IMMUNOPHILINS: NEW BIOMARKERS AND THERAPEUTIC TARGETS FOR NEURODEGENERATIVE DISEASES

by

Mihaela Avramut

MD, "Gr. T. Popa" School of Medicine, Iasi, Romania

Submitted to the Graduate Faculty of

University of Pittsburgh School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2003

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Mihaela Avramut

It was defended on

November 25, 2003

and approved by

Adriana Zeevi, PhD, Professor, Department of Pathology

Robert Bowser, PhD, Associate Professor, Department of Pathology

George K. Michalopoulos, MD, PhD, Professor and Chairman, Department of Pathology

Michael Zigmond, PhD, Professor, Department of Neurology

Cristian L. Achim, MD, PhD, Associate Professor, Departments of Pathology and Neurology
Dissertation Director

IMMUNOPHILINS: NEW BIOMARKERS AND THERAPEUTIC TARGETS FOR NEURODEGENERATIVE DISEASES

M. Avramut, MD, PhD

University of Pittsburgh, 2003

Immunophilins (IP) are receptors for immunosuppressive drugs like cyclosporin A, FK506, rapamycin and their analogs, which are collectively referred to as "immunophilin ligands" (IPL). The receptors for FK506 belong to the family of FK506- binding proteins (FKBP). Previous studies showed that FK506 augments neuronal growth in vitro. In animal models, the drug promotes morphologic and functional recovery following neuronal lesioning. Here we show for the first time that FK506 has neurotrophic effects in human brain primary cultures. Our data support a possible use of FK506 and its analogues in the treatment of neurodegenerative disorders and as adjuvants in neural tissue transplants. The effect of the drug in vivo, in patients, will ultimately depend on the presence and distribution of IP receptors in the normal and degenerating human brain.

FKBP12, the archetypal member of the FKBP family, plays a role in protein folding, protein complexes assembly and intracellular calcium release. Crush injury of facial or sciatic nerves in rat leads to markedly increased FKBP12 levels in the respective nerve nuclei in parallel with nerve regeneration. The presence of FKBP IP has never been demonstrated in the human brain. Using light and immunofluorescent microscopy, laser confocal microscopy and western blotting, we studied FKBP12 expression in a set of archival brain material from Parkinson's disease, Alzheimer's disease, dementia with Lewy bodies, encephalitic and non- encephalitic HIV- positive patients and age matched controls. We show that FKBP12 is present in the human brain, predominantly in neurons. Its levels and distribution are altered in the mid- frontal cortex, deep gray matter and midbrain of patients with neurodegenerative diseases. Moreover, it colocalizes with markers of pathology (Lewy bodies, neurofibrillary tangles and neuritic plaques) in areas of neurodegeneration. Disease- specific and region- specific changes are evident. Alterations in basal ganglia FKBP12 levels are also observed in MPTP- treated primates in association with dopaminergic loss (evidenced using PET functional imaging).

We propose that the altered expression and distribution of FKBP12 is linked to abnormal protein folding and axonal transport. It may also reflect a compensatory regenerative response that renders immunophilins promising diagnostic and therapeutic targets.

TABLE OF CONTENTS

LIST OF FIGURES

1. Background

1.1. Overview of prevalent neurodegenerative diseases: clinical features and pathophysiology

Several human chronic neurodegenerative diseases have at least two common features. First, misfolded and damaged proteins form toxic species that aggregate and cause neuronal death. Second, there are protective chaperone proteins that can mitigate these deleterious effects. This introduction outlines the main characteristics of the degenerative process in several diseases, followed by the functions of several classes of molecular chaperones and folding catalysts, including immunophilins. These are an unusual class of chaperones or adapter molecules that also function as receptors for immunosuppressive drugs and constitute the focus of the work described in subsequent chapters.

1.1.1. Parkinson's disease

Parkinson's disease (PD), the most frequent neurodegenerative movement disorder, is characterized clinically by rigidity, akinesia, resting tremor, and postural instability. The major motor disabilities of PD are associated with the depigmentation and extensive loss of dopaminergic neurons in the substantia nigra (SN) pars compacta (SNc) (Fig. 1-1). This and the presence of eosinophilic inclusions (Lewy bodies, LB) are considered to be the pathologic hallmarks of the disease. Intracytoplasmic Lewy bodies and dystrophic (Lewy) neurites are present mainly in subcortical nuclei and hippocampus and, less frequently, in cerebral cortex. SN cell loss is significantly related to striatal dopamine (DA) deficiency, as well as to both the duration and clinical severity of disease. The two major clinical subtypes of PD show different brain pathology: the akinetic-rigid form has more severe cell loss in the ventrolateral part of SN with negative correlation to DA loss in the posterior putamen, and motor symptoms related to overactivity of the GABAergic "indirect" motor loop (Fig. 1-2), which causes inhibition of the

glutamatergic thalamocortical pathway and reduced cortical activation. The tremor-dominant type shows more severe cell loss in the medial SNc and retrorubral field A 8, which project to the matrix of the dorsolateral striatum and ventromedial thalamus, thus causing hyperactivity of thalamomotor and cerebellar projections (Jellinger, 2002). Experimental data suggesting different pathophysiological mechanisms for the major clinical subtypes of PD may have important therapeutic implications.

While in vivo and tissue culture studies suggest that apoptosis represents the most common pathway in neurodegeneration, the SN of PD brains rarely displays DNA fragmentation, overexpression of proapoptotic proteins and activated caspase-3 (the effector enzyme of the terminal apopoptic cascade). This is consistent with the rapid course of apoptosis and the extremely slow progression of the neurodegenerative process in PD. The biological role of Lewy bodies and other intracellular inclusions, the mechanisms of the intracellular aggregation of insoluble protein deposits, and their importance for neuronal dysfunction are still unresolved.

The main projections from the nigra synapse on GABA dendrites in the dorsal (sensorimotor) neostriatum. The majority of the cells in the neostriatum are medium size spiny projection neurons with a minority of non-spiny interneurons (Chase and Blanchet, 1998). It appears that the organization of the nigrostriatal dopaminergic projections is closely related to the cortico-striatal inputs. The striatal dorsolateral region receives inputs from the sensorimotor cortex, the central striatum receives inputs from the association cortex while the ventromedial striatum is enervated by afferents from the limbic cortex and amygdala. In normal humans it is estimated that at least 10% of striatal synaptosomes are DA and the greatest DA losses in PD are in the putamen (Pu).

Lewy bodies, the morphologic markers of PD, are composed of an ubiquitin- positive core, surrounded by hyperphosphorylated neurofilament proteins, lipids, redox-active iron, ubiquitin, and alpha-synuclein accumulations in the periphery. Alpha-synuclein, a presynaptic protein, is usually unfolded in alpha-helical form. By gene mutation, environmental stress or other factors it can be transformed to beta-folding, which is prone to self-aggregation in filamentous fibrils and formation of insoluble intracellular inclusions. These inclusions may lead to functional disturbances and, finally, to neuronal death.

Neurotransmitter, synaptic vesicle and cytoskeletal protein transport in the dopaminergic pathway is abundant and may be prone to malfunction. The slow axonal transport is responsible primarily for trafficking of nerurofilament and microtubules, while the fast anterograde transport delivers synaptic vesicles and neurotransmitters to the axonal terminals. It is known that protein structures are not very stable in vivo and their conformation can easily change. Accumulation of unfolded proteins may have deleterious effects on cell function and for this reason cells have developed mechanisms to prevent aggregation of unfolded proteins: e.g. refolding or hydrolysis. Accumulation of unfolded proteins is characteristic to many neurodegenerative diseases, including PD. All protein inclusions contain components of the ubiquitin-proteasome degradative pathway and chaperones, the two main mechanisms of cellular protection (reviewed by Moore et al., 2003).

A direct effect of abnormal protein aggregation in PD was reported by Lee et al. (2001) who showed that α- synuclein can bind directly to DAT and promote its membrane aggregation. This aberrant aggregation may result in increased dopamine cytotoxicity due to intracellular accumulation. Nevertheless, the impairment of neuronal transport is not exclusively pre-synaptic. For example, it was recently reported that MAP-2 co-localized with α̃synuclein and ubiquitin (D' Andrea et al., 2001).

Since the physiological changes and biochemical pathways involved in the selective demise of these neurons are still unclear, it is difficult to develop therapeutical approaches to stop the progression of the disease. The best-known treatment to date is based on the use of L-DOPA or dopaminergic agonists. These are merely substitutive therapies and have limitations because of their side effects. Thus, the development of new therapeutical strategies will require a far better knowledge of the mechanism and the consequences of neuronal death in Parkinson's disease.

Until the 1990s, little was known about PD pathogenesis. Two findings brought the little-studied presynaptic protein α- synuclein to the fore. First, a missense mutation in the α-synuclein gene was found to cause a rare, familial form of PD (reviewed by Goedert, 2001). Second, LB in sporadic PD cases were found to be strongly immunoreactive for α- synuclein. Loss of function of the synuclein protein is unlikely to account for its role in neurodegeneration. On the other hand, overexpression of α- synuclein in transgenic fruit flies and mice causes a parkinsonian phenotype and replicates many of the pathological features of PD (Takahashi et al.,

2003). These findings suggest that a- synuclein perturbations could be an important factor in PD pathogenesis.

Significant clues on the mechanisms responsible for PD development have come from additional biochemical, epidemiological, and genetic studies (Betarbet et al., 2002). Mutations in certain genes found in rare, familial cases of PD, such as alpha-synuclein and parkin, suggest a role for the ubiquitin-proteosome system and aberrant protein aggregation. Biochemical analyses have implicated mitochondrial dysfunction in PD (Beal, 2003). Epidemiological and animal model studies point to a role for environmental toxins, some of which are mitochondrial inhibitors. Mitochondrial dysfunction, resulting from either genetic defects, environmental exposures or an interaction between the two, may cause alpha-synuclein aggregation or neurodegeneration through oxidative stress or excitotoxicity.

Parkinson's disease is characterized by a *selective vulnerability* of sub-populations of dopaminergic neurons in the mesencephalon. The fact that the dopaminergic neurons are already sensitive to oxidative stress in control subjects and the reported increased production of oxygen free radicals in Parkinson's disease suggest that oxidative stress may be involved in the mechanism of nerve cell death. Furthermore, oxygen free radicals are also involved in an oxygen-dependent pro-apoptotic pathway stimulated by the inflammatory reaction observed in Parkinson's disease. These data suggest that anti-oxidant or anti-inflammatory treatments may slow down the progression of the disease. On the other hand, new substitutive therapies may be developed by trying to restore the activity of the neurons located downstream from the nigrostriatal pathway. Indeed, the nigrostriatal denervation induces a hyper-activity of the output structures of the basal ganglia (internal segment of the globus pallidus and substantia nigra pars reticulata), as demonstrated in various animal models of the disease. These changes in the activity of the output structures of the basal ganglia seem to be directly induced by the hyperactivity of the glutamatergic afferent fibers from the subthalamic nucleus. The fact that LDOPA treatment or a reduction in the activity of the subthalamic nucleus alleviate the symptoms of the disease and restore the activity of the output structures of the basal ganglia in parkinsonism suggests that these structures play a key role in the pathophysiology of the disease and could represent a potential therapeutic target.

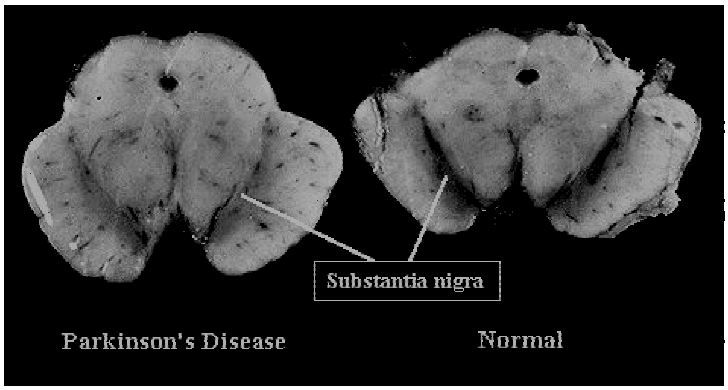


Fig. 1-1 Left: midbrain section showing loss of pigmented cells in the substantia nigra in Parkinson's disease. Right: midbrain section illustrating the normal substantia nigra.

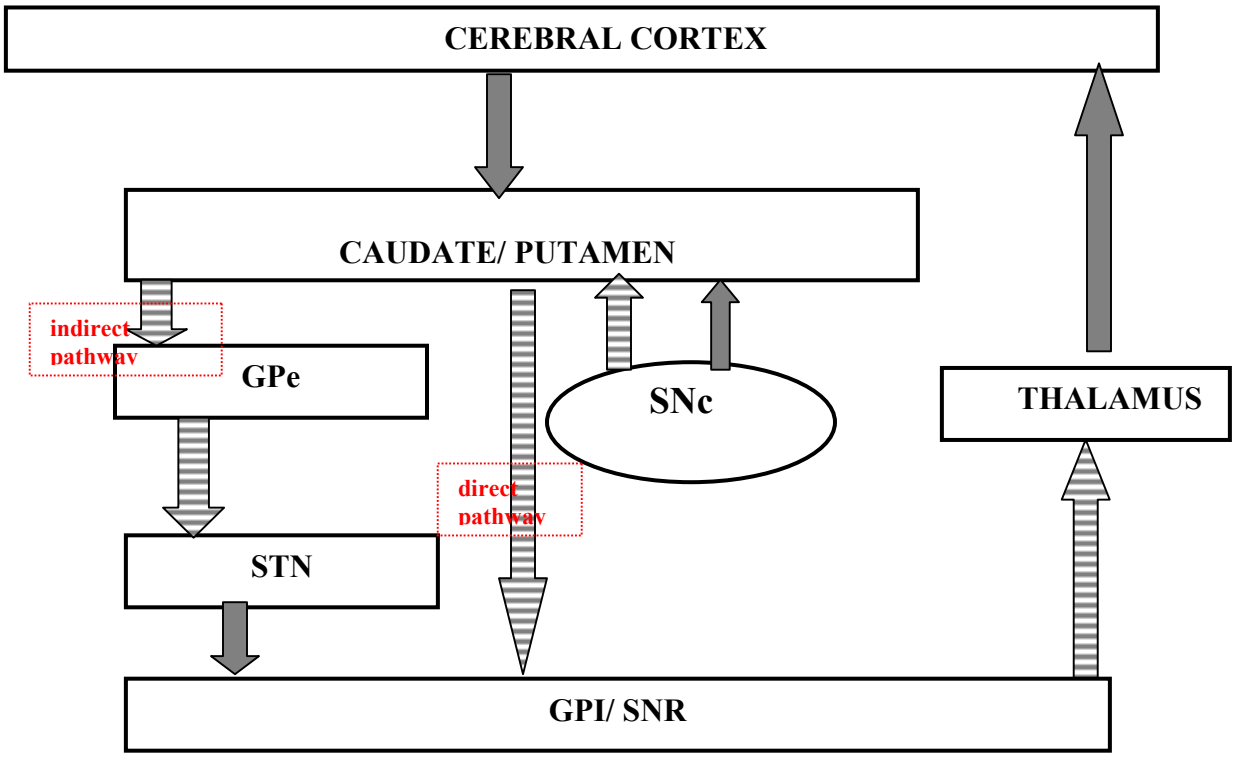


Fig. 1-2 Schema of anatomical structures and pathways involving the basal ganglia. Grey arrows indicate excitation and striped arrows indicate inhibition. STN: subthalamic nucleus; GPe: globus pallidus pars externa; GPi: globus pallidus pars interna; SNc: substantia nigra pars compacta; SNr: substantia nigra pars reticularis. (After Goetz & Pappert, 1999)

Alzheimer's disease

A definite AD diagnosis is made pathologically by examination of the brain at autopsy. Grossly, there is cerebral atrophy, mainly in frontal, temporal, and parietal regions. As a consequence, there is ex vacuo ventricular dilation. The pathognomonic microscopic feature of AD is an increased number of neuritic plaques in the cerebral cortex. These plaques are composed of tortuous neuritic processes surrounding a central amyloid core. They appear as roughly spherical areas of 10-150 nm in diameter. The initial step in the formation of plaques is thought to be amyloid deposition. Reactive astrocytes and microglia may appear at the periphery of these plaques, which may play a role in amyloid synthesis by processing amyloid precursor protein (APP) to form filamentous amyloid (Terry et al, 1994). Though plaques are frequently found in the hippocampus, their presence in increased numbers in neocortex is necessary for a diagnosis of AD. The amyloid core consists primarily of a small peptide known as Abeta which is derived from the larger APP. The extracellular deposition of amyloid is a major component of plaques, comprising 70% of the proteinaceous material in the plaque core. Under physiological conditions, 42 amino acid amyloid peptide isoforms can aggregate to form insoluble filaments about 7-9 nm wide. These consist of anti- parallel beta- pleated sheets. Thus, the peptides found in neuritic plaque cores are a heterogenous mixture of several isoforms.

Plaques that have the amyloid proteins but lack the neuritic processes are known as diffuse plaques, which do not count toward the diagnosis of AD. Since the number of plaques increases with age, the number needed for diagnosis of AD is age-dependent. The distribution of plaques varies widely within cytoarchitectonic units and from one individual to another (Braak and Braak, 1991). However, the sites that tend to have a predilection for amyloid deposits are amygdala, CA1, the subiculum and layers II, III and V of the entorhinal cortex (McKee et al, 1991).

Other histologic features of AD include neurofibrillary tangles (NFT), amyloid angiopathy, and granulovacuolar degeneration. NFTs are considered to be a major pathological hallmark of Alzheimer's disease (AD). The severity of dementia correlates positively with the density of NFTs in the neocortex, but not with neuritic plaque numbers (Arriagada et al, 1992; Samuel et al, 1994). Thus, the formation of NFTs in the neocortex may contribute to cognitive

impairment. NFTs develop within the pyramidal neuronal soma as filamentous inclusions and may extend into dendrites. They may be flame shaped or globoid. After deterioration of the parent cell, the NFT persists as an extraneuronal structure, referred to as a "ghost" or "tombstone" tangle. The alterations that occur within the cell to form NFTs are progressive and gradual (Vickers et al, 1992).

Altered forms of the microtubule associated protein “tau” are generally recognized as a subunit of paired helical filaments (PHFs) (Goedert, 1993; Trojanowski et al, 1993; Terry et al, 1994). All six isoforms of low molecular weight microtubule associated tau proteins seem to undergo abnormal phophorylation (Trojanowski et al, 1993; Goedert, 1993; Greenberg et al., 1994) perhaps due to alterations in intracellular calcium levels. The carboxy terminal repeat region of tau seems to be the major component of the core of PHF (Wrzolek et al, 1992). Therefore, the phosphorylated isoforms of tau which are unable to bind to microtubules, are likely to self- assemble to form some components of PHFs. Current evidence suggest that protein kinases or protein phosphatases with a specificity for serine/threonine-proline residues are involved in this abnormal phosphorylation of tau. Apart from the perikaryal NFTs, PHFs are also found in dystrophic neurites associated with plaques and in neuropil threads (Hue et al, 1989). It was found that NFTs also contain straight filaments of 15 nm diameter and amorphous material of unknown biochemical composition. Perry et al (1992) concluded that beta amyloid protein immunoreactivity could be found essentially in all extracellular NFTs and in most intracellular NFTs. Beta/A4 protein epitopes were localized in a diffuse amorphous layer in NFTs. It was suggested that beta/A4 protein deposition in NFTs is a secondary phenomenon (Yamaguchi et al, 1991).

The major subcortical neuronal populations affected in AD include nucleus basalis; locus coeruleus, dorsal raphe and ventral tegmentum that project to common areas of cortex, while the non-cortically projecting cells within the subcortex remain unaffected. Additionally, neuronal loss in the locus coeruleus is greater in the regions that project to the cortex while those that project to subcortical areas are spared. These observations suggest that the site of the primary lesion in AD lies within the cortex and subcortical neurons are damaged in response to this lesion (Mann, 1988).

Morphological alterations seen in neuronal processes passing through neuritic plaques (McKee 1991; Masliah, 1993a) are associated with accumulation of APP, phosphorylation of

neurofilaments and eventually the accumulation of tau (Masliah, 1993a) thus producing dystrophic neurites (DNs) (Migheli et al, 1992).

1.1.2. Dementia with Lewy bodies

Dementia with Lewy bodies (DLB) is a primary, neurodegenerative dementia sharing clinical and pathological characteristics with both PD and AD. This clinicopathological syndrome may account for up to 20% of all cases of dementia in older patients, typically in their seventh and eighth decades.

There are three major syndromes associated with the appearance of Lewy bodies. These are: the movement disorder known as Parkinson disease, autonomic nervous system failure, and dementia. Parkinson's, the most common syndrome with Lewy bodies, is a disease developing in middle age. In older persons, a mixture of cognitive, autonomic, and motor dysfunction is more common. Some older patients with dementia who are diagnosed with Alzheimer's disease may actually have diffuse Lewy body disease, and some of those subjects may exhibit a movement disorder resembling Parkinson's disease. Conversely, some patients initially presenting with Parkinson's disease may develop manifestations of Lewy body dementia.

The clinical presentation of Lewy body disease varies according to the site of Lewy body formation and associated neuronal loss. In Parkinson's disease, the Lewy bodies are found in the substantia nigra of the midbrain, coupled with the loss of pigmented neurons. In subjects with the dementia associated with diffuse Lewy body disease, there are also Lewy bodies in the neocortex, which often lack the surrounding halo that is characteristic of those in the brainstem. Some patients have the Lewy bodies in both locations. The basal ganglia and diencephalon may also be involved in some cases. Lewy bodies are spherical, intraneuronal, cytoplasmic, eosinophilic inclusions comprising abnormally truncated and phosphorylated intermediate neurofilament proteins, alpha-synuclein, ubiquitin, and associated enzymes. The degree of neuronal loss in the hippocampus is variable. Neuritic changes are noted in the CA2/3 area.

1.1.3. HIV encephalitis (HIV- associated dementia)

Human immunodeficiency virus (HIV)- associated dementia (HAD) is a cognitive, motor and psychiatric syndrome that usually develops in the late stages of the disease (McArthur, 1987). In some patients, though, it is the first AIDS- defining illness (Chiesi, 1996; Lopez, 1999). Basal ganglia dysfunction appears to play an important role in generating the neuropsychiatric manifestations of HIV encephalitis (HIVE), including HAD.

Since the first descriptions, 15 years ago, of the brain pathology found in a large number of AIDS patients at autopsy, the pathophysiologic process that causes HIV dementia has not been clarified. After the initial series of postmortem studies have identified the hallmarks of HIV infection of the brain, the only conclusion widely accepted was that brain macrophages are the cellular host and most likely carrier for the virus into the brain. Since HIV can not be found in neurons, the mechanisms of disease might be mediated by neurotoxic factors secreted by infected and activated macrophages. These macrophages are most abundant in the striatum, consistent with the subcortical symptomatology. The panel of macrophage associated neurotoxins that are studied now by AIDS investigators is rather large and covers the whole range of monokines, other mediators of inflammation, viral proteins, nitric oxide and more recently some still unidentified macrophage secretory factors that may potentiate the neurotoxicity of viral proteins (Garden, 2002). Despite some evidence of cortical synaptic loss in HIVE and patients with mild neurocognitive impairment, no signs of neuronal dropout could be detected in the neocortex (Korbo and West, 2000).

In the new era of efficient antiviral therapies there seems to be, at least temporarily, a significant decrease in the severity of neurologic symptoms in HIV infected patients (Price et al., 1999). The reports about the efficacy of HAART or even less aggressive treatments (e.g. AZT) in preventing neurologic disease are overwhelming and they all suggest that the increased survival in an asymptomatic state and clinical improvement in large cohorts of patients (both children and adults) are most often accompanied by reduced HIV associated pathology in the brain (Pezzotti et al., 1999). The paradigm shift is best illustrated by the decline in HAD. This is primarily the result of efficient systemic control of HIV replication by new generation antiviral drugs. Despite the therapeutic successes, macrophage secreted factors, e.g. viral proteins, cytokines and chemokines, may still present a potential source of neurotoxicity, especially in the patient subpopulation that does not respond to HAART. Furthermore, a concept that has gained momentum in the recent years is “the brain as a viral sanctuary.”

Despite these optimistic predictions, caution is needed. In a recent report by Langford et al. (2002), a new severe form of HIV associated leukoencephalopathy with extensive demyelination was reported in a small series of patients who failed HAART. Furthermore, in another large cohort retrospective study from Europe it was shown that even in patients responding to HAART, the brain is still the second most frequently involved organ (after the lung) (Jellinger et al., 2000). Interestingly, increasing evidence suggests that in many HIV+ patients with neurologic symptoms, the dopaminergic system is affected early and significantly (Berger and Arendt, 2000; Itoh et al., 2000)]

Studies of beta amyloid deposition in the AIDS brains have focused on the human beta amyloid precursor protein APP, that is considered by many to be a marker of neuronal degeneration. Several reports have described a significant increase in brain APP in AIDS, specifically in the axons of the subcortical white matter tracts. While most investigators agree that increased levels of APP suggest axonal pathology, the relationship with HIV presence in the brain is still debated. One leading theory is based on the inflammatory response in HIV infection of the brain parenchyma, where activated microglia are considered by many to be the likely source of mediators of disease that can promote over-production and accumulation of APP (Dickson et al., 1993). In general, overproduction and intra-axonal accumulation of APP is considered to be not only a marker of neuronal degeneration but very likely a mediator of it. Interestingly, in a series of in vivo studies using double transgenic mice overexpressing human APP and the HIV gp120, Mucke and Masliah have demonstrated that the hAPP may protective against gliosis and synaptic loss (Mucke et al., 1995) and also against excitotoxicity (Masliah et al., 1997).

