxiety disorders following juvenile stress (Tsoory et al., 2008). Chronic stress has been shown to induce biphasic PSA-NCAM expression in the adult rat dentate gyrus (Pham et al., 2003). An increased expression of PSA-NCAM has been found in patients with Alzheimer’s disease, however, it is unclear whether the finding is an attempt of the brain tissue to try to restore its structure and function, or to compensate for the damage caused by the disease, or these changes are a part of the disease’s pathologic cascade (Mikkonen et al., 2001). NCAM1 ranked fourth in a met-analysis of schizophrenia susceptibility loci (reviewed in Schmid and Maness, 2008). PSA-NCAM levels are decreased in the hippocampus of schizophrenia patients, however levels of soluble fragments consisting mostly of the extracellular domain of NCAM in the cerebrospinal fluid (CSF) and affected brain regions (prefrontal cortex, hippocampus) are increased, which has been also correlated with disease severity and duration (Senkov et al., 2012). A mouse model with increased NCAM cleavage (NCAM-EC) that has also been observed in schizophrenic patients has been shown to display decreased emotional memory and prepulse inhibition, also increased hyperactivity and enhanced sensitivity amphetamines, as well as stereotypy (Brennaman et al., 2010). Blocking NCAM cleavage has been proposed to restore observed alterations at least partly in these animals (Barbeau et al., 1995; Nothias et al., 1997). Sato et al., (2003) demonstrated that the number of PSA-NCAM positive
cells in bilateral DG as well as marked extension of immunopositive dendrites to the molecular layer increased significantly after repeated exposure to amygaloid kindled general seizures. Pekcec et al., (2008) found that loss of PSA counteracted the status epilepticus-induced increase in neurogenesis.

### 2.5.3.2 Neural adhesion molecule as a potential target for neuroprotection

NCAM is developmentally down-regulated but increases after brain injury and this increase has been linked to potential of brain for regeneration (Becker et al., 2006). A transient up-regulation of NCAM-140 and NCAM-120 mRNA has been reported in response to cortex lesions and ischemia in astrocytes. A NCAM derived peptide binding to and inducing phosphorylation of the fibroblast growth factor receptor (FGFR), acts neuroprotectively after an ischemic insult both in vitro and in vivo (Skibo et al., 2005). The growth factor, FGF-2 associated with NCAM signalling has been described to be neuroprotective against excitotoxicity caused by glutamate (Mattson et al., 1989; Freese et al., 1992; Fernandez-Sanchez and Novelli 1993; Kume et al., 2000).

![Diagram of NCAM-mediated signal transduction pathways](image)

**Figure III**: NCAM-mediated signal transduction pathways: NCAM induces different signal transduction pathways. The structure of physical interaction between two fibronectin type III domains of NCAM and Ig domains 2 and 3 of FGFR has been recently shown (Kiselyov et al., 2003). Dashed lines represent putative interactions. This is a simplified depiction, modified from (Povlsen et al., 2003).
Control of PSA-NCAM expression by NMDA receptor activation has been described in several systems, suggesting a functional link between these two proteins. The NMDARs are a subtype of ionotropic glutamate receptors that are found widely throughout the brain. NMDA receptors exhibit a dichotomy of signaling with both toxic and plastic responses. Recent reports have shown that exposure to subtoxic concentration of NMDA results in a neuroprotective state that was measured when these neurons were subsequently challenged with toxic doses of glutamate or kainite (Singh and Kaur, 2009). Intracerebroventricular administration of the NCAM mimetic FGL peptide increases memory strength in rats and enhances presynaptic function in primary hippocampal neurons (Cambon et al., 2004). These results provide the first evidence for a memory-facilitating effect resulting from a treatment that mimics NCAM function. Upregulation of PSA-NCAM by hyperthermia may have a significant impact on hippocampal plasticity, permitting induction of the complex molecular cascade responsible for neuroprotection where prior exposure to heat shock protects against kainate-induced cell damage in the hippocampus, we show that hyperthermia upregulates PSA-NCAM expression for at least 1 week, without affecting neurogenesis (Duveau et al., 2007).