The immunophilin CyPA is secreted by macrophages in response to endotoxin, suggesting that it may function as a cytokine (Sherry et al., 1992) and it can be incorporated specifically into the HIV-1 virions but not in other lentiviruses (Franke et al., 1994). Incorporation of host-derived CyPA into HIV is critical for infectivity and can be inhibited by neutralizing antibodies (Sherry et al., 1998). Cyclosporins have potent anti-HIV-1 activity in vitro by interfering with translocation of the preintegration complex to the nucleus and production of infectious particles. These activities are mediated by binding to CyPA (Billich et al., 1995). The inhibition of HIV replication by Cyclosporin A depends on its ability to disrupt the interaction between CyPA and HIV Gag (Franke and Luban, 1996). The interaction between

the CyPA incorporated in the virion and cell surface heparans is the initial step of HIV attachment suggesting that it can participate in both entry and post-entry events (Saphire et al., 2002).

1.2. Protein folding, transport and aggregation in neurodegeneration: the role of molecular chaperones

The degenerative disorders discussed in Chapter 1.1. are examples of proteinopathies, characterized by protein deposits in the brain that associate with neuronal degeneration. It is conceivable that stress enhances the progression of these diseases by facilitating protein unfolding and misfolding, which lead to aggregation and deposition.

Chaperones are highly conserved proteins responsible for the preservation and repair of the correct conformation of cellular macromolecules such as proteins and RNAs (Soti and Csermely, 2002). The most prominent representatives are heat shock proteins (Hsp)), small Hsp, crystallins, Hsc70, Grp (glucose- regulated protein)78. Chaperones usually increase the yield, but not the speed of protein folding. Certain chaperones, however, are “folding catalysts”. They accelerate certain steps of protein folding, such as the isomerization of peptide bonds at proline residues (peptidyl- prolyl cis- trans isomerases, including immunophilins) (Hartl, 1996).

Molecular chaperones appear to be a major constituent of the cytoplasmic network (Soti and Csermely, 2002). They form complexes with cytoskeletal elements and attach to a multitude of other proteins. It was shown that disruption of chaperone- protein complexes perturbs the organization of cytoplasmic traffic for several proteins (e.g., steroid receptors) and accelerates cell lysis (Pratt et al., 1999; Csermely, 2001). Accumulation of misfolded proteins in aged organisms is especially pronounced in postmitotic cells, such as neurons. The danger of damaged proteins is amplified if the protein is protease- resistant. The difficulties of degradation, as well as impaired protease and chaperone activities in aging neurons, seem to lead to a massive accumulation of these proteins and cause degeneration (Macario and Conway de Macario, 2001).

Stressors impact on cells directly or indirectly, cause protein denaturation, and elicit a stress response. This is mediated by stress (heat-shock) genes and proteins. Among these are the molecular chaperones that assist other proteins to achieve and maintain a functional shape (the native configuration), and to recover it when partially lost due to stress. Denatured proteins tend to aggregate and precipitate. The same occurs with abnormal proteins due to mutations, or to failure of post-transcriptional or post-translational mechanisms. These abnormal proteins need the help of molecular chaperones as much as denatured molecules do, especially during stress. A cell with normal antistress mechanisms, including a complete and functional set of chaperones, may be able to withstand stress if its intensity is not beyond that which will cause irreversible protein damage. There is a certain threshold that normal cells have above which they cannot cope with stress. A cell with an abnormal protein that has an intrinsic tendency to misfold and aggregate is more vulnerable to stress than normal counterparts. Furthermore, these abnormal proteins may precipitate even in the absence of stress and cause diseases named proteinopathies (including age- related neurodegenerative disorders). It is possible that stress contributes to the pathogenesis of proteinopathies by promoting protein aggregation, even in cells that possess a normal chaperoning system (Macario and Conway de Macario, 2000).

A number of reports in the last few years have described research aimed at elucidating the role of heatshock proteins, molecular chaperones in particular, in the pathogenesis of neurodegenerative disorders. The findings begin to shed light on the molecular mechanism of protein aggregation and deposition, and of the ensuing cell death. The results also begin to elucidate the role of molecular chaperones in pathogenesis. This is a fascinating area of research with important clinical implications. Although there are already several experimental models for the study of proteinopathies, others should be developed using organisms that are better known now than only a few years ago and that offer unique advantages. Use of these systems and of information available in databases from genome sequencing efforts should boost research in this field. It should be possible in the not-too-distant future to develop therapeutic and preventive means for proteinopathies based on the use of heat-shock protein and molecular chaperone genes and proteins.

Polypeptides emerging from the ribosome must fold into stable three- dimensional structures and maintain that structure throughout their functional lifetime. Quality control over protein structure and function is accomplished by molecular chaperones and proteases, both of which

can recognize hydrophobic regions exposed on unfolded polypeptides. Molecular chaperones promote proper protein folding and prevent aggregation, while energy- dependent proteases eliminate irreversibly damaged proteins (Wickner et al., 1999). The kinetics of partitioning between chaperones and proteases determines whether a protein will be destroyed before it folds properly. When both quality control options fail, damaged proteins accumulate as aggregates. After initial folding and assembly, proteins suffer damage in response to various stresses and insults. For such damaged proteins, as for proteins that are misfolded due to mutations in the encoding gene, lack of transcription fidelity or translational errors, a number of fates are possible: rescue by chaperones, destruction by cytoplasmic proteases, or aggregation. The efficiency and cost of the quality control depends on the balance between these processes. While chaperones and proteases do not appear to directly cooperate with each other, both systems appear to be necessary for proper functioning of the cell and can, to a certain extent, substitute for one another.

Protein aggregation associated with amyloid and prion diseases (reviewed in Wickner et al., 1999) can be considered biologically relevant failures of posttranslational quality control. Mutant forms of some proteins and, under unknown conditions, even some wild-type proteins, can form ordered aggregates called amyloid fibrils. These are protease- resistant structures characterized by a high content of beta sheets. To date, up to 20 proteins have been shown to form amyloids, which are associated with Alzheimer's, Parkinson's, Huntington's, Creutzfeld- Jakob diseases, as well as systemic amyloidoses. These proteins escape the quality control systems. This may occur via surface structures that are not recognized by chaperones and proteases or perhaps by a rapid aggregation that eludes degradation and remodeling. The latter possibility seems to characterize cellular prion protein conversion to the scrapie form (Liautard, 1999). Once initiated, fibril formation will not be easily reversed, because the most abundant chaperones and proteases in the cell do not appear to act on aggregates. Not only does the quality control system allow the formation of the amyloid, but evidence suggests that some chaperones may support the generation of amyloid forms. In S. cerevisiae, for example, Hsp104 is required for the maintenance of a prion- like state of a translation termination factor (DebBurman et al., 1997). Conversion to this prionic state results in an inefficient translation termination and therefore suppression of nonsense mutations. Hsp104 may support the formation or stabilization of

prionogenic folding intermediates of the translation termination factor. However, high levels of Hsp104 expression helps to annihilate prionic forms, presumably by dissolving aggregates.
It appears that Hsp104 and GroEL promote conversion of cellular PrP to a protease- resistant form, indicating that, in principle, chaperones are able to regulate conformational transitions in PrP. Association of chaperone and proteasome components with amyloid deposits has been noted in the nonprion disease spinocerebellar ataxia type I and in a model system for spinal bulbar muscular atrophy. In both cases, amyloid inclusions can be reduced by increasing the intracellular concentration of Hsp40 chaperone. This further supports the idea that chaperones fail to keep pace with misfolded proteins, leading to aggregation (Wickner et al., 1999). These and other data suggest that the failure of the quality control mechanisms to repair and remove misfolded proteins can lead to or allow the progression of the disorders associated with protein inclusions.

Hsp70 molecular chaperones and their co-chaperones work together in various cellular compartments to guide the folding of proteins and to aid the translocation of proteins across membranes. Chaperone proteins have been initially identified by their ability to confer cellular resistance to various stress conditions. However, molecular chaperones participate also in many constitutive cellular processes. Mitochondria contain several members of the major chaperone families that have important functions in maintaining its function. The major Hsp70 of the mitochondrial matrix (mtHsp70) is essential for the translocation of cytosolic precursor proteins across the two mitochondrial membranes. Hsp70s stimulate protein folding by binding exposed hydrophobic sequences thereby preventing irreversible aggregation. Hsp40s stimulate the ATPase activity of Hsp70s and target unfolded proteins to Hsp70s. Genetic and biochemical evidence supports a role for cytosolic Hsp70s and Hsp40s in the post-translational translocation of precursor proteins into endoplasmic reticulum and mitochondria (Ngosuwan, 2003).

PD is an age- related disorder. There is increasing evidence that protein folding defects are a key element in its pathogenesis. It was shown that neurons and glia of PD patients express αB- crystallin (Renkawek et al., 1999). Protein aggregates in LB contain Hsp (Jellinger, 2000). TorsinA, a protein with homology to yeast Hsp104, colocalizes to colocalize with alpha-synuclein in LB. McLean et al. (2002) showed that, like torsinA, specific molecular chaperone heat shock proteins colocalize with alpha-synuclein in Lewy bodies. In addition, in a cellular model of alpha-synuclein aggregation, torsinA and specific Hsp molecular chaperones

colocalized with alpha-synuclein immunopositive inclusions. Further, overexpression of torsinA and specific Hsp suppressed alpha-synuclein aggregation, whereas mutant torsinA has no effect. A study by Auluck et al. (2002) found that directed expression of the molecular chaperone Hsp70 prevented dopaminergic neuronal loss associated with α- synuclein in *Drosophila* and that interference with endogenous chaperone activity accelerated α- synuclein toxicity. Furthermore, LB in human postmortem tissue immunostained for molecular chaperones, also suggesting that chaperones play a role in PD progression. These findings support a role for chaperone proteins in cellular responses to neurodegenerative inclusions.

Despite recent advances, data about the expression of chaperones in the AD brain is still scarce. Recent studies have suggested that chaperone proteins are involved in pathogenetic processes leading to AD. Yoo et al.(2001) analyzed the expressional patterns of chaperones in several brain regions of patients and neurologically normal subjects, as this is essential in understanding how folding defects can lead to disease. The study revealed that six chaperone proteins out of nine studied via gel electrophoresis and mass spectroscopy (Hsp60, Hsp70RY, heat shock cognate (HSC) 71, alpha crystalline B chain, glucose regulated protein (GRP) 75 and GRP 94) showed aberrant expression depending on brain region.

The main histological characteristic of AD is the deposition of amyloid beta peptides (Aβ, A4) in extracellular plaques in the central nervous system and in the walls of cerebral blood vessels. The Aβ peptide is derived by proteolytic processing of the amyloid precursor protein (APP), a transmembrane protein of unknown function. The biological activity of Aβ correlates with its conformational state. Monomeric $A\beta_{1-40}$ or $A\beta_{1-42}$ is a random coil or α- helical conformation that stimulates neuronal growth in vitro (Stege and Bosman, 1999). A change into β-sheet conformation leads to the multi- step assembly of fibrils and the concomitant toxic effect on neurons in vitro. In addition to amyloid, the plaques contain several proteins, including the molecular chaperones Hsp27 and αB- crystalline. These were shown to prevent protein aggregation (Fu and Liang, 2003). Stege and collaborators demonstrated that aB- crystalline can prevent the fibrillization of $A\beta_{1-40}$. However, it induces chaperone/Aβ complexes that are highly neurotoxic. These observations provide valuable insight into the role of chaperones in the pathogenesis of AD, both as cellular defenders and facilitators of pathological processes. The fact that the neurotoxic complexes occur extracellularly is particularly interesting. The normally cytoplasmic Hsp have been found extracellularly, expressed on cell surface and colocalizing with

the core of plaques in the AD brain. (Yoo et al., 2001). If chaperone molecules indeed can stabilize non- fibrillar neurotoxic Ab species in the brain, then therapeutic attempts to prevent of fibril formation may augment the pool of smaller toxic species, thus enhancing neuronal damage.

DnaK- and DnaJ- like proteins are two major classes of molecular chaperones in mammals. DnaJ-like family proteins can inhibit polyglutamine aggregation, a hallmark of many neurodegenerative diseases, including Huntington's disease. Although most DnaJ- like proteins are ubiquitously expressed, some have restricted expression (e.g., MRJ, enriched in the CNS), so it is possible that some specific chaperones may affect polyglutamine aggregation in specific neurons (Chuang et al., 2002).The formation of insoluble protein aggregates in neurons is a hallmark of neurodegenerative diseases caused by proteins with expanded polyglutamine (polyQ) repeats. However, the mechanistic relationship between polyQ aggregation and its toxic effects on neurons remains unclear. Two main hypotheses have been put forward for how polyQ expansions may cause cellular dysfunction. In one model, neurotoxicity results from the ability of polyQ-expanded proteins to recruit other important cellular proteins with polyQ stretches into the aggregates. In the other model, aggregating polyQ proteins partially inhibit the ubiquitin-proteasome system for protein degradation. These two mechanisms are not exclusive but may act in combination. In general, protein misfolding and aggregation are prevented by the machinery of molecular chaperones. Some chaperones such as the members of the Hsp70 family also modulate polyQ aggregation and suppress its toxicity. These recent findings suggest that an imbalance between the neuronal chaperone capacity and the production of potentially dangerous polyQ proteins may trigger the onset of polyQ disease.

Intracellular accumulation of insoluble aggregates of ubiquitinated protein, either in the perikarya or dystrophic neurites, is characteristic to chronic neurodegeneration. Deposition of such ubiquitinated proteins and the lack of degradation by the proteosome could disrupt neuronal physiology, particularly axonal transport. It is also possible that, conversely, protein aggregates are part of a protective mechanism that "traps" soluble toxic proteins and ubiquitination contributes to their degradation. In any case, axonal loss is a major cause of symptoms, even in disorders where the primary defect lies somewhere else. This is partly due to the fact that CNS axons seem difficult to regenerate. Even in the PNS, regeneration is only partially successful.

1.3. A new class of chaperones: immunophilin- and non- immunophilin peptidyl- prolyl isomerases

Immunophilins are a class of chaperones with apparently multiple and poorly understood roles in protein folding and transport. In 1984, G. Fischer described a protein isolated from porcine kidney which catalyzed the interconversion of cis and trans rotamers of amide bonds adjacent to proline residues in peptidic substrates (Fischer et al., 1984). The enzyme was termed "peptidyl- prolyl cis- trans isomerase". That same year, while investigating the cellular actions of the immunosuppressant drug cyclosporine A, Handschumacher's team isolated a protein from calf thymus and dubbed it "cyclophilin" (CyP) (Handschumacher et al., 1984). It later became clear that PPIase and CyP were the same protein (Takahashi et al., 1989). S. Schreiber and scientists at Vertex Pharmaceuticals identified and characterized the binding protein for another immunosuppressive drug, FK506 (harding et al., 1989). This new protein was consequently called " FK506- binding protein 12 kDa" (FKBP12). This protein also binds a structurally related immunosuppressive drug, rapamycin (RM). Despite having no structural homology with CyP, FKBP also possesses PPIase activity. CsA, FK506 and RM bind to the proline- binding site of their respective PPIase receptors and inhibit their enzymatic activity. This inhibition, however, does not represent the substrate of the immunosuppressive activity, but rather the interaction of the complex thus formed with subsequent target proteins (Schreiber, 1991). The fact that the pharmacologic actions of these drugs are mediated by two distinct proteins with PPIase activity prompted the study of PPIases. It was found that CyP and FKBP12 each belong to a large family of enzymes which are highly conserved and present in prokaryotes and eukaryotes (Galat, 1993). The enzymes that bind immunosuppressive drugs are called "immunophilins" (IP). They were found in a variety of mammalian tissues, included immune tissue, lung, heart , liver and kidney.

A large body of evidence suggests that the PPIase activity of immunophiIins is responsible for various functions (Galat, 2003). However, the controversy regarding the chaperone function of small immunophilins has yet to be fully settled. It was suggested that only multidomain large IP can exert chaperone- like functions, whereas abundant single-domain small immunophilins are restricted to only PPIase activity.

In mammals, FKBP are a family of at least 20 proteins, all discovered over the past several years. They are designated by appending their apparent molecular weight in kilodaltons to the prefix FKBP. The FKBP known to be present in humans include FKBP 12, 12.6, 13, 25, 37 and 52.

FKBP12 is the archetypal, most abundant member of the family (Harding et al., 1989). It is a soluble protein, present mostly in the cytosol. Although hFKBP12 has a relatively low catalytic efficiency, it exhibits a pronounced substrate specificity. Hydrophobic residues, particularly residues with branched alkyl side chains, are preferred. Succ-Ala-Leu-Pro-Phe-pNA

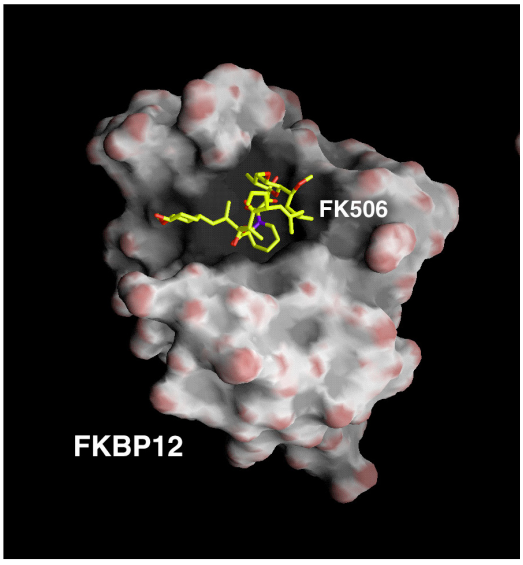


Fig. 1-3 The crystal structure of FK506 in complex with FKBP12. (After Huse et al., 1999)

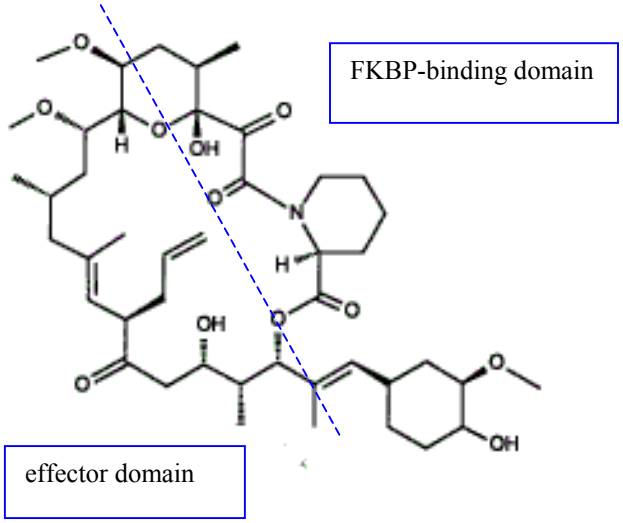


Fig. 1-4 Molecular structure of FK506 (Friedman and Weissman, 1991). The IP-binding domain and the "effector" domain (responsible for mediating the iummunosuppressive effects) are shown.

is the best substrate for FKBP12 and the one used to characterize the enzymatic activity of FKBP PPIases.

The structure of human FKBP12 (Michnik et al., 1991), as determined by NMR and X-ray crystallography, contains a five-stranded antiparallel β- sheet wrapped around a short a-helix, together with flexible connecting loops. Twisting of the b- sheet results in the formation of hydrophobic concave and convex surfaces. The loops comprising residues 39- 45 and 84- 91 (the 40s and 80s loop, respectively) surround the rotamase domain and are rather disordered in the unliganded protein. These residues that make up the FK506 binding site define a common "FKBP domain", which is conserved across species and in higher molecular weight FKBP. Mutagenesis experiments have shown that drug binding and rotamase activity sites might be structurally segregated (Bossard et al., 1994).

Binding of FK506 to FKBP12 does not cause significant change in the overall three-dimensional structure of the protein. In addition to FK506, FKBP12 binds related

immunosuppressive drugs ascomycin, rapamycin and several small molecule inhibitors, as well as non- immunosuppressive analogs (GPI- 1046 and V-10,367) (Hamilton and Steiner, 1998). The FKBP12- binding portions of these compounds are largely superimposable. The remaining parts of the molecules extend into the solvent- accessible region of the protein and are essential in mediating the immunosuppressive effects of the drug- IP complexes. It is believed that the pipecolinyl- ketoamide portion of the ligand functions as a transition- state mimic for the natural peptidyl- prolyl substrate.

FKBP52 (also designated FKBP59 or Hsp56) is an Hsp- binding IP present in various non-lymphoid cells. It contains two FKBP domains with rotamase activity. It is part of the glucocorticoid receptor complex, to which it is linked via Hsp90. It is also implicated in other diverse processes, including regulation of transcription, cation channel activity, and gene transfer efficiency (Scammell, 2003).

Colocalization experiments revealed partial association of FKBP59 with microtubules. During mitosis, the IP segregated from the regions of the chromosomes; it mainly localized with the mitotic apparatus (centrosome, spindle and interzone separating the chromosomes). It appeared again as a fibrous network in the cytoplasm of the two daughter cells.

1.4. Cellular functions of immunophilins

The discovery that immunosuppressants bind with high affinity to IPs and lead to their interaction with calcineurin (CaN) (see 1.6) does not establish physiologic roles for mammalian IP. This implies the existence of endogenous ligands for these molecules. In addition, the fact that immunosuppressants influence CaN activity does not necessarily mean that IPs interact with CaN under normal circumstances. Brain localization studies have suggested a physiological association of FKBP and CaN (see 1.5), however, IP are present in cells that lack CaN. The search for an endogenous ligand for FKBPs, though only partially successful, has revealed that FKBPs interface with a wide range of intracellular signal transduction systems. So far, important roles for FKBP IP have been identified in protein folding and trafficking processes, as well as in assembling and modulating protein complexes. The function of several receptors is also

modulated by FKBP. Although it has been suggested that rotamase activity underlies these functions, it appears that, in many instances, this is not true (Timerman et al., 1995).

Although IPs exhibit "traditional" chaperone activities, they are best described as "folding catalysts". Isomerization about the peptidyl- prolyl amide bond is one of the slower steps in protein folding, therefore it could represent a rate- limiting step in folding and unfolding. FKBP12 has been found to catalyze carbonic anhydrase and RNase T1 folding (Kern et al., 1994).

The role of IP in protein trafficking has been documented mainly for CyP and FKBP52. It was shown that CyP participate in the translocation and maturation of proteins in the secretory pathway. FKBP52 (as well as CyP40) is associated with untransformed steroid receptor heterocomplexes (Bose et al., 1996; Ratajczak et al., 2003), by binding to Hsp90. This interaction does not occur through the rotamase domain and is important for the targeted movement of the receptor.

In E. coli, FkpA, a FKBP- related heat shock periplasmic PPIase, suppresses the formation of inclusion bodies from a defective- folding variant of the maltose- binding protein MaIE31, both in vivo and in vitro (Arie et al., 2001). Experiments using active- site and deletion variants revealed that this chaperone- like activity of FkpA is independent of its prolyl isomerase activity, but requires an intact two- domain structure.

The yeast two- hybrid system was employed by Chambraud et al. (1996) who identified a protein that binds FKBP12 and designated it FAP-48 ("FKBP- associated protein 48"). FAP-48 is associated with both FKBP59 and FKBP12, but the functional significance of this association is not clear.

FKBP12 appears to interact with the cytoplasmic domains of type I TGF- beta receptors in vitro and function as an inhibitor (Wang et al., 1994). FK506 displaces FKBP12 from type I, thus enhancing functional responses induced by TGF-β. However, the role of this IP in TGF-beta superfamily signaling is controversial (Bassing et al., 1998). Physiological studies suggest that FKBP12 is dispensable for TGF-beta signaling, but definitely modulates calcium release activity of both skeletal and cardiac ryanodine receptors.

IP are implicated in several aspects of Ca^{2+} signaling. FKBP12 interacts with two types of intracellular Ca channels: the ryanodine receptor (RyR) and the inositol 1, 4, 5- triphosphate receptor (IP3R).

RyR is localized to the SR of skeletal muscle, cardiac muscle and brain. FKBP12 copurifies with RyR (Collins, 1991), with each of the four receptor subunits bound to an FKBP12 molecule. The skeletal muscle RyR1 is isolated as a hetero-oligomer with FKBP12, whereas the cardiac RyR2 more selectively associates with FKBP12.6. Patch- clamping studies indicate that the role of the IP appears to be stabilization of the channel (both closed and open states) and improvement of its fluxing capacity (Jayraman et al., 1992; Brillantes et al., 1994). High concentrations of FK506 dissociate FKBP12 from RyR, leading to Ca^{2+} leakage through the channel.

Developmental cardiac defects have been reported in FKBP12- deficient mice. Shou and collab. (1998) used embryonic stem cell technology to generate FKBP12 deficient mice and found they have normal skeletal muscle, but severe dilated cardiomyopathy, ventricular septal defects that mimic a human congenital heart disorder, noncompaction of left ventricular myocardium. About 9% of the mutants exhibit exencephaly secondary to a defect in neural tube closure, suggesting the interesting possibility of a role for FKBP12 in nervous system development.

IP3 is another tetrameric Ca^{2+} channel found at the ER and sometimes at the plasma membrane. These receptors respond to hormones and neurotransmitters that act at membrane receptors and activate phospholipase C, thereby inducing IP3 generation. IP3 subunits associate with FKBP12, which functions to stabilize the channel and improve its Ca^{2+} gating properties (Cameron et al., 1995). This association is also disrupted by FK506.

Complexes formed by FKBP12 with both RyR and IP3 associate with CaN. In IP3, the phosphatase controls the phosphorylation state of the channel, thereby modulating Ca flux (Cameron et al., 1995). Again, FK506 disrupts this association, indicating that FKBP12 plays an important role as an adapter that facilitates macomolecular complex assembly.

DuBell et al.(2000) showed that FKBP12 modulates specific mouse K^+ channels, thus the mouse ventricular action potential, by interacting directly with channel proteins or with associated proteins.

These results suggest that FKBP12 may play important regulatory roles in many cell processes, ranging from long-term depression in neurons to contractility in cardiomyocytes. However, the level of involvement of these proteins in the modulation if intracellular Ca channels is still unclear.

Steroid receptors form heterocomplexes with hsp90 that contain at least one of several IP (FKBP52, FKBP51 or CyP-40) or the IP homologue protein phosphatase 5 (PP5). All these bind via tetratricopeptide repeat (TPR) domains to a TPR- acceptor site on the hsp90 homodimer (Silverstein et al., 1999). The steroid receptors move continuously into and out of the nucleus. A considerable body of evidence indicates that both hsp90 and FKBP52 participate in the cytoplasm- nucleus trafficking of the glucocorticoid receptor (Galigniana et al., 2001).

The immunophilin cochaperones cyclophilin 40 (CyP40), FKBP51 and FKBP52 and PP5, a serine / threonine protein phosphatase, have been implicated as modulators of steroid receptor function through their association with Hsp90, a molecular chaperone with a key role in steroid hormone signaling (Ratajczak et al, 2003). Although progress towards a satisfying definition for the role of these components in steroid receptor complexes has been slow, recent developments arising from novel approaches in both yeast and mammalian systems, together with available crystal structures for Hsp90 and some of these cochaperones, are beginning to provide important clues about their function. Hsp90, recently identified as a member of the GHKL superfamily of ATPases, is the central player in receptor assembly, an energy-driven process that allows receptor and the immunophilins to be proximally located, or to interact directly, on a Hsp90 scaffold. Immunophilin structure, relative abundance, their binding affinity for Hsp90 and their ability to interact with specific receptors may all contribute to a selective preference of the immunophilins for individual receptors. Association of receptors with different immunophilins leads to differential functional consequences for receptor activity. Observations of glucocorticoid resistance in New World primates, attributed to FKBP51 overexpression and incorporation into glucocorticoid receptor complexes, have provided the first evidence that these cochaperones can control hormone-binding affinity. Application of a yeast model to FKBP52 function in the glucocorticoid receptor system has now provided crucial evidence that this immunophilin enhances receptor transcriptional activity by increasing receptor avidity for hormone through PPIase- mediated conformational changes in the ligand-binding domain. A recent novel finding suggests that hormone binding may induce a functional exchange of immunophilins in receptor complexes and that the modified complex directs receptor to the nucleus.

In certain systems, FKBP12 can display a different, cytokine-type of action. For example, it can be released from mast cells and activate Ca signaling in neutrophils (Bang et al., 1995).

In conclusion, rather than associating with "small molecule' ligands, FKBP could be conceptualized as adapter molecules for macromolecular assemblies. In addition to having enzymatic activity, they possess rotamase domains that function to recognize molecules.

1.5. FKBP in the normal and injured nervous system

Early studies monitored [^{3}H]FK506 binding and showed that levels of FKBP in immune tissues resemble levels in heart, kidney, liver and lung (reviewed by Snyder et al., 1998). Surprisingly, levels in the rodent brain are up to 50 times higher than in any peripheral tissue, depending on the brain region considered (Steiner et al., 1992; Dawson et al., 1994). Although these studies mentioned only FKBP12, several members of the FKBP class bound radioactive FK506. Additional studies analyzing mRNA levels by in situ hybridization confirmed the fact that FKBP12 levels vary by more than 10- fold among brain regions (Steiner et al., 1992). FKBP12 is sufficiently enriched in neurons that extraneuronal levels are not detected routinely. Highest densities of FKBP12 transcript occured in the corpus striatum and comparable levels were detected in the substantia nigra pars compacta (Dawson et al., 1994). Lesioning studies indicate that these localizations reflect a very high FKBP12 content in the descending striato-nigral pathway. High FKBP12 levels are present in the hippocampus, especially in the CA1 layer. In the cerebral cortex, FKBP12 is most prominent in the superficial layers, while in the cerebellum it is found in all cellular layers and particularly enriched in the Purkinje cells. CaN protein and mRNA display very similar localizations, suggesting a physiological link with FKBP12.

Cyclophilin localization also resembles CaN, for example in the hippocampus and olfactory bulb (Goldner and Patrick, 1996). However, only modest levels are present in the striatum (Dawson et al., 1994; Snyder, 1998).

Very little is known about the role of IP in the mammalian brain. Intracranial administration of nanomolar amounts of CsA significantly impairs long- term memory formation in neonate chicks (Bennett et al., 2003). It appears that the drug selectively disrupts only later stages of memory formation. Analogues that do not bind CyP, nor inhibit CaN, fail to produce

retention deficits. Bennett and collaborators reported strong amnestic effects of non-immunosuppressive, non- CaN inhibiting CsA analogues. This is was the first set of data establishing a physiological role for CyP in the nervous system and showed a "requirement for PPIase activity for successful memory formation." The study did not, however, rule out the possibility that another CyP activity (other than PPIase) might underline the role of these IP in memory formation. Similar studies using FK506 would certainly be worth performing to establish a potential function of FKBP IP in memory formation, that are expressed at relatively high levels in the hippocampal region. A role for IP in memory is suggested by numerous studies showing that protein biosynthesis, and therefore folding and possibly transport, is essential for long- term memory formation in various species (Bennett et al., 1998).

It was suggested that FKBP play a role in axonogenesis (reviewed by Snyder and Sabatini. 1995: Snyder et al., 1998). To our knowledge, two studies found that FKBP12 is expressed in the peripheral nerve nuclei and its levels increase after nerve injury. Lyons et al (1998) showed that FKBP12 levels are upregulated in nerve nuclei following nerve transection and this change parallels regeneration. A study by Sezen et al. (2002) found that FKBP 12 is localized to penile innervation in the rat. Interestingly, it becomes upregulated in the major pelvic ganglion, cavernous nerve and terminals, following cavernous nerve crush injury, independent of FK506 treatment. This suggests that the IP mediates a neurotrophic mechanism. Whether FK506 affords neuroprotection that preserves penile erection through FKBP 12 upregulation is unclear.

Kato and collaborators (2001) investigated immunohistochemically the changes in FKBP12 following cerebral and spinal cord ischemia in the rat. In normal rats, FKBP12 immunoreactivity was the highest in the CA1 subfield of the hippocampus and the striatum, and the localization was primarily neuronal. FKBP12 was at a low level in the spinal cord. Following global cerebral ischemia, FKBP12 immunoreactivity in CA1 neurons decreased after 1 day, and was lost between 2 and 7 days. Many CA1 neurons showed a transient increase in FKBP12 at 2 days. Following focal cerebral ischemia and spinal cord ischemia, FKBP12 immunoreactivity decreased rapidly in the ischemic core, but increased in surviving neurons in the penumbra. Invading leukocytes and macrophages in areas of infarction exhibited FKBP12 immunoreactivity. No FKBP12 immunoreactivity was observed in reactive glia in either paradigm. The findings suggest that FKBP12 plays a crucial role in neuronal damage following ischemia and in inflammatory reactions induced in areas of infarction.

A recent study (Manabe et al., 2002) examined the expression of FKBP-12 and FKBP-52 in the spinal cord of transgenic mice with an ALS-linked mutant Cu/Zn superoxide dismutase (SOD1) gene. The immunoreactivity of FKBP-12 was present predominantly in the cytoplasm, but did not show a difference between age-matched wild type and transgenic (Tg) mice at 25 and 35 weeks. FKBP-52 immunoreactivity was predominantly present in the nucleus and progressively declined in the Tg mice at 25 weeks of age in the anterior horn neurons. In motor neuron disease (MND) patients, however, FKBP12 levels may decrease in the early stages of degeneration in dying neurons (Kihira et al, 2002), which can play a causative role in the development of neurodegeneration in MND. Overall, these results suggest that FKBP alterations may be involved in familial ALS pathogenesis.

By virtue of its folding catalyst properties, FKBP12 might be part of the cellular response against unfolded proteins, thereby preventing aggregation of mutant and damaged proteins, catalyzing protein folding, solubilizing aggregates, promoting ubiquitination and degradation of abnormal proteins. Previous studies reported that CyPA protects cells from death following expression of mutant Cu/ Zn superoxide dismutase (SOD), which is associated with familial amyotrophic lateral sclerosis (Lee et al., 1999). A possible mechanism for mutant SOD toxicity is linked to a common phenomenon in neurodegeneration: protein misfolding and intracellular aggregates formation. The hypothesis that aggregates contribute to ALS pathogenesis is supported by the finding that in murine models of SOD mutant – mediated disease, motor neurons and sometimes surrounding astrocytes feature prominent SOD1- positive intracellular aggregates (Cleveland and Liu, 2000). It has been proposed that aggregates may exert their toxic effects through sequestration of proteins required for neuronal function, reducing the availability of protein folding chaperones or reducing proteosome activity (Bruening et al., 1999; Johnston et al., 2000). It was found that both aggregates and acute toxicity in vitro are reduced by elevating the levels of Hsp-70, a protein folding chaperone. In addition, the overall protein folding activity is slightly diminished in spinal cord extracts from mutant SOD1 mice. The ability of neurons to mount a robust heat shock response is naturally low. Therefore, one possible model for SOD1-positive aggregates toxicity is the entrapment of chaperone proteins and prevention of their folding/ refolding activity. The presence of FKBP12 in surviving spinal cord neurons (Kihira et al., 2002; Avramut and Achim, 2003) might provide a link to a previously unexplored neuronal defense mechanism compensating for the lack of heat shock protein activity.

Brecht and collaborators showed that after transient focal cerebral ischemia in rats, FK506 treatment (1 mg/kg i.p.) reduces the infarct size by 53%. In this study, the expression of FKBP12, 52 and 65 did not significantly change in the peri- infarct area. However, PPIase activity increased in the necrotic infarct core 24 hours after ischemia onset and this activity was blocked in a dose- dependent manner by in vivo FK506 treatment.

In an experimental model of global ischemia in gerbils (Schmidt et al., 2000), FKBP12 and caspase 1 and 3 are upregulated 4- 6 hours post- insult in the perikarya of CA1 pyramidal cells. These alterations parallel neuronal degeneration. Days later these markers are "partially localized in microglia and astroglia". A survival time of 7 days lead to an almost complete destruction of CA1 neurons. FK506 administered intraperitoneally after global ischemia was highly neuroprotective to CA1 pyramidal neurons. These results clearly indicate that FKBP12 is involved in the pathophysiology of post- ischemic neuronal degeneration and suggest that FKBP inhibitors may hold a clinical potential in the treatment of stroke and ischemia.

1.6. **Immunophilin ligands**

The term immunophilin is generally used to designate "receptors for immunosuppressive drugs" such as cyclosporine A (CsA), FK506, and rapamycin. Immunosuppression is used clinically for a variety of purposes. One of the most important applications is the treatment of patients undergoing organ transplantation, to prevent host-versus-graft rejection (Fung and Starzl, 1994). Immunosuppression is also effective in diminishing symptoms of autoimmune diseases such as myocarditis and glomerulonephritis (Thomson and Starzl, 1992).

The discovery of the immunosuppressive potential of cyclosporins has been responsible for the modern revolution in transplantation therapy. Research on CsA ultimately led to the identification of the intracellular calcium/ calmodulin – dependent phosphatase calcineurin as an immunosuppressive target and has helped to clarify the role of autoimmunity in rheumatoid arthritis, diabetes, multiple sclerosis, psoriasis and others.

FK506 is a macrolide compound with potent immunosuppressive activity. Like CsA, it is an effective agent for prevention of solid organ transplant allograft rejection.Both CsA and FK506 exert their major therapeutic effect by inhibiting T- cell activation through similar

mechanisms. Both their efficacy and toxicity appear to be dependent on the formation of ligand-protein complexes with their cognate immunophilins: CsA with cyclophilin and FK506 with FKBP12. The first drug- IP target was discovered by Liu et al. (1991) and Friedman and Weissman (1991), who demonstrated that CyA- CsA and FKBP12- FK506 complexes bind to and inhibit the activity of calcineurin. The relative potencies of CsA, FK506 and analogues in inhibiting CaN are closely correlated with their immunosuppressive potencies, indicating a role for CaN inhibition in their immunosuppressive action.

The immunosuppressive effect of drugs can be theoretically mediated by the direct inhibition of IP rotamase activity or the inhibition of CaN by the drug- IP complex. However, numerous compounds that bind to CsA or FKBP and inhibit their rotamase activity lack immunosuppressive effect (Bierer et al., 1994) Moreover, these immunosuppressive drugs typically act at nanomolar concentrations, whereas tissue levels of some immunophilins are almost micromolar, so that in intact tissues only a very small portion of rotamase activity would be inhibited (Schreiber, 1991; Snyder et al., 1998). It has been concluded, therefore, that the substrate of the immunosuppressive effect is CaN inhibition, rather than rotamase inhibition. The employment of non- immunosuppressive CsA and FK506 analogues can serve to discriminate between PPIase and CaN inhibition.

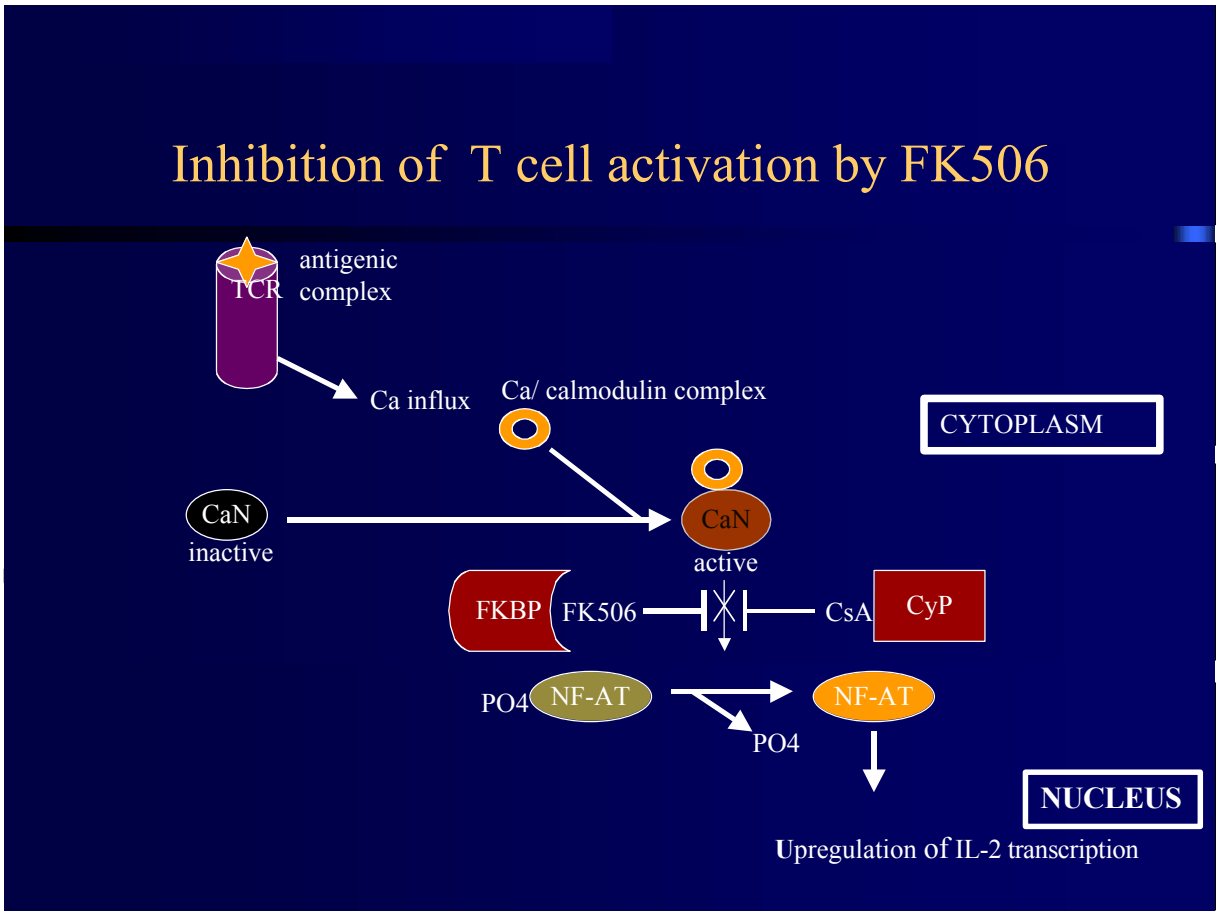


Fig. 1-5 Mechanisms of immunosuppressive effects of FK506 and CsA

After engaging the antigenic complex, the activated T-cell receptor initiates cascades of signaling events leading to Ca^{2+} influx. The increased intracellular Ca^{2+} concentration activates CaN (a calcium- sensitive protein phosphatase) via binding of Ca^{2+}- calmodulin complex. Active CaN dephosphorylates NF-AT and allows its translocation to the nucleus, where it upregulates the transcription of severeal cytokine genes (most notably IL-2). In the presence of immunosuppressive drugs, the FK506- FKBP12 and CsA- CyPA complexes inhibit calcineurin, thereby not allowing NF-AT dephosphorylation.

In turn, CaN inhibition prevents the calcium- dependent translocation of the p88 subunit of the transcription factor NF-AT to the nucleus, where it is required for T-cell activation (Clipstone

and Crabtree, 1992) (Fig. 1-5). Knowledge of this mechanism has proven to be important because, in addition to elucidating one substrate of pharmacological immunosuppression, it also suggests that CsA and FK506 are a useful agents in revealing CaN cellular functions.

Most of the reported effects of CsA and FK506 are attributed to their inhibition of CaN. Emerging evidence, however, suggests that CyP activity inhibition per se can mediate CsA activities, including the neurotrophic one (Steiner et al., 1997). Inhibition of PPIase activity by CsA has been suggested to result in neurotrophic effects, folding of proteins imported into mitochondria and inhibition of HIV-1 replication (Andreeva et al., 1999; Zander et al., 2003).
This HIV-antiviral activity of CsA does not appear to involve CaN inhibition, since antiviral effects are observed with the nonimmunosuppressive analog NIM SDZ811 (Navia, 1996). Nor can FKBP inhibition bring about this effect, since rapamycin does not exhibit an antiviral activity. The ubiquitous IP cyclophilin seems instead to be involved directly in the process of viral entry during the initial infection.

Although it forms complexes with the same IP (FKBP12), Rapamycin differs from FK506 in its immunologic effects. It was found to inhibit the clonal proliferation of T cells by preventing signaling through the interleukin- 2 (IL-2) receptor (Bierer et al., 1990, 1991). Several groups (Sabatini et al., 1994; Brown et al., 1994; Chiu et al., 1994) identified the target of RM- FKBP12 complexes as being RAFT1 ("rapamycin and FKBP12 target 1") or "FRAP" (FKBP and rapamycin associated protein. RAFT1 is the mammalian homolog of two yeast proteins, Tor1 and Tor2 ("target of rapamycin"), which when mutated confer resistance to the lethal effects of RM in yeast.

1.7. Neurotrophic effects of immunophilin ligands

FK506 has been clinically used for more than 10 years to prevent allograft rejection. Meanwhile, therapeutic solutions for neurodegenerative diseases are still scarce and have little efficacy. FK506 and its derivatives elicited much interest, as they hold the promise to a new means of treating these disorders.

The neuroregenerative and protective properties of FK506 are intriguing and were demonstrated by a plethora of studies. However, so far, no IPL are in use for acute (stroke) or chronic neurodegeneration (e.g., Parkinson's disease).

***In vitro* studies**

The cell culture models used to test neuroprotective and regenerative properties of IPL, particularly FK506, include primary neurons as well as immortalized cell lines like PC12, Neuro2A and SH-SY5Y cells. UV light, serum deprivation , H_2O_2 and thapsigargin have been employed as noxious agents.

The relatively high expression of FKBP12 in peripheral nerves and its presence in growth cones of developing neurons suggested a potential role in nerve growth (Snyder, 1998). It was subsequently shown that CsA and FK5O6 potently augment axonal growth in PC12 or SH-SY5Ycells, as well as embryonic rat sensory ganglia and mesencephalic DA neurons in vitro (Lyons et al., 1994; Constantini and lsacson, 2000; Guo et al., 2001; but see Parker et al., 2000). Concentrations of FK506 in the picomolar range produce detectable stimulation in rat and chick dorsal root ganglia (Lyons et al, 1994; Steiner et al, 1997), while half- maximal effects in PC 12 cells occur at 0.1 nM. Rapamycin is neurotrophic for PC12 cells with a potency equal to FK506 (Steiner et al., 1997). Stimulation of neuritic outgrowth in PC12 cells by FK506 occurs only in the presence of NGF and the use of the drug enhances the potency of NGF about 10- fold (Lyons et al, 1994). CsA has also been shown to protect neuronal cells exposed to various insults.

The non- immunosuppressant analog GPI-1046 shows a degree of protective effect on neuronal cell lines after H_2O_2 application (Tanaka et al., 2001). However, it fails to induce neurite outgrowth in PC12 cells (Parker et al., 2000). Another non- immunosuppressive IPL, V-10,367, elongates neurites in SH-SY5Y cells and induces branching in primary dopaminergic neurons (Costantini et al., 1998; Gold et al., 1997). Klettner and collaborators showed that it is strongly protects PC12 cells against H_2O_2, UV light and thapsigargin (Klettner and Herdegen, 2003).

IPL exert neuroprotective actions against β-amyloid- and serum deprivation- induced toxicity (Pasinetti and Aisen, 1997; Hugon et al., 1997). In Neuro2A cells, FK506 and the IPL Vertex 10,367 prevented cytochrome C release following H_2O_2 stimulation.

***In vivo* studies**

Early studies on CsA could not detect a significant neuroprotective effect, most likely because the drug does not cross the blood brain barrier (Sharkey and Butcher, 1994). When applied directly to the brain tissue, a significant effect was present in global ischemia (Uchino et al., 2002) and glutamate stimulation (Ankarcrona et al., 1996). In traumatic brain injury, the drug

exerts protective effects that are not shared by FK506 (Buki et al., 1999). Similarly, hypoglycemic injury is ameliorated by CsA, but not by FK506 (Ferrand- Drake et al., 1999). FK506, however, has protective and regenerating effects in many models of neurodegeneration. Early studies found that FK506 enhances the rate of axon regeneration in adult rat sciatic nerve following crush injury (Gold et al. 1994, 1995), and improves functional recovery after spinal cord injury (Madsen et al., 1998; Bavetta et al., 1999). A study by Rosenstiel et al. (2003) provided evidence that FKBP ligands FK506 and V-10,367 protect (otherwise dying) retinal ganglion cells from optic nerve crush-induced cell death, promote neurite outgrowth in vitro and that locally applied FK506 enhances the sprouting of axotomized central intrinsic neurons such as retinal ganglion cells in vivo after optic nerve crush.

By far the most extensive in vivo experiments have been conducted with dopaminergic neurons. Animal models of Parkinson's disease are created using 6-OHDA lesions in rats or MPTP lesions in mice and primates. As early as 1994, FK506 was shown to protect against MPTP- induced striatal DA depletion (reviewed by Hamilton and Steiner, 1998; Klettner and Herdegen, 2003). Non- immunosuppressive FK506 derivatives V-10,367 and GPI-1046 (also L685818 and V-13,661) have been used in various PD models and found to exert a significant effect on the survival and growth of affected neurons. According to Steiner's group (Steiner et al., 1997), GPI-1046 administration before, during or after MPTP lesioning restores the damaged dopaminergic neurons, as evidenced by recovery of tyrosine hydroxylase staining and reversal of haloperidol- induced catalepsy and akinesia. GPI-1046 also elicited morphological and functional recovery in 6-OHDA lesioned rats.

It is not always clear whether the beneficial effect of the compounds is due to protective or regenerative effects. Most likely both mechanisms come into play, as FK506 and GPI-1046 enhance dopaminergic cell survival and promote the retention of processes in TH- expressing neurons (Guo et al., 2001). Moreover, GPI-1046 ameliorates the 6-OHDA – induced behavioral deficits without cytoprotection, by enhancing the sprouting of remaining neurons (Zhang et al., 2001). The results of the studies using GPI-1046, however, could not be reproduced by some groups and are still controversial (e.g., Emborg et al., 2001).

Following serotonin neurons lesioning, animal receiving GPI-1046 displayed twice the density of cortical serotonergic fibers compared to untreated animals (Steiner et al., 1997). A similar effect was observed in an animal model of cholinergic lesion (Guo et al., 1997).

FK506 is protective against ischemia in several organs. The earliest reports of anti-ischemic properties of FK506 and CsA came from studies of ischemia in heart, kidney, liver and lung (Kawano et al., 1991). Numerous in vivo studies in rodent brains indicate that FK506 and CsA exert antistroke effects, some of them involving NMDA- mediated signaling. In their pioneering study, subsequently replicated by several groups, Sharkey and Butcher (1994) demonstrated that FK506 administration diminishes neuronal damage following middle cerebral artery occlusion (i.e., focal cerebral ischemia) in rats. This effect is blocked by RM, indicating that CaN inhibition plays a role in neuroprotection. The finding that CaN inhibition prevents apoptosis (Asai et al., 1999) supports this hypothesis.

Mechanisms of neurotrophic effects

FK506 exerts its immunosuppressive actions via binding FKBP12 and subsequent CaN inhibition. It has been a matter of dispute whether or not the neuroregenerative and protective effects of FK506 are also dependent on CaN inhibition. Initial efforts to explain the neurotrophic actions of IPL focused on CaN substrates, especially GAP-43 (Snyder et al., 1998). This protein ("growth- associated protein of 43 kDa) is selectively localized to developing and regenerating neurons. Phosphorylation enhances its function. FK506 administration increases its phosphorylated levels (Steiner et al., 1992). In addition, GAP-43 mRNA levels increase in the facial nucleus following nerve crush, with a similar time course as FKBP12 mRNA (Lyons et al, 1995).

The idea that CaN inhibition underlies IPL neurotrophic actions has come under questioning following the development of FK506, CsA and RM non- immunosuppressive derivatives that do not bind the phosphatase, yet they bind FKBP12 and appear to possess neurotrophic properties. Several studies emerged, most notably from researchers at Guilford Pharmaceuticals, who developed these derivatives. GPI-1046, for example, inhibits rotamase activity (Ki= 7.5 nM) and fails to inhibit T cell proliferation even at micromolar concentrations (Steiner et al., 1997). The compound elicits neuritic outgrowth in chick sensory ganglia at 1pM concentration, with 50% maximal effect at 58 pM. Similar findings emerged regarding other nonimmunosuppressive FK506 analogs such as V-10,367 (Gold et al., 1997). Nonimmunosuppressive derivatives also appeared to be effective in stimulating the regrowth of damaged neurons, either in the peripheral or central nervous system. The increased potency and

lack of apparent immunosuppressive effects lead many researchers (Snyder et al., 1998) to believe these compounds are the true candidates for treating neurologic disorders. However, subsequent studies conducted mostly in vivo failed to detect any beneficial effects of these nonimmunosuppressive drugs (e.g., in animal models of Parkinson's disease: Kordower et al). Clinical studies in PD employing an Amgen- synthesized nonimmunosuppressive IPL yielded unsatisfactory results (although this can be due to the particular pattern of IP distribution in the human brain in vivo, as outlined in Chapter 3). In conclusion, contrary to the opinion voiced by several groups over the past decade, CaN inhibition might be important for the neurotrophic, especially neuroprotective, actions of IPL.