2.5.4 Heat Shock proteins in cell survival and stress

Chaperone proteins and heat shock proteins (HSP) are essential components of cellular protein folding systems under normal conditions; their expression and activities are upregulated during stress. There are several chaperone mechanisms based on the inducible HSP70 and constitutive HSC70 members of the HSP70 family. Some of these machinery serve for the correction of the structure of misfolded proteins, while others involving HSP70 and HSC70 with Bag-1 and C-terminal HSC70 interacting protein (CHIP) proteins are focused on the proteolytic degradation of irreversibly damaged polypeptides (Meacham et al., 2001). HSPs have well-characterized roles in facilitating protein folding in de novo protein synthesis and during refolding of partially denatured proteins that arise after cellular stress (Morimoto et al., 1997; Hartl and Hayer-Hartl 2002). They are a group of highly conserved and ubiquitous molecular chaperones conventionally subdivided into the following major families: HSP110, HSP90, HSP70, HSP60, HSP40, and small HSPs. Their ability to recognize and bind to denatured or partially unfolded proteins allows HSPs to counter denaturation, misfolding, and irreversible aggregation of proteins.
Family members have been implicated in the solubilization of aggregated proteins (Stege et al., 1995). Because alteration of aggregation kinetics can affect the progression of the neurodegenerative disease (Wolozin and Behl, 2000), upregulation of HSPs could alleviate neurodegeneration by modulating protein misfolding in affected neurons. This concept has led to a quest for pharmacological agents that can induce HSPs in neuronal cells as a therapeutic approach for combating neurodegeneration. The 70 kDa family of stress proteins is one of the most extensively studied. Included in this family are HSC70 (heat shock cognate, the constitutive form), HSP70 (the inducible form, also referred to as HSP72) and GRP-75 (a constitutively expressed glucose-regulated protein found in the endoplasmic reticulum) (Yenari et al., 1999, Calbrese et al., 2006).

2.5.4.1 HSP70 mediated neuroprotection in neurodegenerative disorders

The HSP70 has been demonstrated to have a neuroprotective role both in animal and cell culture models of neurotoxicity such as ischaemia (Ferriero et al., 1990; Xu et al., 2006), trauma (Brown et al., 1989), seizures (Uney et al., 1988; Vass et al., 1989) and Alzheimer’s disease (Hamos et al., 1991). Only recently, the availability of transgenic animals and gene transfer allowed us to over-express the gene encoding for HSP70, thus demonstrating that overproduction of this protein leads to protection in several different models of nervous system injury (Kelly et al., 2002). Following focal cerebral ischemia, HSP70 mRNA is synthesized in most ischemic cells except in areas of very low blood flow, due to scarce ATP levels. HSP70 proteins are produced mainly in endothelial cells, in the core of infarcts in the cells that are most resistant to ischemia, in glial cells at the edges of infarcts and in neurons outside the areas of infarction (Hata et al., 2000). It has been suggested that this neuronal expression of HSP70 outside an infarct can be used to define the ischemic penumbras, which means the zone of protein denaturation in the ischemic areas (Hata et al., 2000). As mentioned above, HSPs are induced in many neurodegenerative disorders mainly in the view of its cytoprotective function. HSP72 was overexpressed in post-mortem cortical tissue of AD patients and an increase in HSP70 mRNA was found in cerebellum hippocampus and cortex of AD patients during the agonal phase of the disease (Yoo et al., 1999). Recently Kakimura et al. demonstrated that HSP70 induces IL-6 and TNF-a in microglial cells and this event is associated with an increased phagocytosis and clearance of Ab peptides (Kakimura et al., 2002).
Overexpression of HSP70 has been reported to be associated with a decrease in apoptotic cell death and a reduction in matrix metalloproteinases (Yenari et al., 2002). Thus HSP70 is a multifaceted protein capable of protecting brain cells from injury through a variety of mechanisms. HSPs provide a line of defense against misfolded aggregation prone proteins and among the most potent suppressors of neurodegeneration in animal models (Merrin and Sherman 2005; Brown et al., 2007). Neurons may rely on their constitutive levels of HSC70 as a 'pre-protection' mechanism for defense against protein misfolding and aggregation that is induced by stressful stimuli or associated with neurodegenerative diseases.