Recent studies employing FKBP12- deficient mice revealed that FKBP12 is dispensable for the *neuroregenerative* effects of FK506 in vitro. Neuroregenerative properties are likely to be elicited through FKBP52 (Gold et al., 1999; Guo et al., 2001), not FKBP12/ CaN. Although progress towards elucidating the role of this IP and the associated Hsp90 in steroid receptor complexes has been slow, recent developments arising from novel approaches in both yeast and mammalian systems are beginning to provide important clues about their function. While the single-domain PPIases FKBP12 and FKBP12.6 are shown to interact with receptor protein kinases and calcium channels at their active sites, heterooligomeric nuclear receptors contain multi-domain PPIases like FKBP51, FKBP52 or cyclophilin 40. These directly interact with the chaperone hsp90 via the tetratricopeptide repeat modules of the folding helper enzymes and play a critical role in the functional arrangement of components within receptor heterocomplexes (Schiene and Fischer, 2000).FKBP52 enhances receptor transcriptional activity by increasing steroid receptor avidity for hormone through PPIase-mediated conformational changes in the ligand-binding domain. A recent novel finding suggests that hormone binding may induce a functional exchange of FKBP51 and FKBP52 immunophilins in receptor complexes and that the modified complex directs the receptor to the nucleus (Ratajczak, 2003). FK506 was shown to inhibit glucocorticoid receptor- mediated transcription (Le Bihan, 1998). The manner in which this can contribute to neuronal growth is unclear. Steroid hormones (glucocorticoids, estrogen, androgens) have neurotrophic, including neuroregenerative, properties in vitro and in vivo (Jones, 1993; Gold et al., 1999; Sakamoto et al., 2003). The effects of FK506 in these circumstances are difficult to explain. A suitable model could be analogous to the one proposed by Klettner and Herdegen in connection with neuroprotection (2003), which is based on the

release Hsp90 and p23. However, there are also many reports of detrimental effects involving atrophy and loss of synaptic contacts, most notably in the hippocampus (reviewed by Sousa and Almeida, 2002). A reduction of receptor- mediated transcription by FK506 would be beneficial, although in this case it could represent a means of *protecting* brain cells against glucocorticoid aggression.

The exact downstream components of the pathway leading to neuritic outgrowth are unidentified. GAP-43 and c-jun are likely candidates, both of which show a markedly increased expression during nerve regeneration, that is further amplified by FK506 administration (Gold et al., 1999).

For *neuroprotection*, several views on the role of CaN have been expressed. One of them is related to anti- NMDA protection. Treatment of brain homogenates with FK506 lead to increased phosphorylation levels of numerous proteins, one of them being neuronal nitric oxide synthase (NOS) (Steiner et al., 1996). This enzyme is activated by the excitatory neurotransmitter glutamate via NMDA receptors. High levels of glutamate released in vascular stroke cause neurotoxicity. NOS inhibitors and NOS gene knockout significantly reduce neuronal damage in NMDA- treated cultures and in vascular stroke models (Yun et al., 1998). These findings indicate a role for NOS in glutamate- mediated excitotoxicity. In phosphorylated state, NOS is inactive. This appears to be the basis for numerous observations that FK506 and CsA possess antistroke effects by reducing ischemic neuronal damage (Sharkey and Butcher, 1994). However, Butcher and collaborators observed that the drugs are not protective in models of quinolinate- induced excitotoxicity. In addition, the effect of FK506 after direct NMDA stimulation could not be replicated in vivo.

The recent work of Klettner and collaborators rightfully suggest a differentiated view of the importance of CaN in neuroprotection (described in Klettner and Herdegen, 2003). In PC12 and Neuro2A cells treated with H_2O_2, FK506 and its non- CaN inhibiting analog V-10,367 possess similar protective effects. V-10,367 fails to rescue SH-SY5Y cells. The non- CaN inhibiting GPI-1046 protects against H_2O_2- induced cell death in Neuro2A cells. One explanation might be that CaN inhibition is one of several options for neuroprotection. CaN can be important for neuronal cell death, but not all death pathways rely on its activation. A novel CaN inhibitor, Lie20, does not appear to have any protective effects in H_2O_2- treated PC12 cells. This is a strong argument against the involvement of CaN in neuronal cell survival .

The influence of other cells and organs might play an important role in generating the protective effects of FK506. Calcineurin effects on microglia and immune cells of the blood might contribute to its beneficial effects. Cellular inflammation occurs after focal ischemia, and is characterized by the invasion of polynuclear leukocytes and macrophages into the otherwise "immunologically privileged" brain. Vascular and non-vascular cells generate cytokines and chemokines, leading to activation of endothelial and inflammatory cells and resulting in neurotoxicity (e.g., IL-1 and TNFα) (Yamasaki et al., 1992). The post-ischemic inflammatory response can contribute to secondary brain injury in several ways. Therefore, the calcineurin inhibiting properties of FK506 might be beneficial in acute stroke by suppressing the immune system. Inhibition of IL-1ß or TNFα has been shown to reduce the infarct volume (Shohami et al., 1999). FK506 and Cyclosporin A exert a direct inhibitory effect on microglia activation in the brain after ischemia (Wakita et al., 1995). In myocardial infarction, the immunosuppressive action of FK506 has been directly linked to its protection of myocardiocytes (Wakita et al., 1998). In rat liver and kidney, FK506 reduces TNFα production when given prior to ischemia (Ishii et al., 1994). It is not known whether inflammation after stroke is always detrimental to neuronal tissue. Transgenic mouse models lacking certain cytokines, for example, TNFα- or IL-6- deficient mice, show more extensive injury and less favorable long-term recovery compared to wild type (Bruce et al., 1996). This issue has to be considered in connection with the influence of calcineurin inhibition on neuroprotection.

Finally, FK506 can be neurotoxic in humans, inducing tremor, neuralgia and peripheral neuropathy. More severe side effects include psychosis, hallucinations, blindness, seizures, cerebellar ataxia and motoric weakness. Also, due to hypertensive effects, FK506 induces hemorrhages in the brain (Bechstein, 2000). Additionally, FK506 is nephrotoxic. These side effects of FK506 are shared by Cyclosporin A and most likely due to its calcineurin inhibiting properties.

Additional effectors potentially mediating FK506 neurotrophic effects are discussed below.

The c-Jun N-terminal kinases (JNKs), also called stress activated protein kinases) are involved in neuronal cell death. One of the prominent substrates is the transcription factor c-Jun. FK506 attenuates cell death in neuronal cell cultures deprived of serum and antagonizes c-Jun expression (Yardin et al., 1998). In axotomized nigral neurons, FK506 prevents cell death and

inhibits c-Jun expression and c-Jun phosphorylation (Winter, 2000). These data indicate an inhibition of JNK activation, which has directly been shown for FK506 in B-cells and PC12 (Klettner et al., 2001; Klettner et al., 2003). Inhibition of JNK activity can protect neurons and may underlie the protective actions of FK506. However, inhibition of JNK induced by FK506 did not contribute to neuroprotection (Klettner et al., 2001). FK506 inhibits JNK in neuronal cell death and the relevance of its inhibition for protection might be analogous to that of calcineurin, which is important in some, but not all models of neurodegeneration.

The FK506 effects on apoptosis vary according to the experimental paradigm and are likely to occur through several mechanisms. In vitro experiments using H2O2, found that FK506 inhibits cytochrome c release, caspase 3 activation and Parp cleavage, and abolishes p53 expression (Klettner et al., 2001). After serum deprivation in SHSY-5Y cells, however, FK506 does not inhibit caspase 3 activation (Macleod et al., 1998). FK506 induces Bcl-2 expression in SHSY-5Y cells, but not in PC12 cells (Klettner et al., 2001). In cerebral ischemia, FK506 interferes with the receptor pathway of cell death, suppressing the induction of TRAIL and CD95 ligand (Martin- Villalba et al., 1999). Following rat spinal cord injury, FK506 prevents the dephosphorylation of Bad and the activation of caspase 3 in the spinal cord parenchyma and in oligodendrocytes (Nottingham et al., 2002). In keeping with its immunosuppressive properties, FK506 augments cell death in T-cells and mononuclear blood cells (reviewed by Letko et al., 1999).

FK506 induces Hsp70 expression in selected neurons in the brain (cortex, hippocampus and amygdala) (109). The induction of Hsp70 has been linked with the protective effect of FK506 in ischemic/hyperfusion injury in the liver (Kaibori et al., 2001). According to Klettner and Herdegen (2003), FK506 and V-10,367 are strong inducers of Hsp27 and Hsp70 expression in PC12 cells, indicating that this effect is independent of calcineurin. Inhibition of this Hsp expression with quercetin completely abolishes the protective effects of both ligands.

In order to help clarifying the molecular basis of the neuroprotective properties of IPLs, a thorough study by Tanaka and Ogawa (2003) examined the anti-apoptotic effects of IPLs in U251 cells. In this system, GPI1046 and V10367, which are non-immunosuppressive IPLs, as well as FK506, had neuroprotective effects against hydrogen peroxide (H2O2)-induced apoptotic cell death. They analyzed the messenger RNA expression of apoptotic activators and found that H2O2 increased both the ratio of bax/bcl-2 and the p53 mRNA expression. Pretreatment with

FK506 and V10367 prevented significantly the increase of ratio and p53 mRNA. GPI1046 also reduced to the normal level of bax/bcl-2 ratio. However, FK506 only prevented the H2O2-induced increase of three caspase activities: caspase-3, caspase-8 and caspase-9. In U251 cells, three IPLs except for rapamycin had GSH-activating effects. Thus, this study suggested that IPLs have multiple beneficial properties such as anti-apoptotic effect and a GSH-activating one. The study also implies that the immunosuppressive effect is not essential for their neuroprotective properties.

An anti- oxidative effect of FK506 has also been proposed. As early as 1993, FK506 was shown to be protective after ischemia- reperfusion injury in the canine heart. In this paradigm, FK506 protects the mitochondrial function and inhibits the decrease of glutathione (GSH) induced by ischemia- reperfusion (Nishinaka et al., 1993). Although the protective effect in other tissues might have different substrates, a general mechanism of protection against ischemia-reperfusion is possible. In neuronal cell cultures exposed to H_2O_2, the neuroprotective effect of FK506 is accompanied by increased cellular concentration of GSH (Tanaka et al., 2001). PD is characterized by a dramatic depletion of cellular GSH in the nigral region (reviewed by Jenner, 2003). After 6OHDA administration in mice, FK506 increased striatal GSH and DA levels (Tanaka et al., 2002). Therefore, the ability of FK506 to enhance GSH levels might be of clinical significance.

It is evident from the plethora of studies described above that the therapeutic potential of immunophilins and their ligands in neurodegeneration, as well as the mechanisms of their neuroprotective and regenerative properties, still need to be defined. The effects of IPL often depend on the employed in vitro or in vivo model. Ultimately, what matters most is the effect on human neurons. One way to start would be to look at the effect of IPL, especially FK506, on human brain cells. Surprisingly, until now this important aspect has been largely overlooked. The presence of IP receptors in the human brain also needs to be demonstrated.

1.8. General hypotheses and study aims

The IP FKBP12 has been implicated in a multitude of cellular processes, including protein folding and trafficking, intracellular Ca^{2+} receptor stabilization, regeneration and growth of

rodent peripheral neurons. Its PPIase activity may partly underlie these functions. The presence of FKBP12 in the normal and degenerating human brain has not been demonstrated. The role of this IP in neuronal death or survival processes has never been explored.

Hypothesis 1: FKBP12 levels are altered in the brain of patients with neurodegenerative disorders in response to protein misfolding and axonal transport perturbations that characterize chronic brain neuronal death.

Aim 1 (Chapter 2): Analyze the expression of FKBP12 in the normal human brain and in the brain of patients with neurodegenerative diseases involving the dopaminergic system (PD, DLB, HIVE) and non- primarily dopaminergic degenerative conditions (AD).

Aim 2 (Chapter 3): Analyze the expression patterns of FKBP12 in a primate model of PD.

The FKBP ligand FK506 has been shown to exhibit neurotrophic effects in animal models. These effects have not been demonstrated in primary human brain cultures.

Hypothesis 2: FK506 increases neuronal survival and growth in human fetal brain cultures.

Aim3 (Chapter 4): Study the neurotrophic effects of the immunosuppressive drug FK506 in human fetal brain cultures.

2. Changes in immunophilin (FKBP12) levels and distribution in chronic brain degeneration

2.1. Introduction

Immunophilins (IP) were originally identified as receptors for immunosuppressive drugs like cyclosporin A, FK506, rapamycin and their non- immunosuppressive analogs, which are collectively referred to as "immunophilin ligands" (IPL). These drugs are used clinically in preventing allograft rejection and in the therapy of autoimmune diseases (Schreier, 1993; Bierer, 1994; Fung and Starzl, 1994). Consequently, initial research on immunophilins focused on their roles in lymphocytes (Schreiber and Crabtree, 1992; Hollander et al., 1994).

Cyclosporin A binds to a class of IP called cyclophilins, whereas the receptors for FK506 and rapamycin belong to the family of FK506- binding proteins (FKBP). The latter are designated according to their molecular weight: FKBP12, 25, 52 etc. Both FKBP and cyclophilins have enzymatic activity: they function as peptidyl- prolyl cis- trans isomerases (rotamases). Isomerization about peptidyl- prolyl amide bonds is one of the slower steps in protein folding, possibly a rate limiting step in protein folding and unfolding (Hamilton and Steiner, 1998). So far, FKBP12 has been shown to catalyze the folding of carbonic anhydrase and RNase T1 (Tropschug et al., 1990; Kern et al., 1994). Macrophage- tropic and T cell- tropic V3 loop peptides of HIV-1 gp120 protein bind specifically to the active site of FKBP12 and cyclophilins A and B (Endrich and Gehring, 1998) and inhibit the rotamase activity of these IP. A role for FKBP as conformases or docking mediators has therefore been suggested. FKBP12 has also been found to associate with intracellular calcium channels localized to the endoplasmic

reticulum (ER) and improve calcium fluxing capability (Jayraman et al., 1992). However, its precise intracellular functions are still unknown.

The finding that FKBP levels in the rat brain are up to 50 times higher than in the immune system prompted the study of the effects of FK506, rapamycin and their analogs on the nervous system (Steiner et al., 1992; Dawson et al., 1994). Moreover, FKBP12 levels increase in nerve nuclei following nerve transection and this increase parallels regeneration (Lyons et al., 1995). Over the past decade, an increasing body of evidence has indicated that immunophilin ligands have neurotrophic effects in a variety of in vitro and in vivo systems (for review, see Snyder et al., 1998). FK506, rapamycin and their analogues increase neuronal survival and neuritic outgrowth in various cell lines and in rodent dopaminergic neurons, both in vitro and in vivo (Lyons et al., 1994; Gold et al., 1999; Castilho et al., 2000;Costantini and Isacson, 2000). In addition, FK506 and its non- immunosuppressive analogues promote morphological and functional recovery in animal models of Parkinson's disease (Steiner et al., 1997; Costantini et al., 2001). The mechanisms underlying these trophic effects are unclear. Studies using FKBP12 $^{-/-}$ mice indicate that the neuroregenerative effects of FK506 are not mediated by FKBP12, but probably by FKBP52 (Guo et al., 2001). However, FKBP12 is a likely candidate for mediating the neuroprotective activities of IPL.

Since previous studies suggested that IP play a role in protein folding and intracellular trafficking (Freskgard et al., 1992; Gothel and Marahiel, 1999; Schiene and Fischer, 2000), we hypothesize that FKBP12 mitigates axonal transport perturbations and protein misfolding that characterize chronic brain neuronal death. In addition, decreased FKBP12 levels can promote or accelerate the degenerative processes.

This study investigates the presence and expression of FKBP12 in various brain regions of patients with neurodegenerative diseases and normal controls. It describes and quantifies the distribution of FKBP12 in paraffin embedded tissue from HIV- positive patients with or without HIV encephalitis (HIVE), as well as patients with Parkinson's disease (PD), Alzheimer's disease (AD), dementia with Lewy bodies (DLB), and from neurologically normal controls. This is the first study to demonstrate that FKBP12 is present in the human brain and that its levels and distribution change in the brain of neurodegenerative diseases patients, in areas of pathology.
We conclude that FKBP may constitute a new biomarker and therapeutic target for neurodegenerative diseases.

2.2. Materials and methods

2.2.1. Human subjects and neuropathological diagnosis

Paraffin embedded archival material was obtained from the London Health Science Center, London, Ontario, Canada; University of Pittsburgh Alzheimer's Disease Research Center and University of California at San Diego, Department of Pathology. Human brain tissues obtained at autopsy were fixed in formalin within 24 hours of death, for 7 days, and then embedded in paraffin. Sections of 4μm thickness were cut and mounted onto microscopic slides (Fisher Scientific, Springfield, NJ). The present study used brain tissue from a cohort of 20 non-HIV positive neurodegenerative diseases patients and normal controls, as follows: PD (five cases, one familial and four idiopathic; average age: 75.8, range: 59- 99), DLB (five cases, average age: 80; range: 72- 86) and AD (seven cases; average age: 80; range: 77- 89), as well as from three age- matched control subjects free of neurological disease (average age: 72; range: 64- 78). In addition, brain tissue was obtained from patients in the following categories: HIV encephalitis (eighteen cases; average age: 49), HIV- positive non- encephalitic patients (ten cases; average age 42.6) and age- matched seronegative controls (three cases; average age: 40).

The diagnosis of PD was based on the clinical history and typical histopathological findings including neuronal loss, gliosis, pigmentary incontinence and Lewy body formation in the substantia nigra and locus coeruleus. Neocortical regions were unremarkable. There was no evidence of AD, DLB, progressive supranuclear palsy or corticobasal degeneration. DLB was defined on the basis of a history of cognitive impairment, coupled with loss of pigmented neurons in the substantia nigra and cortical LB (according to the consensus guidelines for the clinical and pathological diagnosis of DLB; McKeith et al., 1996). All AD cases were "definite" by CERAD criteria (Braak and Braak stage V or VI).

All HIV patients in this study died of respiratory failure due to bronchopneumonia and the general autopsy findings were consistent with AIDS. The associated pathology was most frequently due to systemic CMV, Kaposi sarcoma and liver disease. The majority of autopsy cases eventually selected for statistical analysis are males. The main neuropathologic diagnoses

were: microglial nodules (MGN; n = 10), multinuclear giant cells (MNGC; n = 12), CMV encephalitis (n = 3), CNS lymphoma (n =2), and PML (n =2), while 10 cases were "normal."

Control patients died of complications following myocardial infarcts and their brain tissue was histologically normal.

We analyzed the regional, cellular and sub- cellular expression of the immunophilin FKBP12 in the substantia nigra (SN), deep gray matter (striatum, pallidum and amygdala), hippocampal formation and mid-frontal cortex. The results were assessed independently by three investigators.

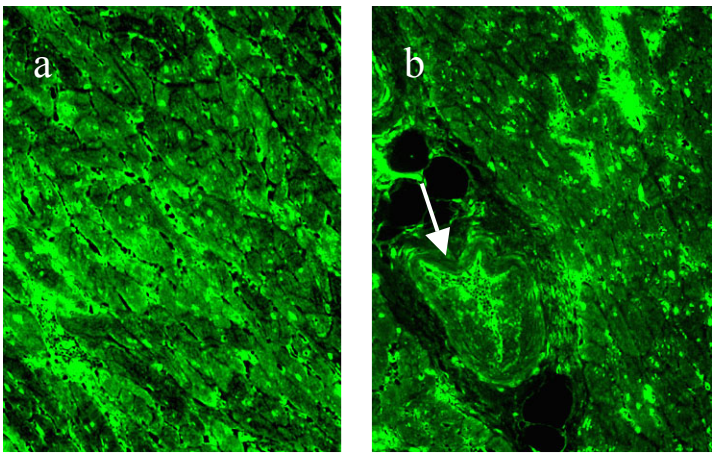
2.2.2. Immunohistochemistry and laser confocal microscopy

The IP FKBP12 was detected with a rabbit affinity purified antiserum (Alexis Biochemicals) raised against a peptide corresponding to aminoacid residues 1- 13 from human FKBP12, at a concentration of 1:400. Antibodies against the neuronal markers MAP-2 (1:100, clone HM2, Sigma, St. Louis, MO) pan- neurofilament (mouse monoclonal, 1: 50, Zymed Laboratories, San Francisco, CA), synaptophysin (rabbit, 1:50 Dako, Carpinteria, CA) were used in single- and double labeling studies. Anti- alpha- synuclein (mouse monoclonal, 1:1000, Zymed), anti- neurofibrillary tangles (NFT) (rabbit polyclonal, 1: 200; Chemicon) and anti-ERAB (Chemicon) antibodies were used to detect markers of pathology. RCA-1 and anti-HAM56 antibodies were used to identify cells of macrophage- microglia lineage.

Paraffin embedded sections were incubated at 55°C for 15 min and deparaffinized by immersion in Histoclear (3 x 10 min) (National Diagnostics, Atlanta, GA). Sections were rehydrated in 100% ethanol (2X10 min), followed by 95%, 90% and 70% ethanol (5 min. each), rinsed in H_2O and immersed in 3% H_2O_2/ methanol (30 min) to inactivate endogenous peroxidase activity. After rinsing, antigen unmasking was achieved by placing slides in 0.4 % pepsin (37°C, 10 min) and blocking was performed using 10% normal donkey serum in phosphate buffered saline (PBS) (30 min, room temperature). Primary antibody anti- FKBP12 (rabbit) was applied at a concentration of 1: 400 overnight at 4° C. Secondary antibody (biotinylated donkey anti- rabbit, Jackson Immunoresearch Laboratories, West Grove, PA) was then used at a concentration of 1:200 for a 30 min. incubation. Sections were subsequently incubated with an ABC complex (Vector Elite kit, Vector Laboratories, Burlingame, CA) for 30 min, rinsed and subjected to chromogen incubation for 5 min (Vector Nova Red substrate kit,

Vector Laboratories), the dehydrated and mounted with Permount. All sections (controls and patients) were processed simultaneously. For immunofluorescence histochemistry, sections were prepared as described above. The commercially available kit "Tyramide Signal Amplification" (NEN Life Science Products, Boston, MA) for FITC, Cy3 or Cy5 was used in the experiments described below as appropriate, according to the manufacturer's protocols. The immunofluorescence sections were analyzed with a Zeiss Axioplan confocal laser-scanning microscope. CY5 is excited with a wavelength of 647 and collected through a long pass filter (660LP). DTAF is excited with light at a wavelength of 488 and collected with a narrow band filter (530DF30). Each image was scanned along the Z axis for a total of 10 sectional planes with a 0.5 μm step (512×512 pixels per sectional plane, 0.34x 0.34 μm per pixel). Confocal images are 10-section projections. Orthogonal sectioning was performed on selected areas.
Image analysis and 3D reconstruction were performed with the integrated software Visual Basic Programming, using the NT 4.0 operating system optimized for the Zeiss LSM 510 machine.

Selected sections were processed without primary or without primary and secondary antibodies, to serve as negative controls for antibody specificity. Adsorption controls were performed by preincubating the affinity-purified antibody with an excess of peptide. Peripheral blood mononuclear cells immunostaining served as internal positive control and myocardial tissue was used as an external positive control, as shown below (a and b: cardiac muscle fiber FKBP12 labeling using a FITC- conjugated secondary antibody; arrow in b: immunopositive blood cells and blood vessel endothelia):



2.2.3. Light microscopic scoring and statistical analysis

Each section was scored for staining intensity based on the following system: 0- absent; 1- present, but faint; 2- moderate; 3 - strong. Staining characteristics of cellular localization and overall distribution were recorded. Semiquantitative data derived from the histological scoring were analyzed using the Kruskal- Wallis test, followed by paired Mann- Whitney analyses. $P \leq 0.05$ was considered statistically significant. Data are presented as mean ± SEM.

2.2.4. Western blots

Frozen tissue was ground and homogenized in RIPA buffer, then incubated on ice in 10 mg/ml PMSF for 30 min. After centrifugation (12,000*g* for 10 min at 4°C), removal of supernatant and re- centrifugation, protein concentration was determined using the bicinchoninic acid (BCA, Sigma) method. Optical densities were determined by reading the absorption at 570 nm and comparing them to a standard curve obtained using known BSA (Sigma) concentrations.

Proteins were separated by SDS-PAGE (BioRad). Samples (30 µl per lane for each subject) were separated at 125V/ 85 mA constant current in 0.05 M Tris-buffer (pH 8.3) containing 0.38M glycine and 0.1% SDS using 4% stacking gels and 10-20% resolving gels cast in a minigel chamber (BioRad). Electrical transfer onto 0.45 mm-pore-size polyvinylidene difluoride (PVDF) membrane was performed at 100V/ 250 mA (RT, 1 hr) in transfer buffer containing 0.2 % SDS. All blots were blocked with 5% non- fat milk powder in PBST for 1 hr. After washing in PBS supplemented with 0.05% Tween 20, membranes were incubated with primary antibody overnight (anti- FKBP12, 1:10,000; Alexis Biochemicals). The membrane was subsequently washed with PBST and a secondary, biotin-conjugated anti-rabbit- antibody was applied (1:10,000) for 1 hr at room temperature, followed by incubation with ABC complex in PBS for 30 min. After washing, FKBP12 protein was detected using a chemiluminescence reaction (kit; BioRad) and developed. Duplicate and triplicate runs were performed.

2.3. Results

2.3.1. FKBP12 expression in the substantia nigra

Normal subjects

FKBP12 is expressed at relatively low levels in the mesencephalic region of neurologically normal subjects, in grey matter (GM) and white matter (WM). It is present predominantly in the SN pars reticularis (SNr) and pars compacta (SNc). Higher levels can be detected in the lateral SNc fibers (Fig.2-1, 2-2).

PD patients

The overall IP levels in the normal SN, although higher than in other brain regions, are lower than in the SN of one patient with PD (familial Parkinson's disease, FPD).The neurites surrounding remaining neurons are often characterized by increased FKBP12 (Fig. 2-4 (g); 2-5 (c)). In familial PD (FPD), the increase in FKBP12 is concentrated in the medial two thirds of the SN. However, the histological scoring indicates that, overall, the SNc of PD patients contains lower levels of FKBP12 in fibers of the lateral part and also in cell bodies (Fig. 2-2). This difference does not reach statistical significance. In the SNr of PD patients, there is a tendency towards a decrease in FKBP12 in the medial part and an increase in the lateral sector (Fig. 2-1 (b), 2-3). In PD patients, numerous neurons in the SN have IP- positive neuronal inclusions (Fig. 2-5 (c), 2-6 (a), (b)). FKBP12 is present in distinct non- laminated inclusions and in extracellular globular deposits. Extracellular deposits are frequent in FPD (Fig. 2-4 B).

The distribution of the labeling suggests that the immunophilin is contained in plexiform terminals of fibers projecting to SN and abnormal, degenerating processes. Most often, FKBP12 is present in synaptophysin- positive (Fig. 2-4 (d)-(f)) and NF- labeled fibers (results not shown), which indicates the localization of this IP in axonal processes. In processes, FKBP12 rarely colocalizes with the dendritic marker MAP-2.

In PD (as in DLB) cases, FKBP12 is present in α- synuclein - positive LB and Lewy threads (Fig. 2-5 (c), asterisk; medial SNc). Groups of cells and especially neurites strongly positive for both proteins are observed in PD patients (Fig. 2-5 (d), asterisk)). Alpha- synuclein – positive, FKBP12- negative processes and midbrain- type LB are also present (Fig. 2-5- (d), arrow), as

well strongly FKBP12- positive neurons that do not display extensive α- synuclein aggregates (Fig. 2-5 (c), arrow).

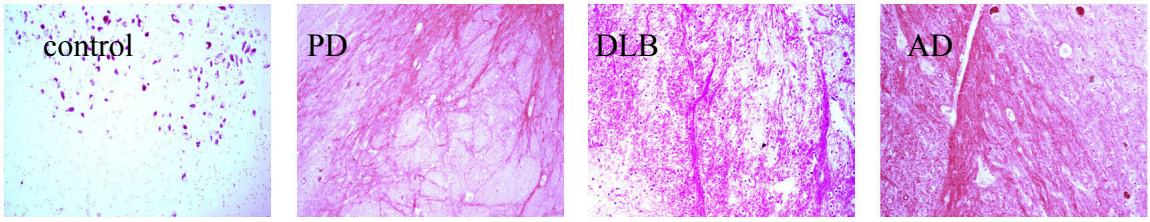


Fig. 2-1: FKBP12 expression in the substantia nigra of control subjects and neurodegenerative diseases patients. Nova- Red chromogen and haematoxylin counterstaining. Magnification: 200×

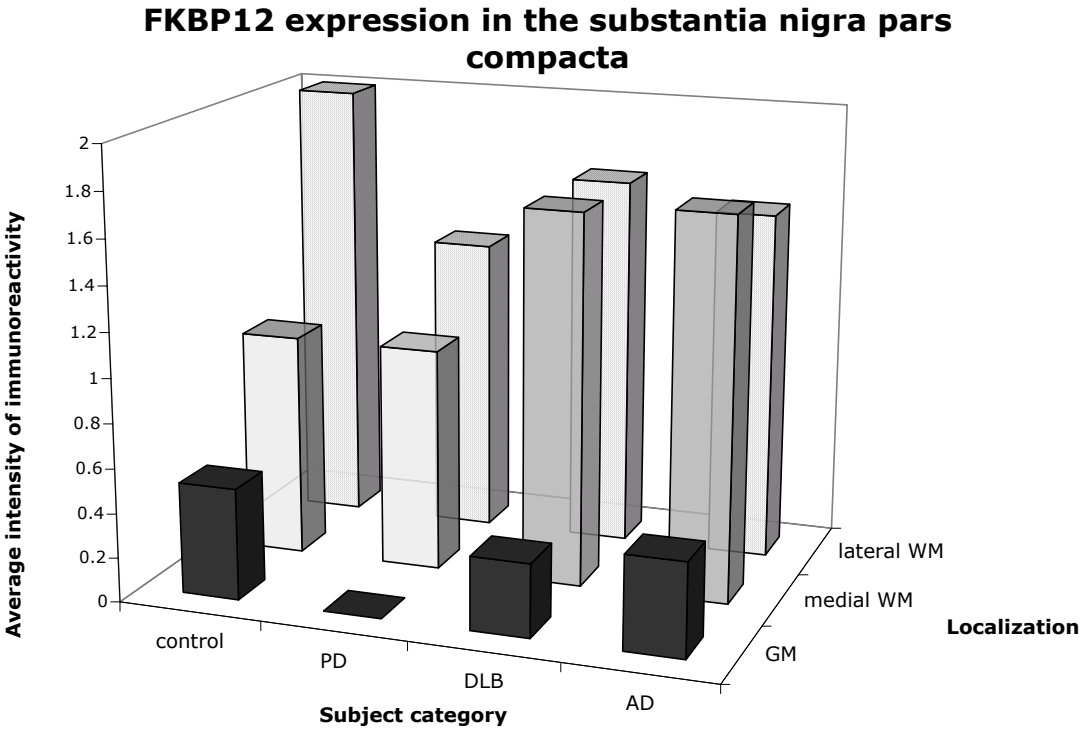


Fig.2-2: Changes in average FKBP12 immunoreactivity levels in the substantia nigra pars compacta of neurodegenerative diseases patients

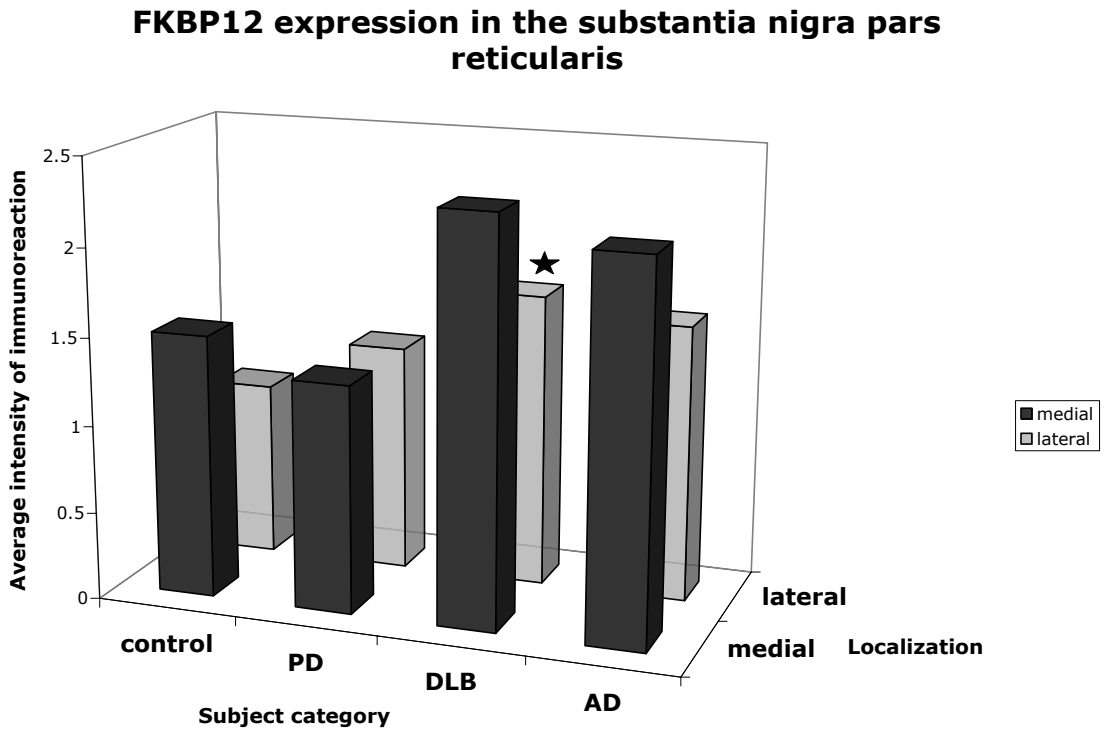
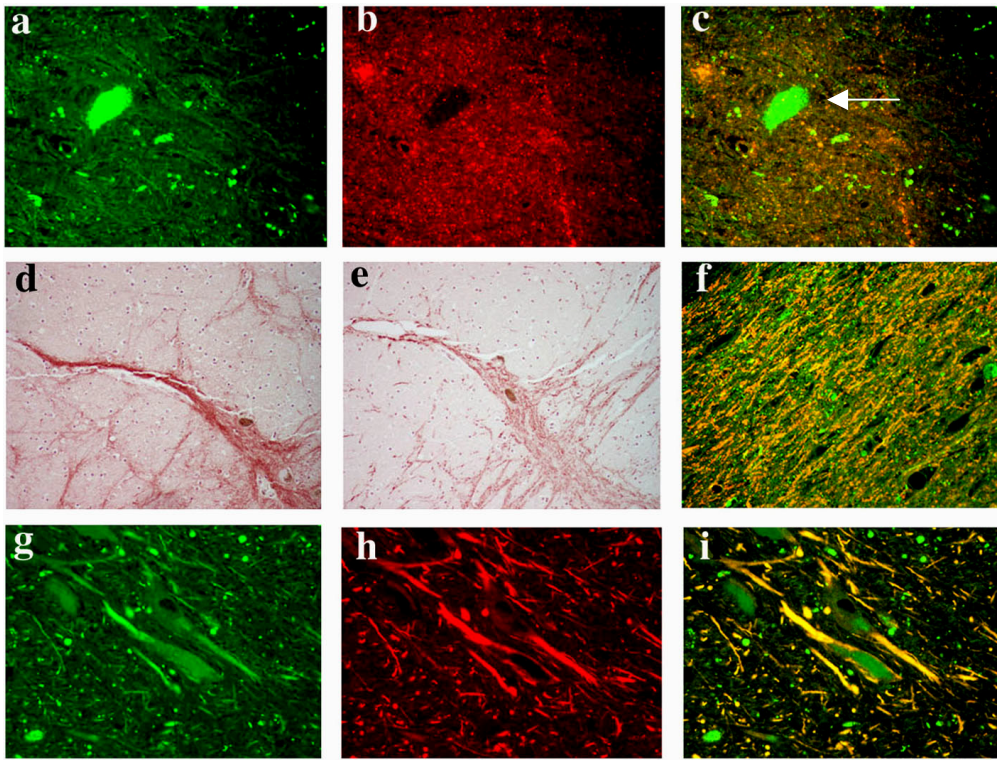


Fig. 2-3: Changes in average FKBP12 immunoreactivity levels in the substantia nigra pars reticularis of neurodegenerative diseases patients. $^{*}p < 0.05$

A)



B)

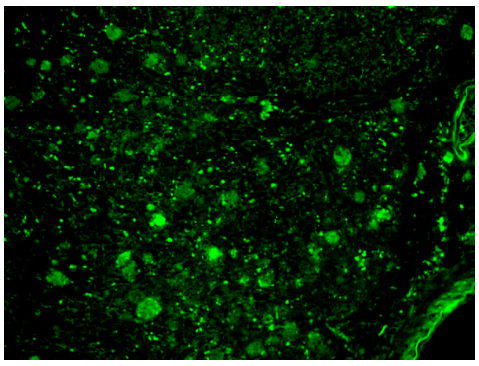


Fig. 2-4: Substantia nigra A) FKBP12 colocalization with MAP-2 (a-c, AD patient), synaptophysin(d- f, PD patient) and NF (g-i). a: FKBP12 (FITC); b: MAP-2 (Cy3); c: overlay (arrow: FKBP12- positive aggregate); d-e: adjacent sections stained for FKBP12 and synaptophysin, respectively (Nova Red); f: double labeling for FKBP12 (FITC) and synaptophysin (Cy3); g: FKBP12 (FITC); h: NF (Cy3); i: overlay. Magnification: 200× (d-f), 400× (a-c, g-i) B) FKBP12 (FITC) deposits in an FPD patient. Magnification: 200×

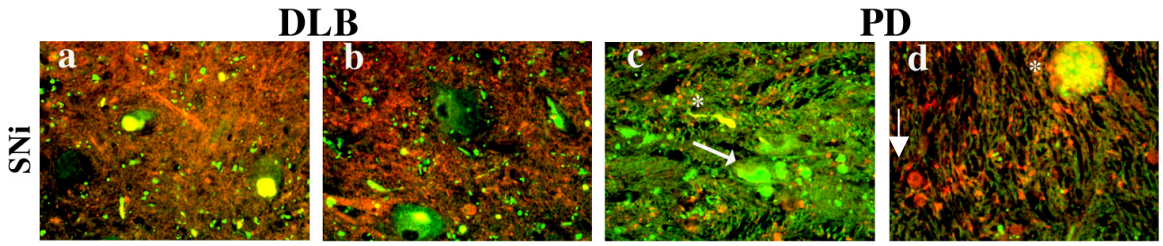
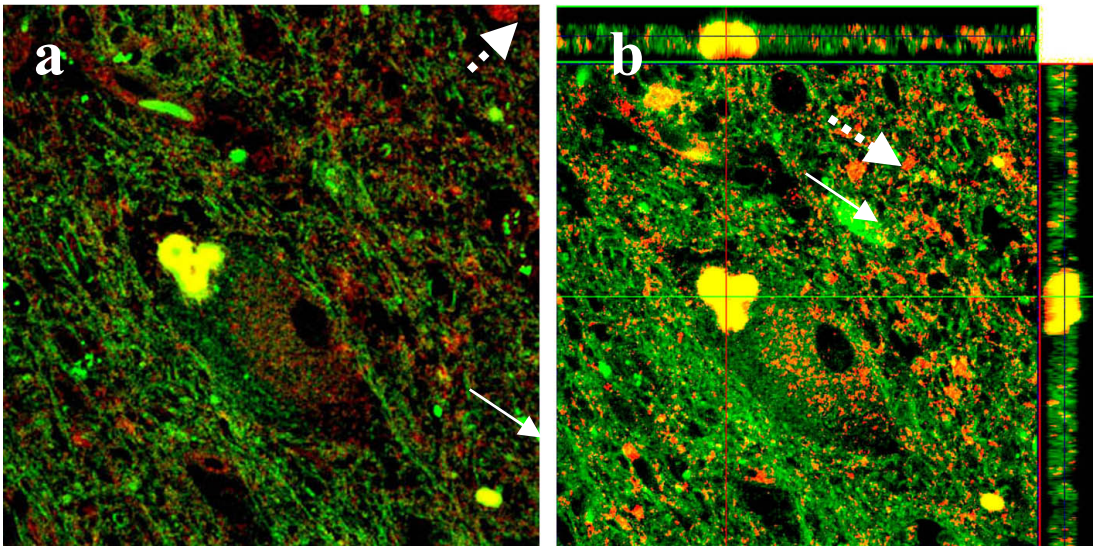


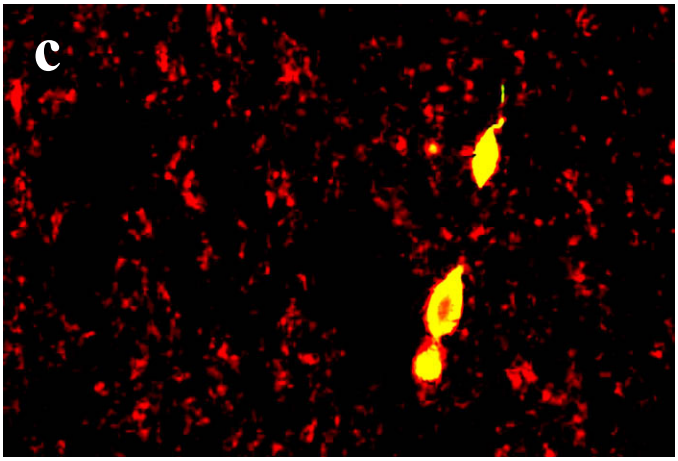
Fig. 2-5 FKBP12 is present in α- synuclein - positive LB and Lewy threads in PD and DLB patients (FKBP12: FITC; α- synuclein: Cy3). Magnification: 200×.

DLB patients

FKBP12 immunoreactivity is enhanced in fibers of medial part of the SNc and the entire SNr, with a statistically significant increase in the lateral SNr (Fig 2-1 (c), 2-2, 2-3). IP levels tend to decrease in cell bodies and lateral SNc.

Numerous neurons have IP- positive neuronal inclusions, as well as diffuse FKBP12 (Fig. 2-5 (a), (b) respectively). FKBP12 is present in distinct non- laminated, spherical inclusions, also in extracellular globular deposits. It often colocalizes with α- synuclein. Fig. 2-6 (d) illustrates the colocalization (arrow) of FKBP12 (left) and α- synuclein (right) (light micrographs of adjacent sections). Laser confocal micrographs (Fig. 2-6 (a), (b)) indicate extensive colocalization of FKBP12 (red, Cy5) and α- synuclein (green, FITC) in LB. Fig. 2-6 (a) and (b) depict a SNi neuron bearing confluent α- synuclein- and FKBP12- positive LB, with a thin layer of FKBP12 on the surface (as indicated by orthogonal sections visible on the upper and right side of image (b)). Image (a) illustrates the same neuron as (b) and shows the LB at a different level, with a synuclein- positive center. FKBP12 is present outside the neuronal soma and could be part of extracellular aggregates or neuronal processes (dashed arrow). Intracellular FKBP12 immunostaining is observed mainly around the nucleus. Serial sectioning (b) indicates the punctate perinuclear staining is indeed intracytoplasmic, as opposed to belonging to axo- somatic synapses. FKBP12- negative α- synuclein aggregates are also observed (arrow in 2-6 a, b). Many dystrophic, swollen neurites are also positive for both synuclein and FKBP12 (Fig. 2-6 (c)). The immunophilin colocalizes with α- synuclein mostly in the region adjacent to the axolemma. A central streak and an outer neuritic layer containing FKBP12 are often visible.


a
b


c

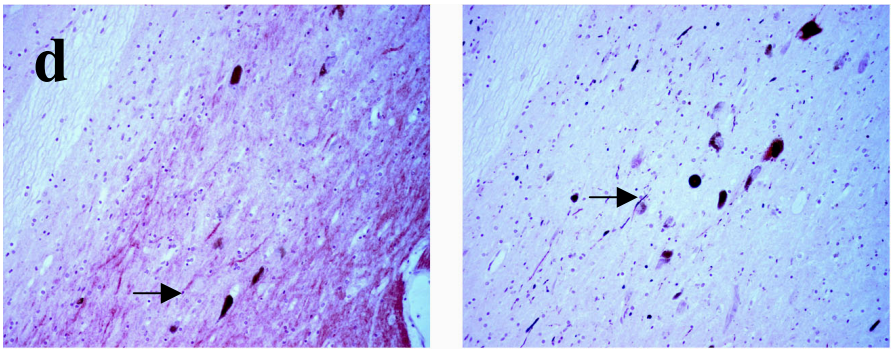

d

Fig. 2-6: Substantia nigra (DLB) a-c: Laser confocal micrographs indicating extensive colocalization of FKBP12 (red, Cy5) and α- synuclein (green, FITC) in LB and threads; d: Light micrographs of adjacent sections showing colocalization (arrows) of FKBP12 (left) and α- synuclein (right). Magnification: 600 × (a-c), 200 × (d)

AD patients

The increase in FKBP12 levels in AD patients is observed predominantly across the ventral part of the SN, (especially medial part of the SNc and the entire pars reticularis) (Fig. 2-1). FKBP12 levels tend to be lower in fibers of lateral pars compacta (Fig. 2-2, 2-3).

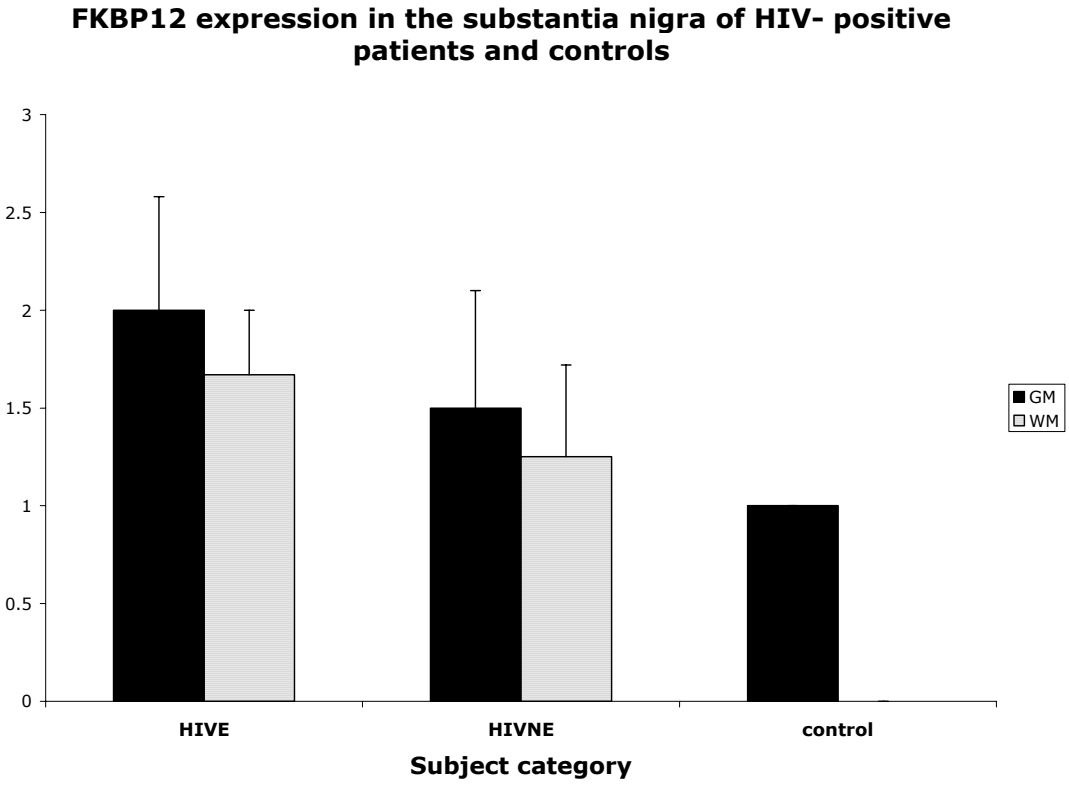
The distribution of the labeling suggests that, in AD also, the immunophilin is contained in plexiform terminals of fibers projecting to SN and abnormal, degenerating processes (Fig. 2-1(d)). Double- labeling immunofluorescence studies indicate that FKBP12 is rarely located in MAP-2 positive processes (Fig. 2-4 (a)- (c)), i.e. dendrites and occasionally in DAT- positive processes (results not shown). Most often, FKBP12 is present in synaptophysin- (results not shown) and NF- positive fibers (Fig 2-4 (g)- (i)), which indicates the localization of this IP in axonal processes.

AD cases with increased FKBP12 levels in the mesencephalon and deep GM also exhibit FKBP12- positive nigral aggregates (Fig. 2-4 (a)-(c) arrow). In these patients, the intracellular IP immunostaining appears diffuse in the medial SN (as opposed to perinuclear in DRN, results not shown) (Fig. 2-4 (g)- (i)), while in normal subjects it exhibits a predominantly perinuclear, polarized distribution in both regions. The IP deposits are sometimes found to line the cell membrane.

HIV- positive patients

Higher levels of FKBP12 are present in the nigral region of HIVE patients compared to non- encephalitic patients and normal controls (Fig. 2-7). HIV- positive patients without overt encephalopathy have higher FKBP12 levels than normal controls.

A)


FKBP12 expression in the substantia nigra of HIV- positive patients and controls
3
2.5
2
1.5
1
0.5
0
GM
WM
HIVE
HIVNE
control
Subject category

B)

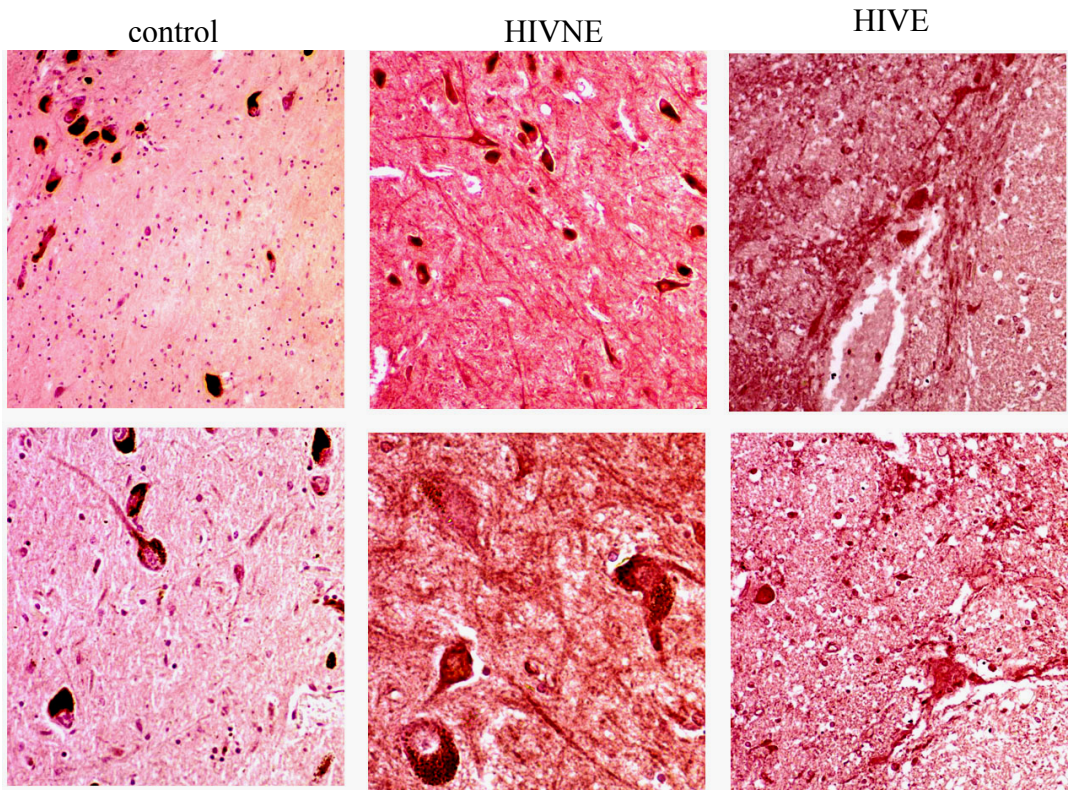


Fig. 2-7: A) Changes in FKBP12 immunoreactivity levels in the substantia nigra of HIV- positive patients. **B)** Increased FKBP12 immunoreactivity in neuronal cell bodies and processes in the substantia nigra of HIVE and HIVNE patients. Nova-Red chromogen and haematoxylin counterstaining. Magnification: 200× (seronegative control, HIVE); 400× (HIVNE).