2.5.5 Modulators of extracellular matrix (ECM) - matrix metalloproteinases (MMPs)

MMPs, a family of zinc ion-dependent endopeptidases, are capable of digesting a broad spectrum of substrates, including collagen types I, II, III, and IV and stromelysin, and are divided into subgroups that include collagenases, stromelysins, and stromelysin-like matrilysins, gelatinases, and membrane-type MMPs. These protease activities of MMPs in the tissue are regulated by tissue inhibitors of metalloproteinases (TIMPs). In particular, of more than 20 different MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) play key roles in the degradation of the main components of ECM, collagen type IV and gelatin. Their natural inhibitors are TIMP-2 and TIMP-1, respectively. In recent years, MMPs are reported to act as regulators of synaptic activity in the adult CNS, especially in the hippocampus. In a recent review, a possible role for MMPs in synaptic function, with an emphasis on MMP substrates in and around synapses has been discussed. Briefly, Ethell and Ethell (2007) review the roles of known MMP substrates in synaptogenesis, synaptic plasticity, and long-term potentiation. They further emphasize the impact of MMP-directed cleavage of various proteins including brevican, tenascin-R, laminins, BDNF, cadherins and ephrins in the formation and function of synapses within the healthy adult CNS (Ethell and Ethell, 2007).
Figure IV: Multiple role of MMPs in CNS (adapted from Agarwal et al., 2008).

Abnormally expressed MMPs are implicated in CNS diseases. Indeed, all neurological disorders have representation of elevated MMP members. Human immunodeficiency virus (HIV), and spinal cord injury all have specific roles of MMPs in inflammatory, infectious and traumatic injuries, respectively, to the CNS (reviewed in Agrawal et al., 2007). MMP inhibitors have useful roles in neurological treatments. Acutely after insults such as SCI, stroke, or following an MS relapse, many MMP members are highly upregulated. The abundance of proteolytic activity immediately following injury is damaging, and using MMP inhibitors at these acute stages can likely lead to improvements (Viappiani et al., 2006; Fingleton, 2007).

2.6 Cellular proteins as oncology markers:
2.6.1. Glial and neuronal interfilament proteins:

GFAP expression has been associated with the growth of gliomas (Rutka et al., 1997), more prominent in high-grade than in low-grade gliomas (Chumbalkar et al., 2005), and serum GFAP has proven a valuable diagnostic marker for glioblastoma multiforme (Jung et al., 2007). In astrocytic neoplasms, the number of cells expressing GFAP is inversely proportional to the extent of anaplasia. The loss of GFAP expression, the principal marker of astroglial cells, in these tumors has been proposed to constitute a step in their development and progression (Wilhelmsson et al., 2003). Malignant astrocytic tumors are often GFAP negative, and many high-grade gliomas seem to lose GFAP expression (Tascos et al., 1982). In addition,
GFAP-negative cells proliferate more rapidly than GFAP-positive cells in the same tumor (Hara et al., 1991; Kajiwara et al., 1992). Thus loss of GFAP expression could represent secondary loss of a differentiation marker or it could be a step in tumor development. Neurofilaments are associated with a number of neural, neuroendocrine and endocrine tumors, neuromas, ganglioneuromas and neuroblastomas. Neurofilaments are also present in paragangliomas and pheochromocytomas, carcinoids, neuroendocrine carcinomas of the skin and oat cell carcinomas of the lung also express neurofilaments (Dehghani et al., 2000). The NF proteins are good markers for pheochromocytoma, and their presence is of basic tumor biologic interest and of potential diagnostic value in other neuroendocrine neoplasms (Miettinen, 1987). NF genes are constitutively active in F9 teratocarcinoma cells and upregulated by RA and cAMP induced differentiation (Murtooki et al., 1999). NF methylation is a novel mechanism for head and neck chemoresistance (HNC) and may represent a candidate biomarker predictive of chemotherapeutic response and survival in patients with HNC (Chen et al., 2012).