2.3.2. FKBP12 expression in the striatum and pallidum

Normal subjects

In normal subjects, FKBP12 is expressed in the rostral and caudal segments of striatum and pallidum. It is present mostly in the WM, but also in GM. (Fig. 2-8 A (a), B (a))

PD patients

FKBP12 immunoreactivity is enhanced in medium- sized striatal neurons (mainly in the ventral putamen) and in white matter tracts (Fig. 2-8 A (b); 2-9 A). Increased levels are also present in white and grey matter of globus pallidus (Fig. 2-8 B (b); 2-10). The increase is significant in fibers of pars externa (Fig. 2-10A). The FPD case is characterized by the highest IP levels.

In the pallidum of PD patients, FKBP12 is mostly expressed in fiber tracts that are not positive for synaptophysin (Fig. 2-11 (d)- (f)). Occasionally, FKBP12 can colocalize with this synaptic vesicle marker, especially in large neurons of GP pars interna of normal controls (Fig. 2-11 (g)-(i)) and in the striatum of normal subjects and PD patients (Fig. 2-11 (a)-(c)).

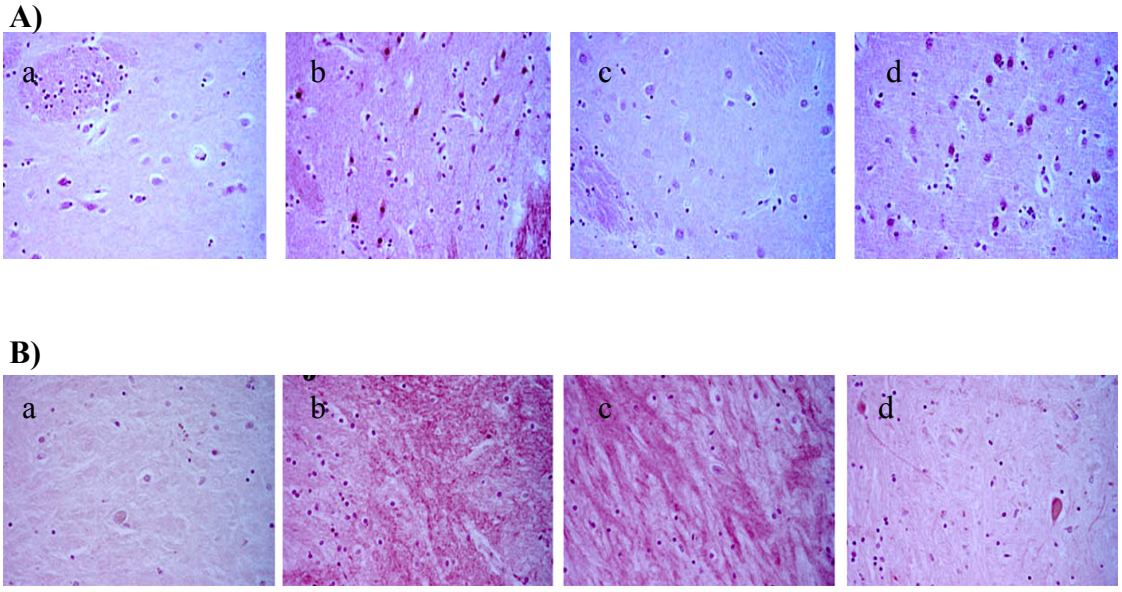
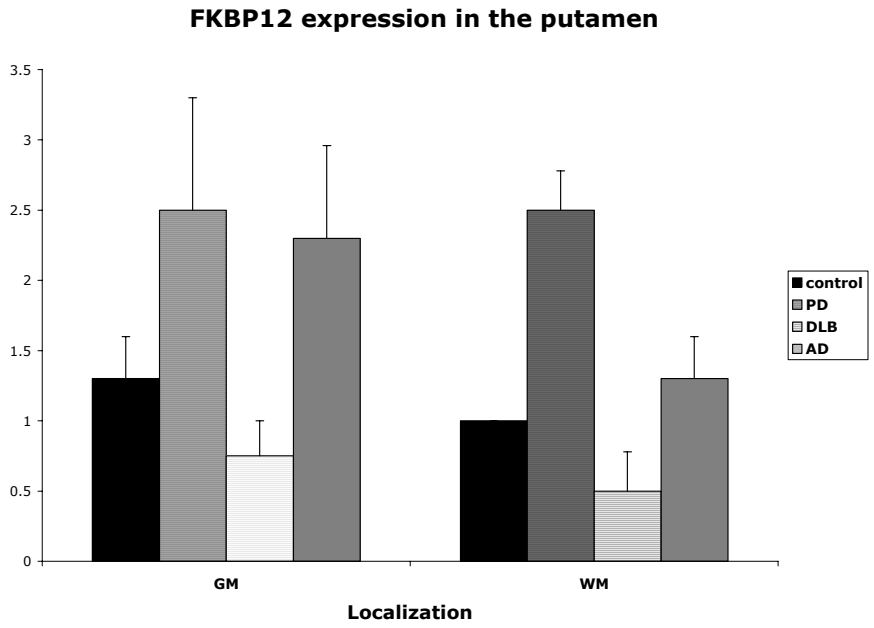


Fig. 2-8 Changes in FKBP12 immunoreactivity in the putamen (A) and globus pallidus (B). Nova-Red chromogen and haematoxylin counterstaining. Magnification: 200×. a: normal control, b: PD, c: DLB, d: AD.

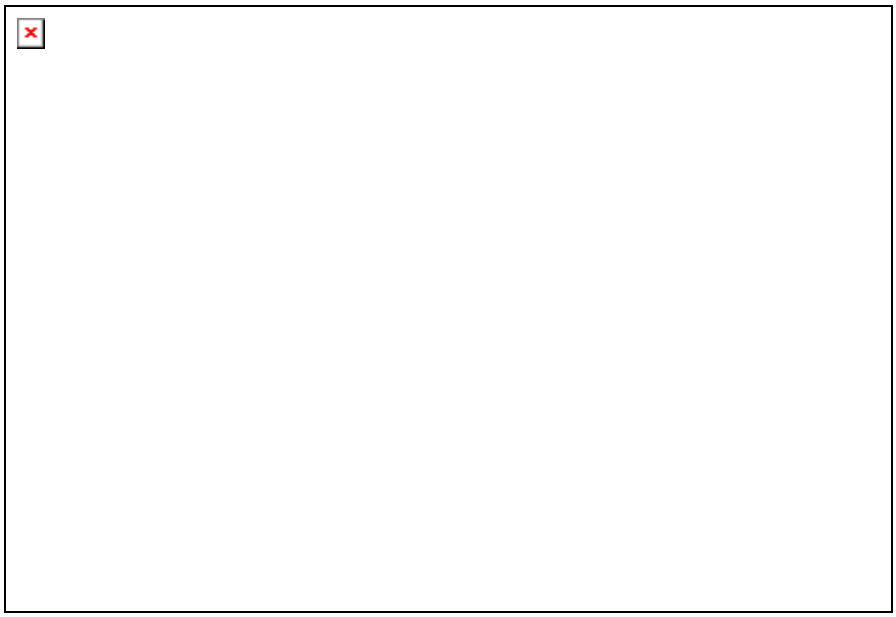
A)



B)

Fig. 2- 9 A) FKBP12 immunoreactivity levels in the putamen of neurodegenerative diseases patients and controls B) Western blot detection of FKBP12 in the putamen, indicating increased amounts of FKBP12 protein in five AD patients compared to the neurologically normal control.

A)



B)

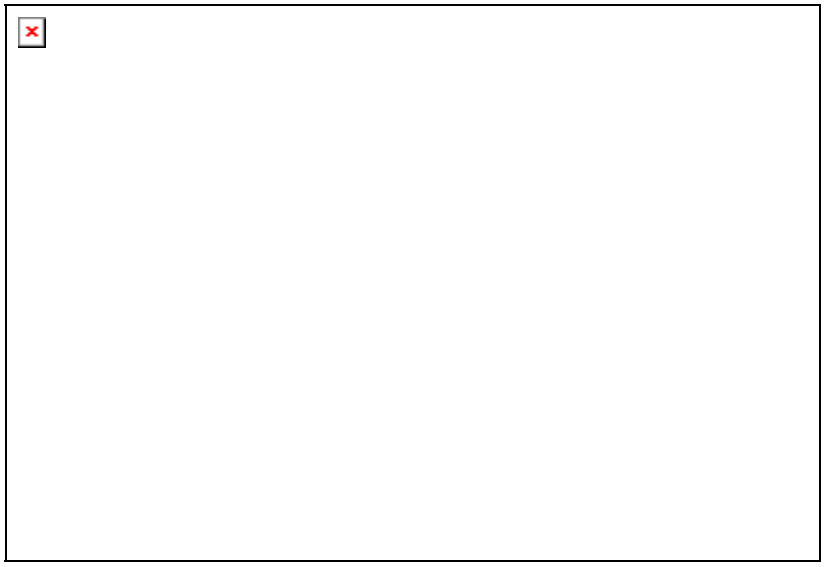


Fig. 2-10 FKBP12 expression in the globus pallidus pars externa (A) and globus pallidus pars interna (B) of neurodegenerative diseases patients and controls. $^{*}p< 0.05$

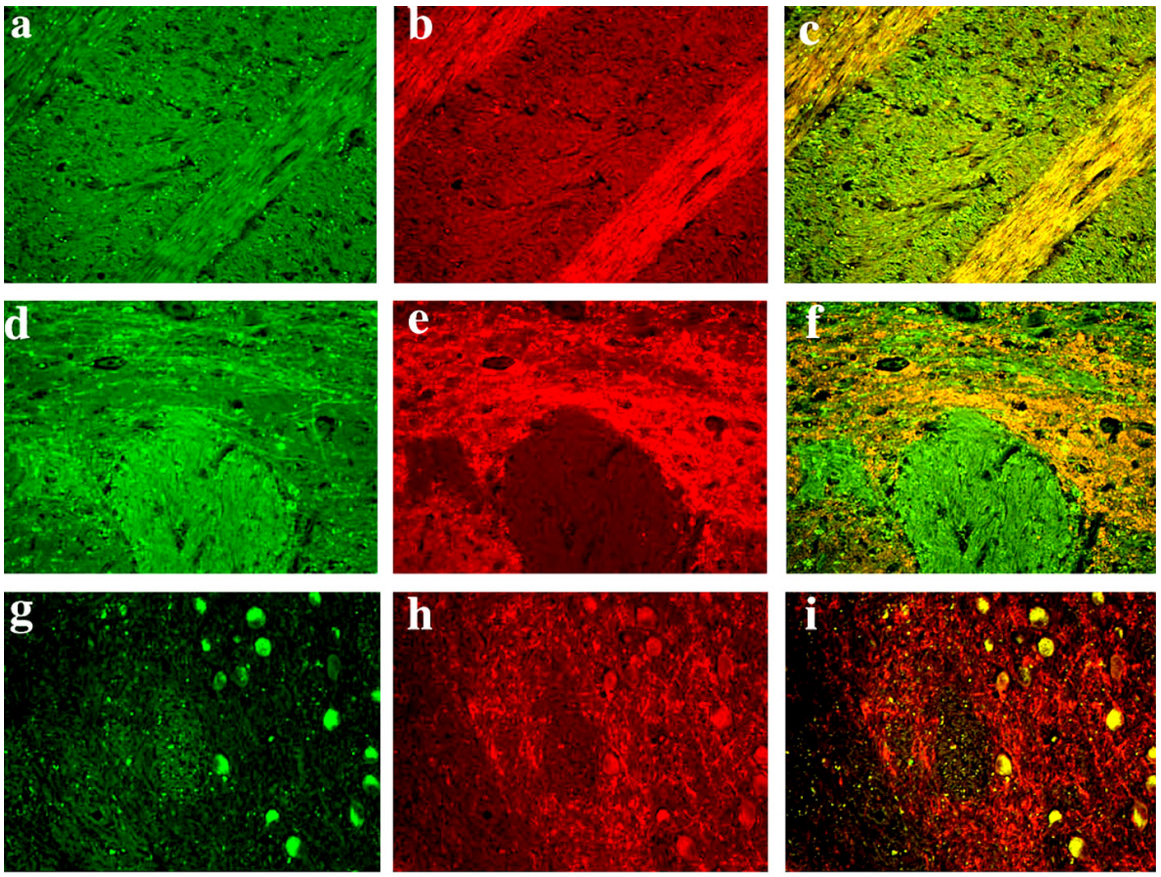


Fig. 2-11 FKBP12 (FITC) and synaptophysin (Cy3) double labeling in the striatum (a- c) and pallidum of PD patients (d- f, g- i). Magnification: 200×

DLB patients

Putaminal FKBP12 levels tend to be low in these patients, in neuronal soma and processes (2-8 A (c); 2-9A). A significant increase in FKBP12 expression occurs in GM and WM of GP pars interna, as well as in WM of GP pars externa (Fig. 2-8 B (c), 2-10A). The IP is present predominantly in white matter tracts, but also in medium- sized neurons of globus pallidus.

AD patients

Increased FKBP12 levels are present in putaminal GM (Fig. 2-8 A (d)), 2-9A) as well as in GP GM and WM (Fig. 2-8 B (d); 2-10). However, this increase does not reach statistical significance (Fig. 2-9A, 2-10).

The AD patient showing the most prominent increase in FKBP12 levels in SNi also exhibits increased IP levels in a region encompassing the putamen and GP (mainly pars interna). FKBP12- positive large neurons are also noted in the pallidum of AD and PD patients, which might represent an extension of nucleus basalis in this area (Fig. 2-11 (g)- (i)).

Western blotting studies using AD putaminal tissue (Fig. 2-9B) reveal an increased amount of protein in AD patients compared to the neurologically normal control.

HIV- positive patients

The putamen of HIV- positive patients, regardless of their encephalitic status, displays mildly increased FKBP12 in the putaminal GM (Fig. 2-13A). However, the difference between normal subjects and patients does not reach statistical significance. FKBP12 levels in WM in globus pallidus are significantly higher in HIVE patients than in non-HIV controls (Fig. 2-12, 2-13B). Patients without detectable markers of HIVE only display a modest increase.

In HIV- positive patients FKBP12 expression is not only detected in neurons, but also in microglia. Fig. 2-14 illustrates the colocalization of FKBP12 with RCA-1.

Seronegative control HIVE

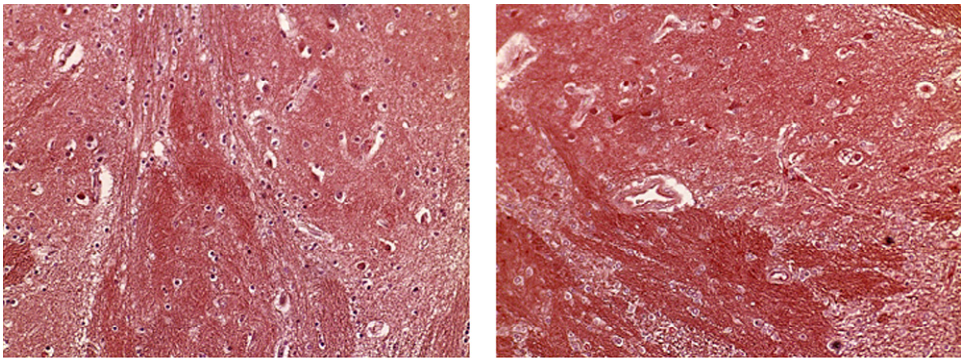
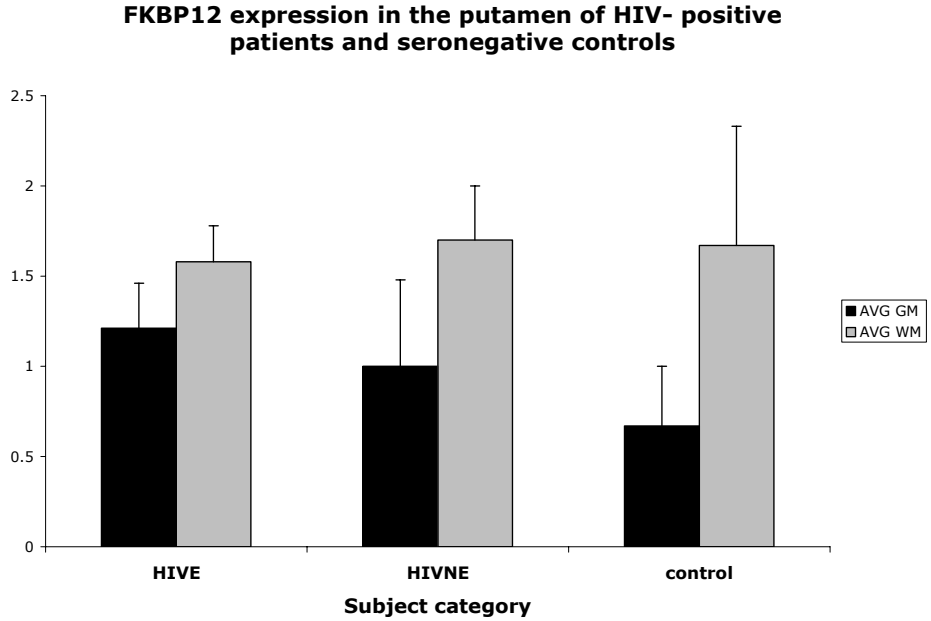


Fig.2-12 FKBP12 immunoreactivity in the globus pallidus of HIVE patients and seronegative controls. Nova- Red chromogen and haematoxylin counterstaining. Magnification: 200×

A)



B)

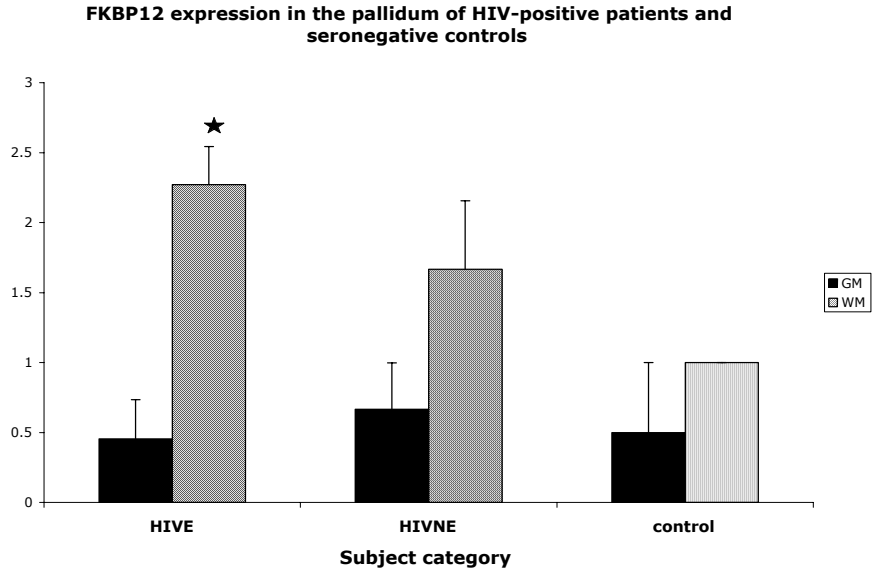


Fig. 2-13 FKBP12 immunoreactivity levels in the putamen (A) and pallidum (B) of HIVE, HIVNE patients and seronegative controls. $^{*}p< 0.001$

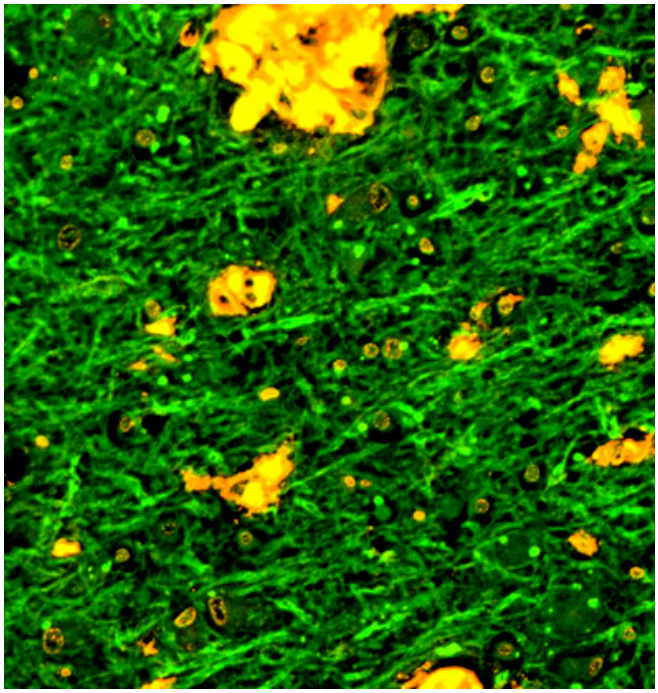


Fig. 2-14 FKBP12 (green) is present in RCA-1 – labeled microglia/ macrophage cells (red) in HIV-positive patients. Magnification: 400×

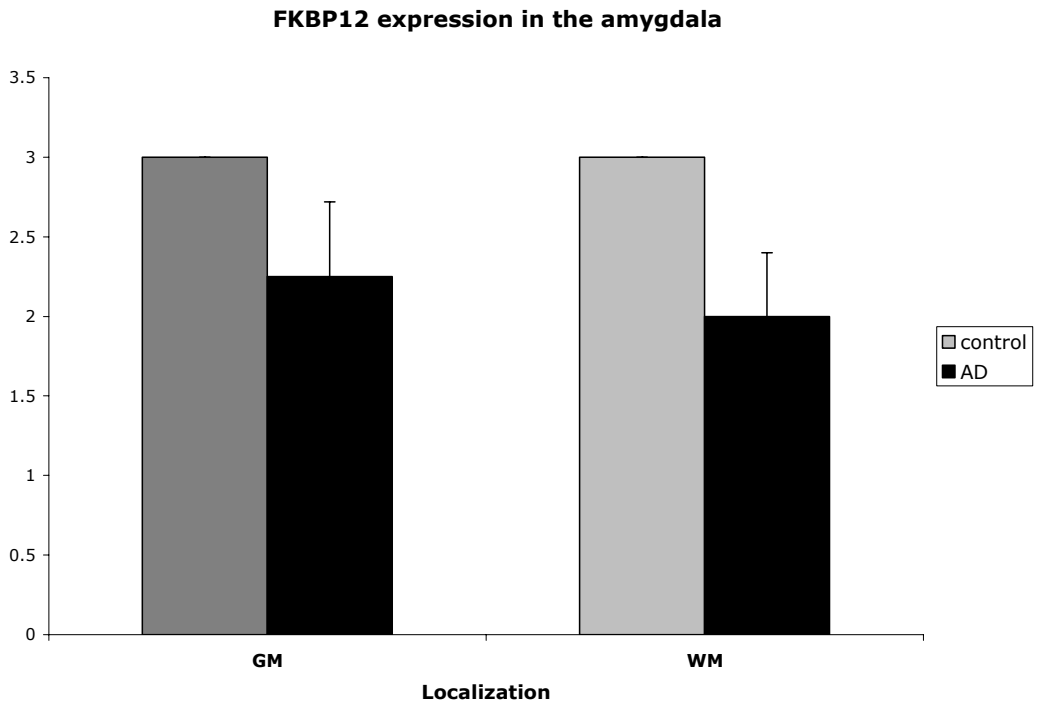


Fig. 2-15 FKBP12 immunoreactivity in the amygdala of AD patients and controls

2.3.3. FKBP12 expression in the amygdala

Normal subjects

FKBP12 is expressed in GM and WM of the amygdaloid nuclear complex (Fig. 2-15, 2-16 A).

Alzheimer's disease patients

In 3 out of 4 cases, we noticed decreased FKBP12 levels in neurons and neuropil of AD patients compared to normal controls (Fig. 2-16 A), especially in processes. One case displays similar levels of FKBP12 with the control (in soma, processes and neuropil). Both the control and this particular AD case have extensive FKBP12- positive plaques and degenerating processes in the amygdala and adjacent rudimentary cortex.

However, Western blot analysis reveals a reversed FKBP12 expression pattern (Fig. 2-16 B; molecular weight marker indicated). One patient has similar FKBP12 levels with the control, whereas the other displays increased levels. In the two patients with elevated FKBP12 levels, the IP is present as a ~14 kD band. The nature of this high apparent molecular size band is unclear.