2.6.2. **NCAM and PSA-NCAM in cancer biology:**

Changes in expression of NCAM and PSA-NCAM have been associated also with different malignant processes. Alterations in NCAM expression in human brain tumor suggest that it can either counteract or promote cancer malignancy depending on the tumor type. Early stage astrocytic tumors express all three major NCAM isoforms whereas its expression decreases with progression towards malignant glioma (Sasaki et al., 1998; Michotte et al., 2004). In colon carcinoma, pancreatic cancer, and astrocytoma, NCAM expression is markedly down-regulated, and the loss of NCAM correlates with poor prognosis. In contrast, in neuroblastoma and certain neuroendocrine tumors, cancer progression correlates with increased NCAM expression (Crnic et al., 2004). Hence, in these tumor types, the role of NCAM would be consistent with that of a tumor-promoting factor. Since a high degree of NCAM polysialylation is observed in neuroblastoma and neuroendocrine tumors (Figarella-Branger et al., 1990; Gluer et al., 1998). The results concerning the role of PSA are more consistent demonstrating that PSA is re-expressed during the progression of several malignant human tumors including neuroblastoma and glioma. In these tumors, polysialylation of NCAM appears to increase the metastatic potential and has been correlated with tumor progression and poor prognosis (Seidenfaden et al., 2003).
Since polysialylation induces NCAM-dependent invasion, it is conceivable that such a post-translational modification determines the role of NCAM in cancer cells (Demuth and Berens, 2004; Suzuki et al., 2005).

2.6.3. HSP70 and Mortalin in oncogenesis:

Elevated expression of members of the HSP70 family has also been reported in high-grade malignant tumors (Ciocca et al., 1993; Ralhan et al., 1995). In tumor cells, the intricate balance between proliferation and cell death shifts toward continued cell growth as a result of the expression of antiapoptotic proteins. Such proteins include members of the Bcl-2 family, members of the inhibitory of apoptosis protein family, and members of the HSP family - in particular, HSP70 and HSP27—that render tumor cells resistant to apoptosis (Wei et al., 1995; Jaattela, 1999). Inducible HSP70 has been suggested to have a multiple roles in cytoprotection against apoptosis; indeed, abrogation of HSP70 expression by use of antisense oligonucleotides leads to inhibition of tumor cell proliferation and apoptosis (Wei et al., 1995). Consistent with this proposal, high levels of HSP70 prevent stress-induced apoptosis. Elevated levels of HSP70, attained in transient transfections or under the control of tetracycline-inducible promoters, reduce or block caspase activation and suppress mitochondrial damage and nuclear fragmentation (Mosser et al., 1997; Buzzard et al., 1998).

Mortalin/mthsp70/PBP74/Grp75 was first identified as a member of the HSP70 family of proteins present in the cytoplasmic fractions of normal fibroblasts from CD1-ICR mouse (Wadhwa et al., 1993). Mortalin is a heat-uninducible, novel member of HSP70 family of proteins initially identified from the cytoplasmic fractions of normal mouse fibroblasts (Deocaris et al., 2006). Whereas normal cells have pancytoplasmic staining, transformed cells showed 4 types of nonpancytoplasmic staining patterns that distinguished complementation groups of human transformed cells (Pereira-Smith and Smith 1988; Wadhwa et al., 1995). The abundance of mortalin in neurons and its involvement in the processes of stress-resistance, bioenergetics and cell proliferation advance this mitochondrial chaperone as a candidate player in the phenomenon of neurogenesis. Massa et al. (1995) discovered Grp75, which later turned out to be mortalpin, from rat brain after exposure to metabolic stress. Mortalin immunoreactivity correlated strongly with the proliferative nature of astrocytic tumors: lowgrade astrocytoma, anaplastic
astrocytoma and glioblastoma. Other types of brain tumors, such as meningiomas, neurinomas, pituitary adenomas and metastases, also had invariably high level of mortalin expression compared to normal brain tissues (Takano et al., 1997).

2.6.4 The Cell cycle regulatory protein - Cyclin D1

Cyclin D1 (CD1) is considered an oncogene with an important tumorigenic role in breast cancer and other human tumors (Arnold and Papanikolaou, 2005). Emerging evidence suggests that CD1 may act through novel pathways that do not involve its widely accepted function on the cell cycle. Instead, it may also exhibit such novel activities independently of its function as Cyclin-dependent kinase (CdK) regulatory subunit (Inoue et al., 1998; Horstmann et al., 2000; Wang et al., 2004). Cyclin D1 is synthesized in the early G1 phase and plays a key role in the initiation and progression of this phase. Genetic aberrations in the regulatory circuits that govern transit through the G1 phase of the cell cycle occur frequently in human cancer. Overexpression of cyclin D1 is one of the most commonly observed alterations (Sicinski et al., 1995; Fu et al., 2004).