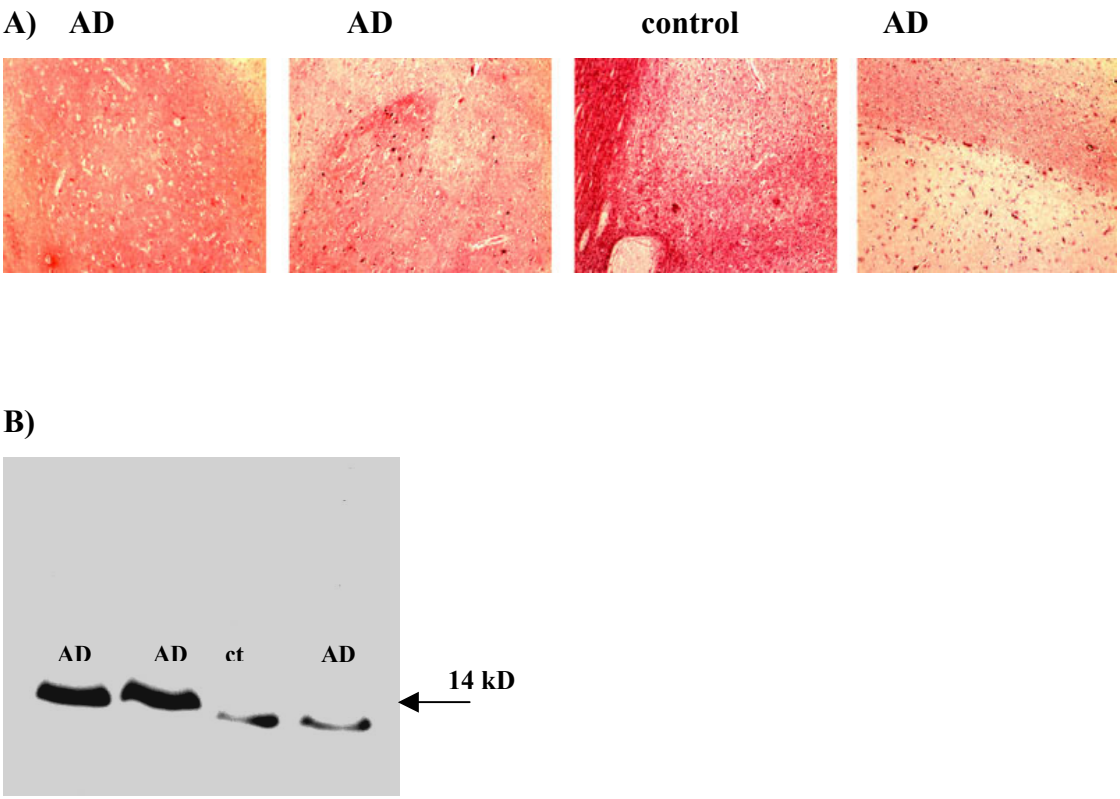


Fig. 2- 16 A) FKBP12 expression in the amygdala of AD patients and neurologically normal control. Nova- Red chromogen and haematoxylin counterstaining. Magnification 200× B) Western blot detection of FKBP12 protein in the amygdala of AD patients and control.

2.3.4. FKBP12 expression in the hippocampus

Normal subjects

In neurologically normal subjects, there is a low or undetectable FKBP12 expression in the dentate gyrus (DG) and CA2- CA4 regions (Fig. 2- 17, 2-18, 2-19). The granule cells of the dentate gyrus appear weakly FKBP12- positive. The entire granular cell layer is often surrounded by FKBP12- positive processes belonging to the inner molecular layer. Occasionally, labeled cell bodies and processes of entorhinal and subicular neurons are present.

PD patients

FKBP12 levels are increased in the FPD case, in the DG, CA4 and CA2/3 subfields (Fig. 2-18, 2-19). A modest increase in FKBP12 immunolabeling is evident in PD subjects in general, in GM of DG, CA4, CA1 and in GM and WM of CA2/ CA3 areas (Fig. 2-17). Nuclear or perinuclear FKBP12 is present (Fig. 2-18).

Alpha- synuclein- positive spherical inclusions are often positive for FKBP-12 (Fig. 2-20 a)- CA4 and (c)- CA2/3). FKBP12 colocalizes with synuclein in LB and Lewy threads, mostly in CA regions (Fig. 2-20 (b); 2-21, laser confocal micrograph). FKBP12 colocalized with α- synuclein can be found in neuritic threads arising from or contacting cell bodies containing synuclein- positive aggregates (Fig. 2-21 arrows). A modest level of expression is also noted in the neuropil and soma of pyramidal neurons in the subicular and entorhinal cortices, where the immunophilin can co-localize with α- synuclein (Fig. 2-20 (e)). Fig. 2-20 (d) illustrates the presence of FKBP12 in a plaque in the parahippocampal cortex of a PD case: the core of the plaque (arrow) is FKBP12- positive, while the periphery is α- synuclein – immunoreactive.

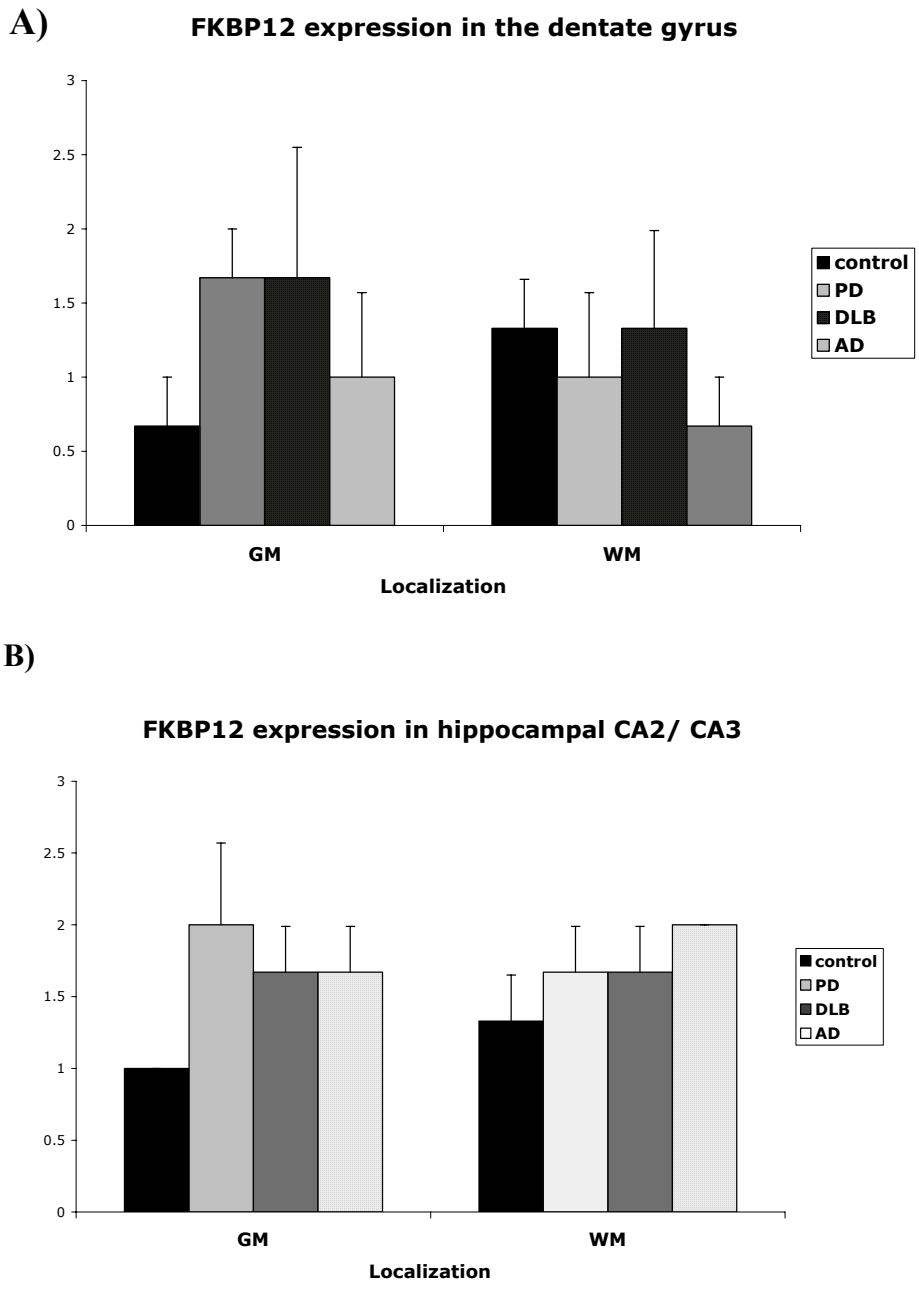


Fig. 2- 17 FKBP12 immunoreactivity levels in the dentate gyrus (A) and hippocampal CA2/ CA3 (B) in neurodegenerative diseases patients and controls.

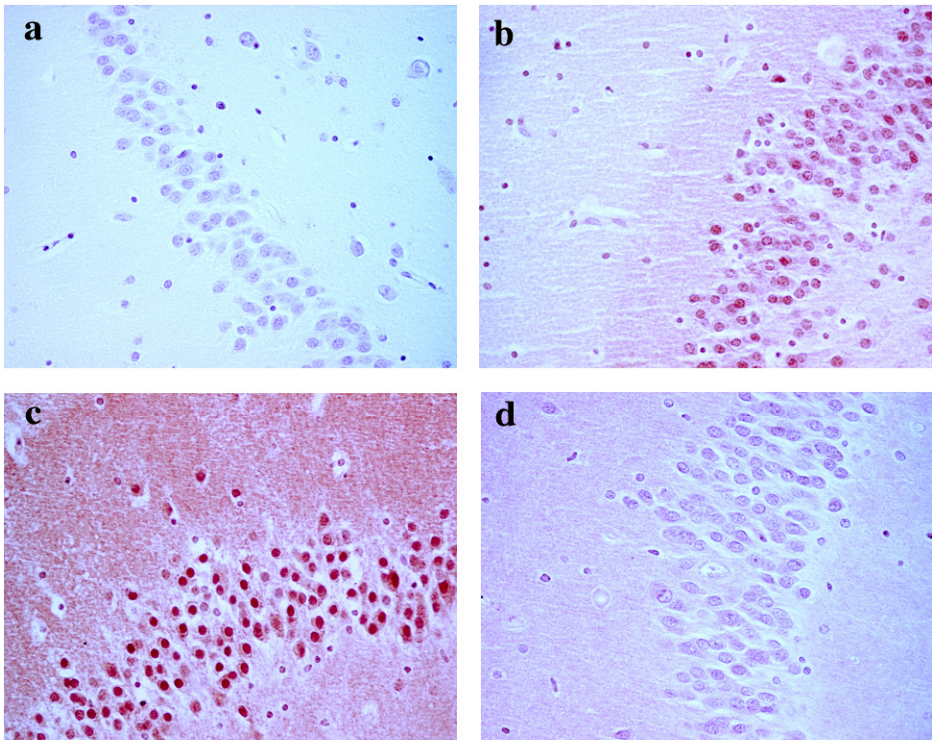


Fig. 2-18 FKBP12 immunostaining in the dentate gyrus of control subject (a), PD (b), DLB (c) and AD (d) patients. Nova- Red chromogen and haematoxylin counterstaining. Magnification: 200×

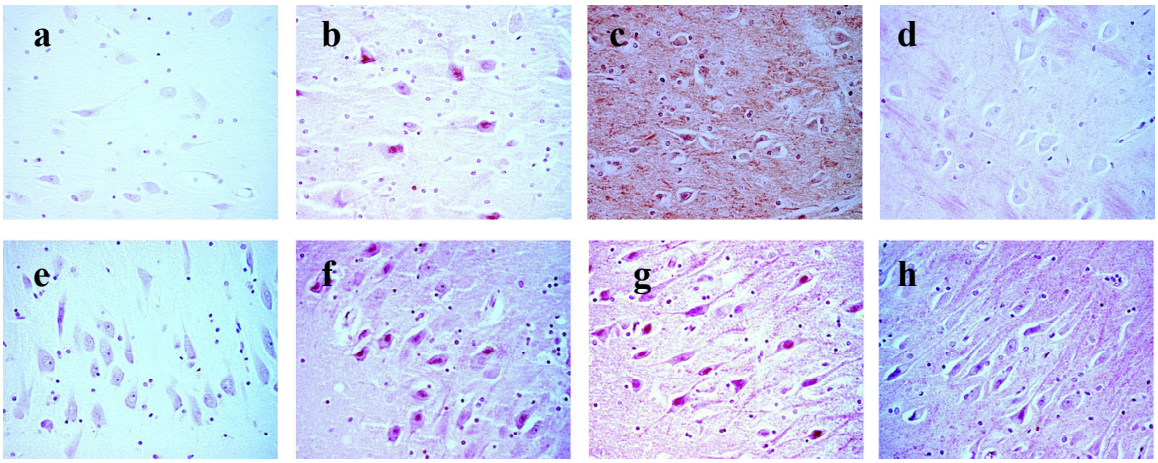


Fig. 2-19 FKBP12 immunostaining in the CA4 (a- d) and CA2 (e- h) of control subject (a, e), PD (b, f), DLB (c, g) and AD (d, h) patients. Nova- Red chromogen and haematoxylin counterstaining. Magnification: 200×

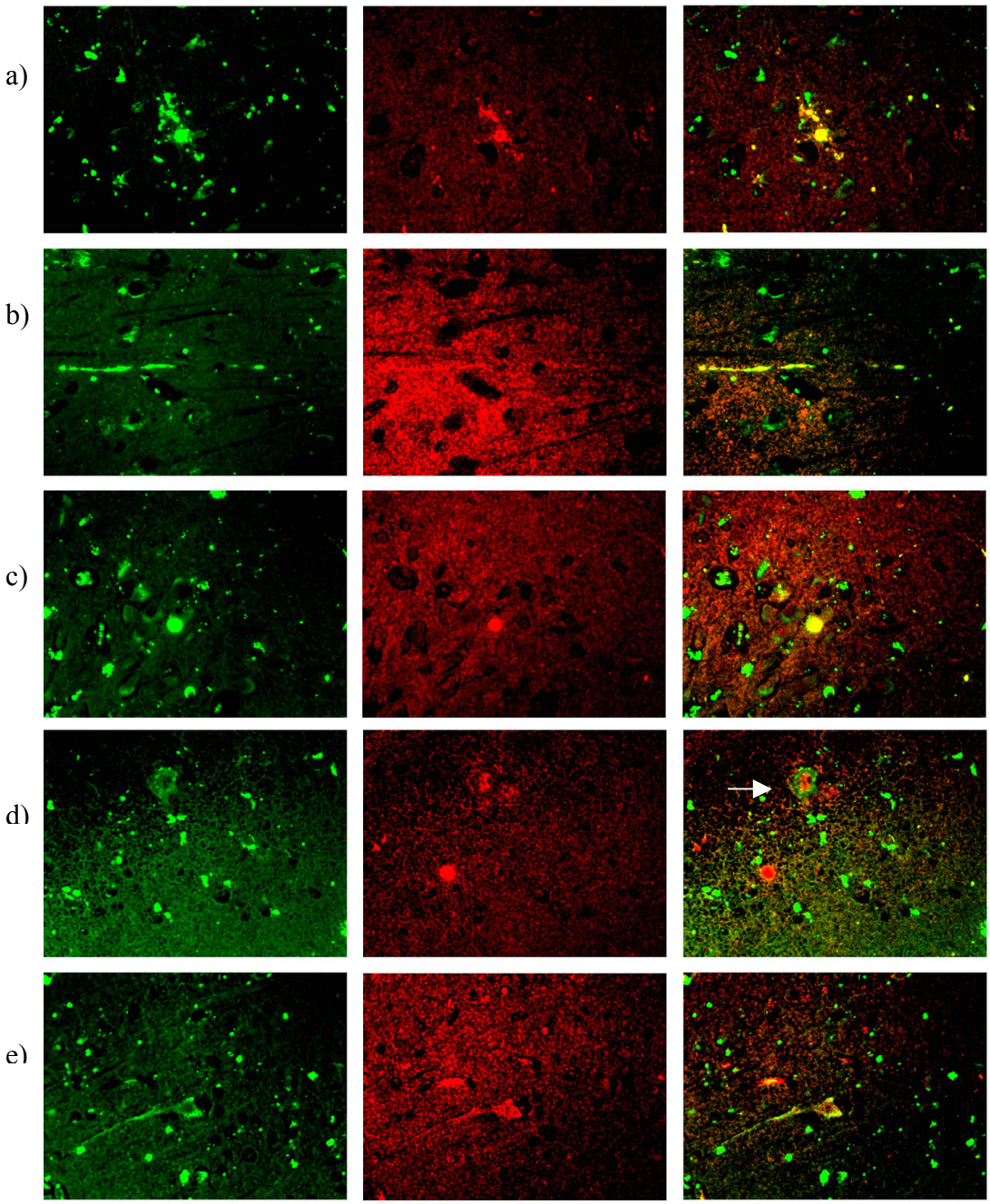


Fig. 2- 20 Double labeling for FKBP12 (Cy3) and α- synuclein (FITC) in the hippocampal formation (PD patient). a- CA4; b, c - CA2/3; d- parahippocampal cortex; e- entorhinal cortex. Magnification: 200×.

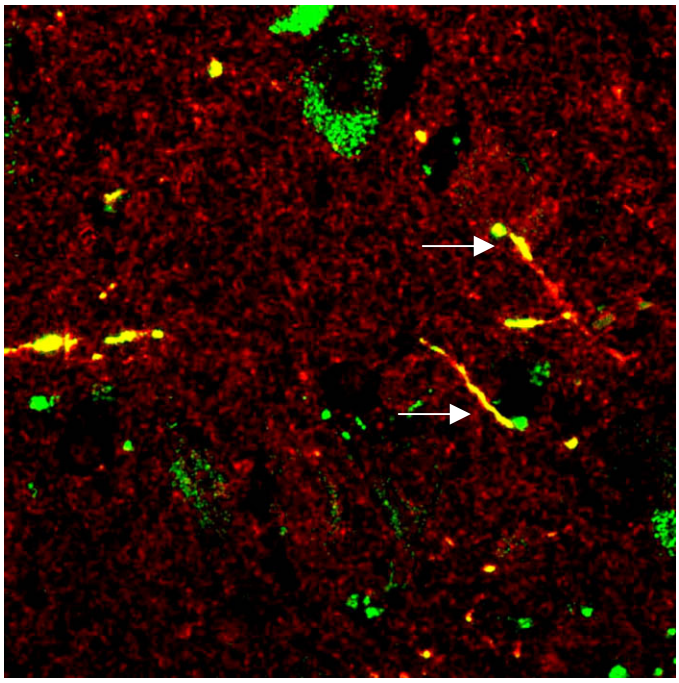


Fig. 2- 21 Laser confocal micrograph illustrating FKBP12 (Cy3) colocalization with α- synuclein (FITC) in LB and Lewy threads in a CA region (arrow). Magnification: 400×.

DLB patients

FKBP12 immunostaining is increased in DLB in the GM of DG and GM and WM of CA2/ CA3 (Fig. 2- 17, 2-18, 2-19). A "halo" of FKBP12- positive processes is present in the inner molecular cell layer (Fig. 2-18). Severe hippocampal atrophy and cell loss in one DLB patient is accompanied by decreased FKBP12 levels. The very low degree of hippocampal atrophy and the absence of NFT in one case associates with an absence of FKBP12 alterations.

Increased IP levels are also noted in the subicular and entorhinal cortices.

Intra- and extracellular FKBP12- positive aggregates are numerous in the DG hilus and CA areas (Fig. 2-22 (a), (b)). Interestingly, many cell nuclei appear to be strongly FKBP12-positive in DG and CA regions of these DLB patients (Fig. 2-18 (c)). However, the location of the protein is just perinuclear, as suggested by the absence of significant nuclear positivity in laser confocal images (Fig. 2-23).

In the DG, the FKBP12- positive processes in the inner molecular cell layer are immunoreactive for the synaptic vesicle marker synaptophysin. This suggests a colocalization of the two proteins and the presence of FKBP12 in axons (Fig. 2-22 (a)). A high degree of colocalization is also present in fibers of the DG granular cell layer, polymorphic layer and hilus, CA4 subfield**.** (Fig. 2-22 (a), (b), (c); 2-23).

In DLB patients, FKBP12 colocalizes with α- synuclein in LB and Lewy threads, mostly in CA and cortical regions (Fig. 2-25, 2-26 (a)-(c); (d)-(f)). Fig. 2-25 illustrates the expression of FKBP12 (a) and α- synuclein (b) in adjacent sections of CA2/3 region. Abundant synuclein reactivity in the soma and dystrophic processes does not translate in a marked IP expression. However, many cell bodies containing synuclein are rich in FKBP12. This is not true for neuronal processes. Fig. 2-27 (A) (laser confocal image) depicts a α- synuclein- and FKBP12-positive Lewy body, with a thin layer of FKBP12 on the surface, surrounded by strongly FKBP12- immunoreactive processes. FKBP12- negative α- synuclein aggregates are observed (arrow in 2-27). FKBP12- positive extracellular aggregates are also present. Many enlarged neurites are positive for both synuclein and FKBP12 (Fig. 2-27 B). The immunophilin colocalizes with α- synuclein mostly in the region adjacent to the axolemma, whereas the rest of the axoplasm is FKBP-12 positive.

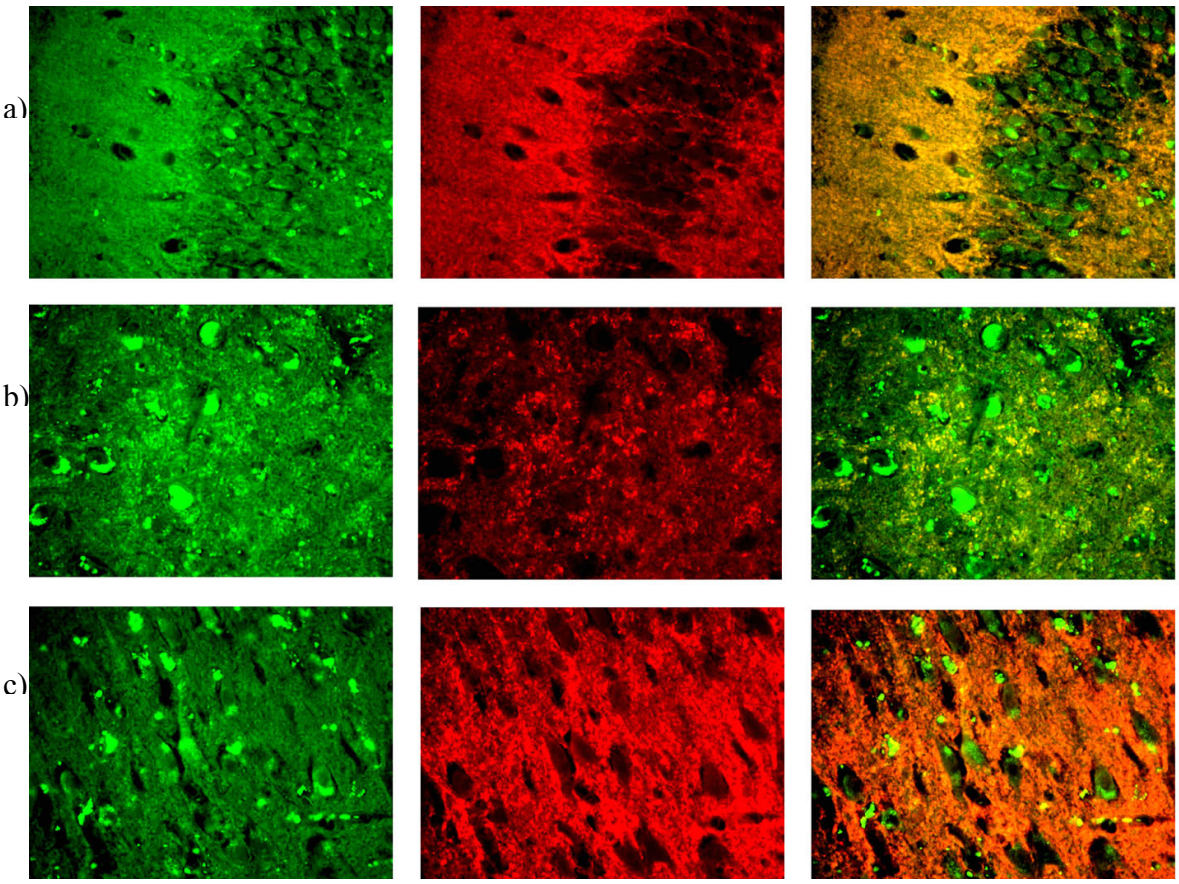


Fig. 2-22 DLB patient: a) FKBP12- positive (FITC) processes in the inner molecular cell layer are immunoreactive for the synaptic vesicle marker synaptophysin (Cy3); b)- c) colocalization in fibers of the DG hilus and CA4 subfield, respectively. Magnification: 200×.

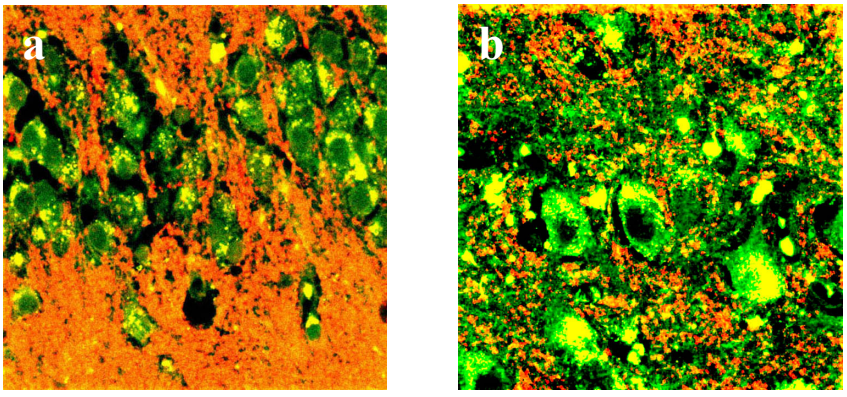


Fig. 2- 23 Laser confocal micrograph illustrating the colocalization of FKBP12 (FITC) and synaptophysin (Cy3) in the DG of a DLB patient. Magnification: 400× (a), 600× (b).