2.6.5 The anti-apoptotic protein Bcl-xl and its cellular functions

Bcl-2 is the prototype of a family of proteins containing at least one Bcl-2 homology (BH) region. In humans and mice, the Bcl-2 family is split into anti-apoptotic multi-domain proteins (prototypes: Bcl-2 and Bcl-XL), which contain four BH domains (numbered BH1 to BH4), pro-apoptotic multi-domain proteins (prototypes: Bax and Bak), which contain three BH domains (BH1, BH2 and BH3), and the pro-apoptotic BH3-only protein family (which has more than a dozen members) (Adams and Cory, 2007; Kroemer et al., 2007). Different combinations of Bcl-2 family proteins are expressed in a cell type-, differentiation- and activation state-dependent fashion. The principle site of action of apoptosis regulation by Bcl-2-like proteins is probably the mitochondrial membrane. The oncogenic potential of Bcl-2 family members has been attributed to disabled apoptosis (Adams and Cory, 2007), which is one of the hallmarks of cancer. However, autophagy—which turns out to be regulated by Bcl-2 proteins as well - has recently emerged as a cellular pathway that is essential for the maintenance of genomic stability and tumor suppression (Mathew et al., 2007). Thus, overexpression of Bcl-2/Bcl-XL (or loss of BH3-only proteins) may not only participate in oncogenesis by inhibiting apoptosis,
which results in improved survival of tumor cells in adverse conditions of endogenous (metabolic) or exogenous (chemotherapy-associated) stress (Pattingre and Levine, 2006).

2.6.6 Cell survival and proliferation – role of Akt

Akt/PKB is a serine/threonine protein kinase that functions as a critical regulator of cell survival and proliferation. Akt/PKB family comprises three highly homologous members known as PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 in mammalian cells. Similar to many other protein kinases, Akt/PKB contains a conserved domain structure including a specific PH domain, a central kinase domain and a carboxyl-terminal regulatory domain that mediates the interaction between signaling molecules (Altomare and Testa et al., 2005). Akt/PKB plays important roles in the signaling pathways in response to growth factors and other extracellular stimuli to regulate several cellular functions including nutrient metabolism, cell growth, apoptosis and survival.

Akt regulates many cellular processes including metabolism, proliferation, cell survival, growth and angiogenesis. (Altomare and Testa et al., 2005). It is well established that Akt plays an important role in the ability of growth and neurotrophic factors to suppress neuronal cell death (Datta et al., 1997; Burke, 2007). The invasion of neoplastic cells into brain parenchyma and fast proliferation are hallmarks of glioblastomas, the most malignant brain tumors (Nakada et al., 2007; Styli et al., 2008). Invasiveness and migration are complex processes which are regulated by phosphoinositide 3-kinase (PI3K), downstream Akt kinase and focal adhesion kinase (FAK) signaling pathways (King et al., 1997; Kim et al., 2001; Natarajan et al., 2003). Binding of ECM proteins or growth factor receptor activation triggers focal adhesion kinase phosphorylation initiating focal adhesions turnover (Hsia et al., 2003; Schlaepfer et al., 2004) and allows PI 3-kinase recruitment to the membrane and stimulation of Akt signaling (Hsia et al., 2003; Styli et al., 2008).

2.6.7 MMPs in tumor migration and invasion

Regardless, though it is well known that the expression of MMPs and TIMPs is closely related to tumor invasion. MMP-9 and -2 are differentially expressed in retinoblastoma cells, whose expression closely depends on the proliferation and differentiation of retinoblastoma cells (Kim et al., 2010). At the transcriptional level,
MMP expression is precisely controlled by various cytokines, including tumor necrosis factor-a (TNFa), acting through positive or negative regulatory elements of its genes and by phorbol esters (Sato et al., 1993; Ries and Petrides, 1995; Gottschall and Deb, 1996). During the multistep processes of tumor progression, the degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) is a critical step in disrupting the barrier restricting tumor growth and invasion. MMPs play crucial roles in invasion and metastasis and regulate signaling pathways that control cell growth, survival, invasion, inflammation and angiogenesis. Of the various MMPs thought to be involved in cancer, attention has focused on the gelatinases because (i) they are overexpressed in a variety of malignant tumors and (ii) their expression and activity are often associated with tumor aggressiveness and a poor prognosis. Elevated levels of MMP-2 and/or MMP-9 are found in breast, brain, ovarian, pancreas, colorectal, bladder, prostate and lung cancers and melanoma (Roy et al., 2009, Klein and Bischoff, 2011).

2.7. Cell culture based in vitro model system for neurooncology and neuroprotection studies

2.7.1 C6 glioma cell line:

The C6 glioma cell line has been used as an in vitro model for the study of glial cell properties. This clonal cell line was acquired from brain tumors induced by N-nitrosomethylurea (Benda et al., 1968). C6 glioma cell line has been used as in vitro glial model system because beside expressing its normal glial like properties (including production of glial marker antigens and glial fibrillary acidic protein), it also releases neurotrophic and neurite promoting factors and show hormonal responsiveness similar to those in normal glial both in vivo and in vitro (Vernadakis et al., 1992; Zhang et al., 2001). C6 glioma cell line differentiates into astrocytes like cells that express the astrocytic markers under specific culture conditions (Parker et al., 1980; Zhang et al., 2001). The C6 clonal cell line has been used to study the production of neurotrophic factors (Matsuoka et al., 1986; Westermann et al., 1988) and extracellular proteins released by astroglia (McKeever et al., 1986; Quarless and Lam, 1989). It has been reported to be a bipotential cell line that gives rise to both oligodendrocyte or astrocyte phenotypes. At late passages, however, this cell line becomes more committed to an astrocyte phenotype (Parker et al., 1980; Mangoura et al., 1989; Lee et al., 1992). C6 glioma cells have been widely used as a model system
for human glioblastoma related studies (Barth, 1998; Ling et al., 2012; Ozeki et al., 2012; Tseng and Wei et al., 2012). RA differentiated C6 cells are well established model system due to its morphological and physiological resemblance like astrocytic lineage (Bianchi et al., 2008; Singh and Kaur et al., 2009).

2.7.2 Human neuroblastoma cell line IMR-32

The human neuroblastoma cell line, IMR32 cells, was obtained from neuroblastoma tissue. The cells have a small neuroblast-like appearance (Carbone et al., 1990) and also have properties of tumor cells with approximately 48 hours doubling time (Tumilowicz et al., 1970). The IMR-32 cells are a typical of N-type neuroblastoma cells which tend to form clumps and poorly adhere to the culture dish (Rossino et al., 1991). It has been identified that the N-myc gene is amplified in IMR32 cells (Kohl et al., 1983; Schwab et al., 1984) and nerve cell adhesion molecule 140 isoform (NCAM140) are abundant in IMR-32 cells (Poongodi et al., 2002). Undifferentiated IMR-32 cells have been used as model system for neuroblastoma related studies in previous reports (Tanaka and Fukuzawa, 2008; Yadav et al., 2010). Neuroblastoma cells can be differentiated by exposing the neuroblastic cells with differentiation agents, which induce cellular morphological, biochemical and electrophysiological changes (Gotti et al., 1987; Cosgrove and Cobbett, 1991). A large number of differentiation factors have cell-type specific effects. 5-bromo-2'-deoxyuridine (BrdU or BUdR), N6-O2-dibutyryl cyclic adenosine 3'-5' monophosphate (Bt2 cAMP or cAMP), 5-azacytidine, neuron growth factor and retinoic acid (Gotti et al., 1987; Rossino et al., 1991; Hartman and Hertel, 1994; Crosland, 1996) are normally used to differentiate neuroblastoma cell line and many have been used on IMR-32 cells (Gotti et al., 1987; Rossino et al., 1991; Hartman and Hertel, 1994). Several studies have demonstrated that the human neuroblastoma IMR32 cell line is a suitable cell model of neuronal phenotype (Gotti et al., 1987; Sher et al., 1989) and exhibit a variety of calcium channel activities (Carbone et al., 1990; McEnery et al., 1997). RA differentiated IMR-32 cell cultures are well established model system for neuronal studies (Guevara-Lora et al., 2011).