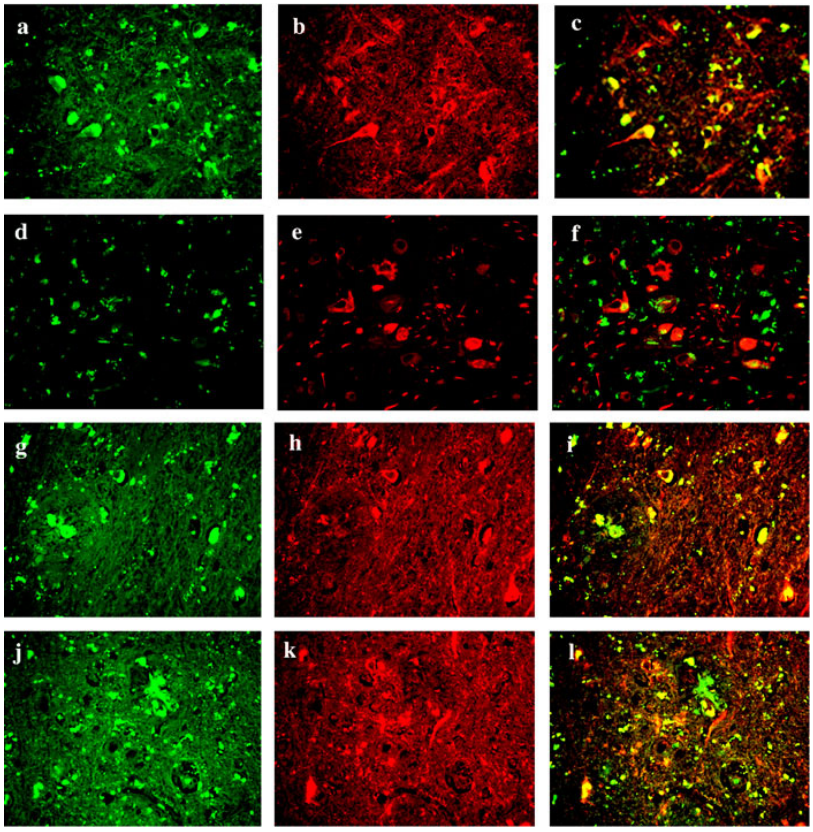


Fig. 2- 24 AD: Double labeling for FKBP12 (FITC) and NF (a- f) or MAP-2 (g- l) (Cy3) in the hippocampal formation. Magnification: 200×.

a **b**

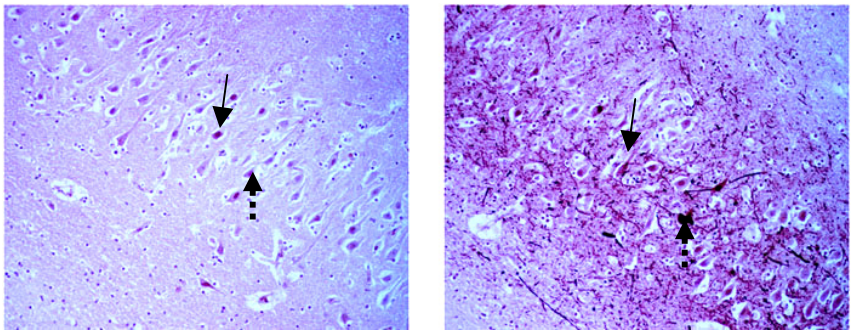


Fig. 2- 25 Expression of FKBP12 (a) and α- synuclein (b) in adjacent sections of the CA2/3 region (DLB patient). Nova- Red chromogen and haematoxylin counterstaining. Magnification: 200×.

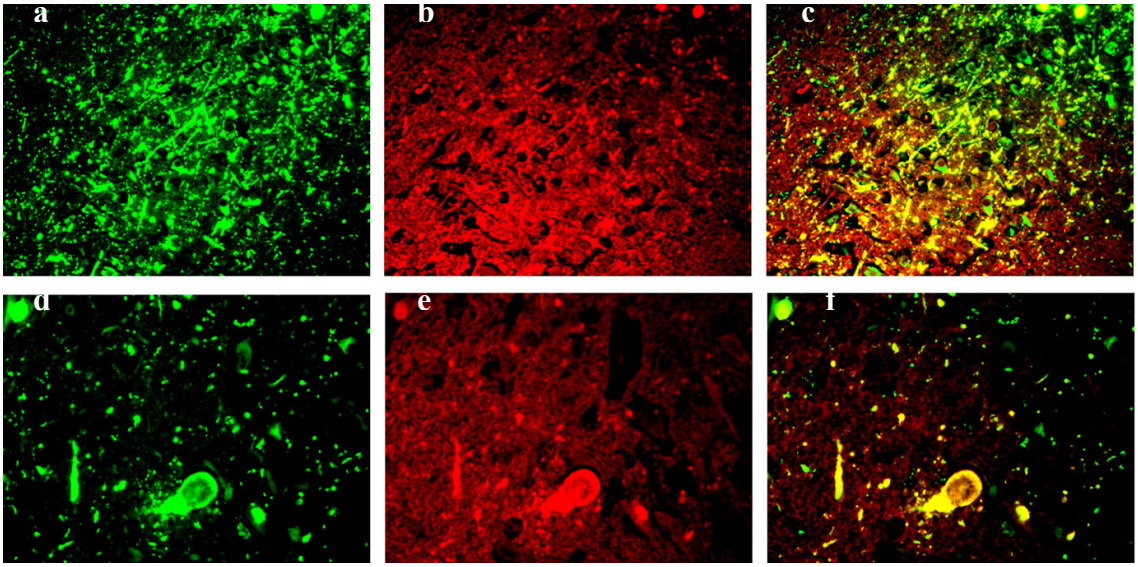


Fig. 2- 26 FKBP12 (Cy3) colocalizes extensively with α- synuclein (FITC) in LB (d- f) and Lewy threads (a- c), in cortical regions and CA, respectively. Magnification: 200×.

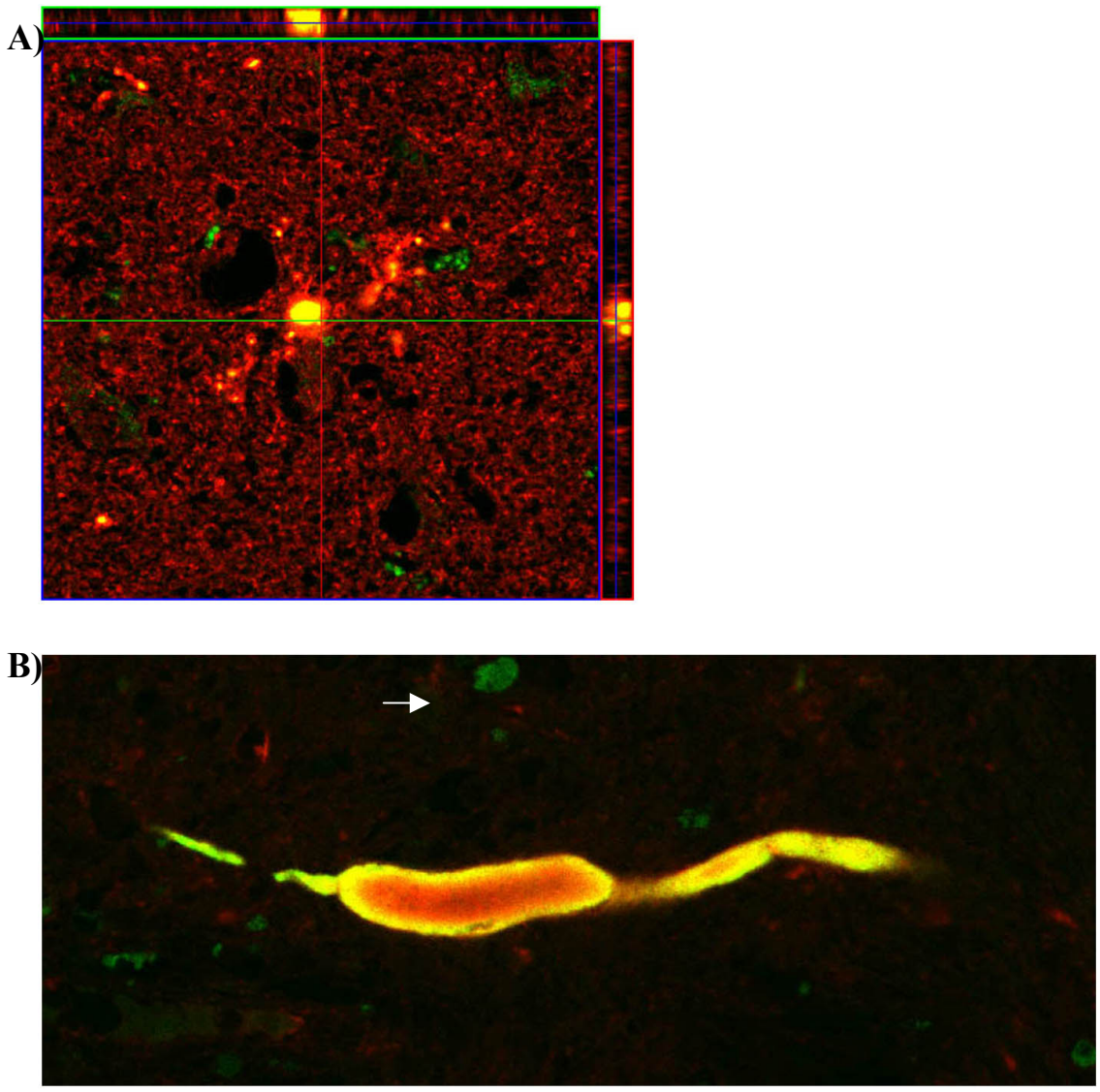


Fig. 2- 27 Laser confocal images depicting α- synuclein- (FITC) and FKBP12 (Cy3) colocalization. A: Lewy body, with a thin layer of FKBP12 on the surface, surrounded by strongly FKBP12-immunoreactive processes. B: enlarged neurite positive for both synuclein and FKBP12; the immunophilin colocalizes with α- synuclein mostly in the region adjacent to the axolemma, whereas the rest of the axoplasm is FKBP-12 positive. FKBP12- negative α- synuclein aggregates are observed (arrow). Magnification: 400× (A), 600× (B).

AD patients

All AD patients exhibit elevated FKBP12 levels in the GM of DG, as well as GM and WM of CA4/ CA3, but rarely in neurons of subicular and entorhinal areas (Fig. 2-17, 2-18). Higher levels of IP are present in plaques (Fig. 2-28). Immunophilin levels in fibers of DG and in the CA1 region are either similar to control subjects or reduced. FKBP12- positive aggregates and neuritic processes are present in the DG hilus, CA4 subfield and pyramidal cell layer of CA3 area (Fig. 2-28; 2-29), as well as occasional nuclear immunopositivity (Fig. 2-19). FKBP12 exhibits little colocalization with NF in axonal processes in the DG. It can, however, colocalize with MAP-2 in neuronal processes in various regions of the hippocampal formation (Fig. 2-24, (g- l)).

Many NFT present in the hippocampus of AD patients are positive for FKBP12 (Fig. 2-28 (a) and (c): DG/ CA4; (b), (d)- (f): CA2/ 3 and Fig. 2-29). FKBP12 is often present in the core of mature neuritic plaques (NP) (Fig 2- 28 (a)), as well as in the surrounding radiating cells (microglia or astrocytes), and tau- positive neurites. Fig. 2-28 (c-f) illustrates the presence of FKBP12 in flame- shaped NFT of various hippocampal areas, especially in the apical dendrite. The IP colocalizes with tau in early- stage NFTs, characterized by accumulation of dispersed tau. In this type of tangles, there may be a perinuclear accentuation of tau immunoreactivity (Fig. 2-28 (b)), which coincides with areas where FKBP12 tends to accumulate. Globose NFTs also contain FKBP12 (Fig. 2-28 (d)- (f)). Fig. 2-29 illustrates the presence of FKBP12 in NFT and diffuse plaques in the vicinity of a blood vessel (which is a common location for plaques), as well as the colocalization with tau in the perivascular space.

In the CA1 area, little FKBP12 is present (mostly in paranuclear inclusions), which rarely colocalizes with NF (Fig. 2-24 (a)- (c)). In the AD case characterized by an intense destruction of CA1 area architecture, little FKBP12 is noted in NP and NFT (Fig. 2-28 (b), (c)); when present, it colocalizes with tau. In this region, NP are strongly immunoreactive for tau, but often lack the FKBP12- positivity. Non- NFT FKBP12 immunoreactive aggregates are present (Fig. 2-28 (b)-(d)).

In adjacent cortical layers, FKBP12 is present in primitive NP, where it can associate with NF- positive processes, as well as in maturing plaques. Here, the IP is localized both in the

amyloid core and in the surrounding dystrophic neurites. The entorhinal cortex exhibits FKBP12- positive aggregates, which generally do not colocalize with NF (Fig. 2- 24 (d)- (f)).

Control and PD subjects exhibit similar, relatively low levels of IP in the wall of blood vessels (endothelium and tunica media). In AD patients, cerebral amyloid angiopathy (CAA) leads to thick- walled arterioles that are consistently and strongly positive for FKBP12 (Fig. 2-30; a: artery with increased FKBP12 expression in the endothelium and possible splitting of the internal elastic lamina- alteration characteristic of CAA; b: arteriole with intense amyloid deposition in the tunica media and strong IP immunoreactivity). In addition to the presence of FKBP12 in the amyloid core of plaques, this suggests the association of this chaperone protein with amyloid aggregates. Indeed, double labeling for FKBP12 and ERAB indicates that FKBP12 co- localizes with amyloid (Fig. 2-31). The intracellular polypeptide ERAB, (endoplasmic reticulum- associated binding protein) binds amyloid- beta and is expressed in normal tissues, but is overexpressed in neurons affected in Alzheimer's disease. The colocalization of FKBP12 and ERAB is extensive, with the two markers associating in cell bodies and dendrites of DG granular and molecular cell layers. FKBP12- positive, ERAB- negative aggregates are present (Fig. 2-31 (a)). Cells and processes in the profound granular layer are FKBP12- positive only (Fig. 2-31 (b)). The two markers colocalize occasionally in the blood vessel wall or perivascular space.

HIV- positive patients

In HIVE patients, FKBP12 levels are elevated in the DG (compared to HIV- positive non-encephalitic subjects and seronegative controls) (Fig. 2-32, 2-33: DG granular and molecular layer, hilus), CA4 and CA2/3 regions (with respect to seronegative subjects) (Fig. 2-32). In DG, FKBP12 is expressed at high levels in processes and cell bodies, especially surrounding the nucleus (Fig. 2-33).

Limited colocalization of FKBP12 with ERAB is observed in the hippocampus (Fig. 2-34; note the high expression of FKBP12 in soma).

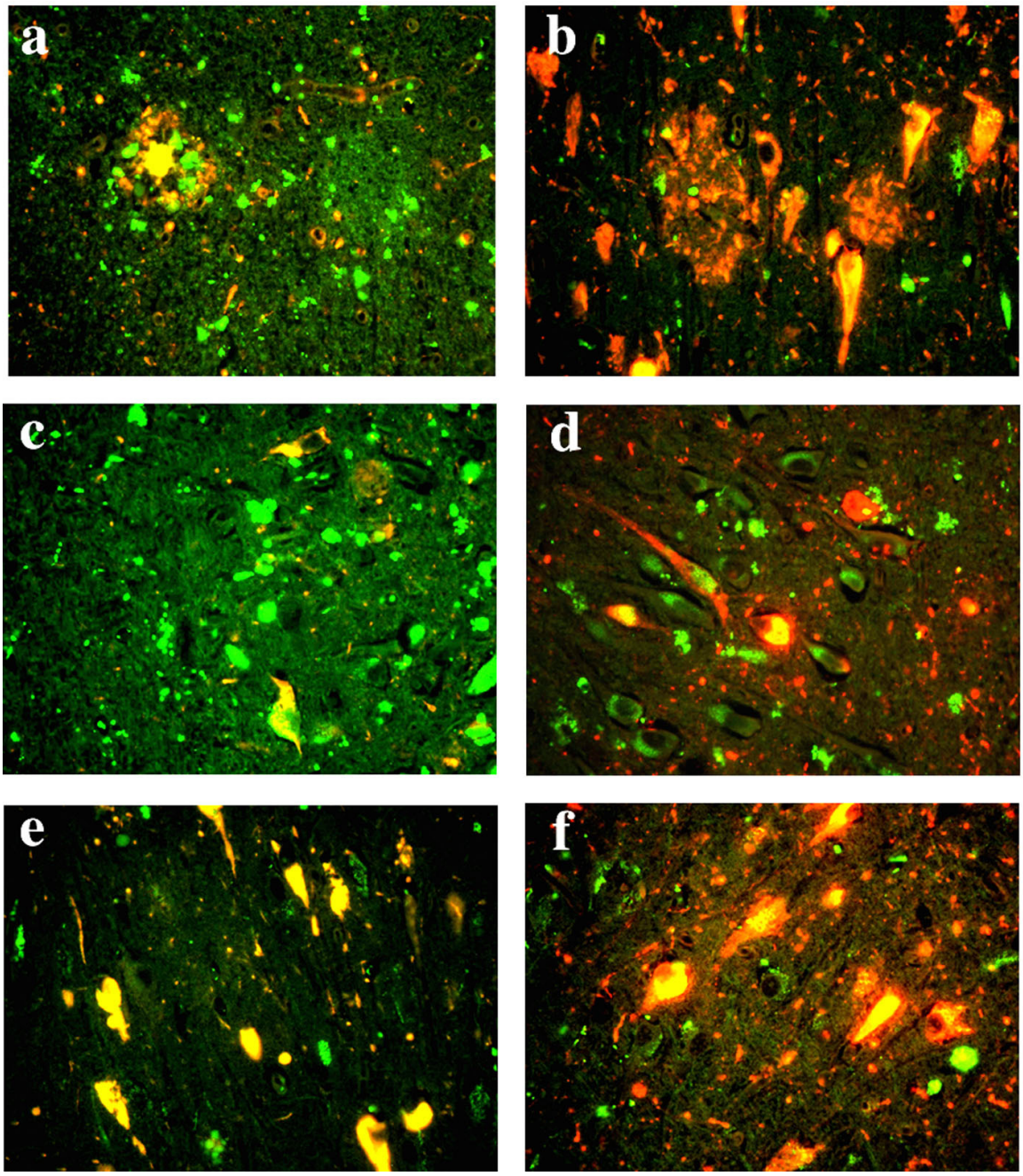


Fig. 2- 28 NFTs (Cy3) in the hippocampus of AD patients are positive for FKBP12 (FITC). (a) and (c): DG/ CA4; (b), (d)- (f): CA 2/ 3. Magnification: 400×.

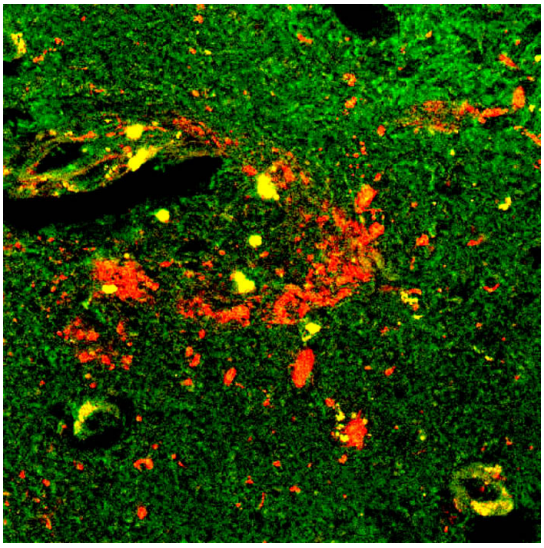


Fig. 2-29 FKBP12 (FITC) is present in NFT (Cy3) and diffuse plaques in the vicinity of a blood vessel. Magnification: 200×.

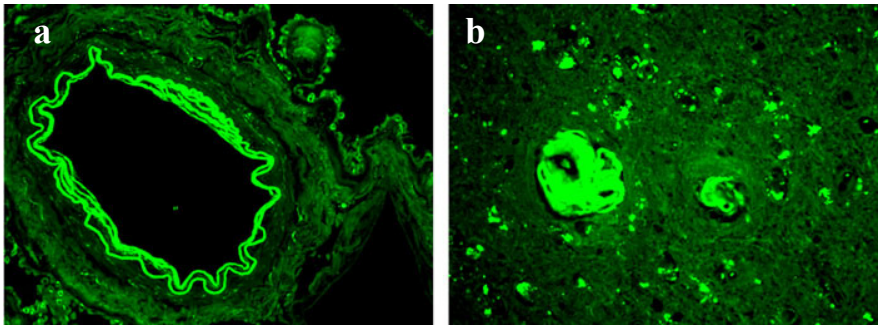


Fig. 2-30 FKBP12 expression in blood vessel walls (AD). a: artery with increased FKBP12 expression in the endothelium and possible splitting of the internal elastic lamina; b: arteriole with intense amyloid deposition in the tunica media and strong FKBP12 immunoreactivity. Magnification: 200×.

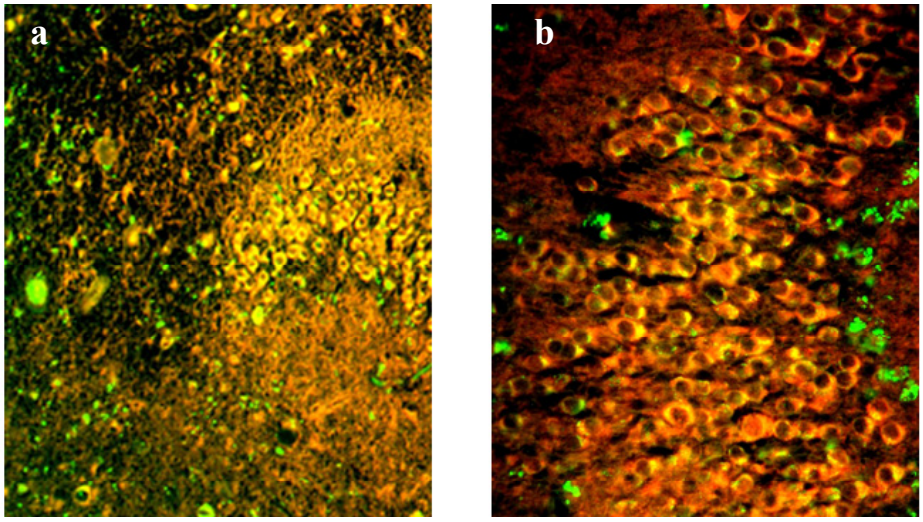


Fig. 2-31 Double labeling for FKBP12 (FITC) and ERAB (Cy5) in AD DG indicates extensive colocalization. Magnification: 100× (a), 200× (b).

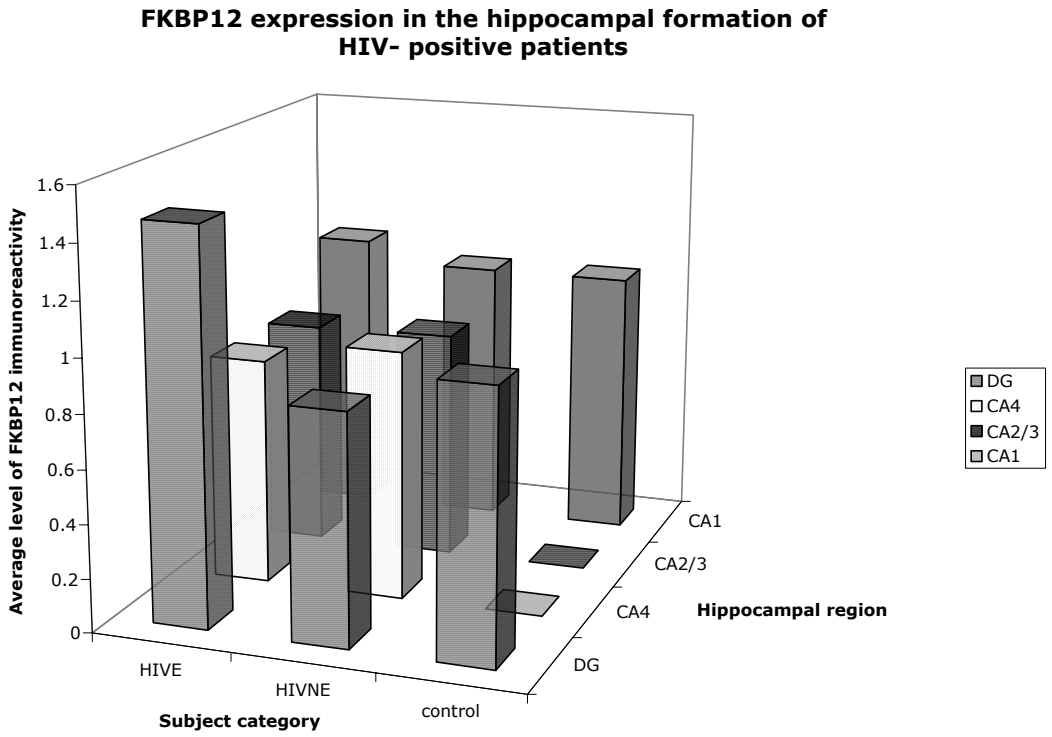


Fig. 2- 32 FKBP12 immunoreactivity levels in hippocampal regions of HIV- positive patients and controls.

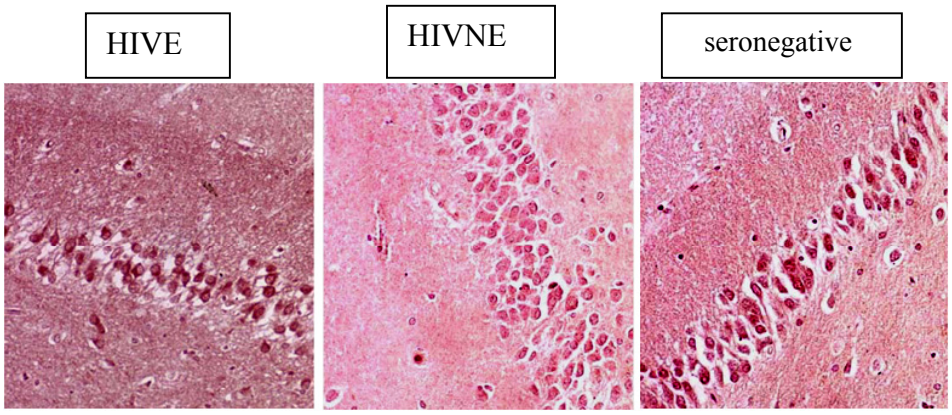


Fig. 2- 33 FKBP12 immunostaining in the DG of HIV- positive patients and controls. Nova- red chromogen and haematoxylin counterstaining. Magnification: 200×.

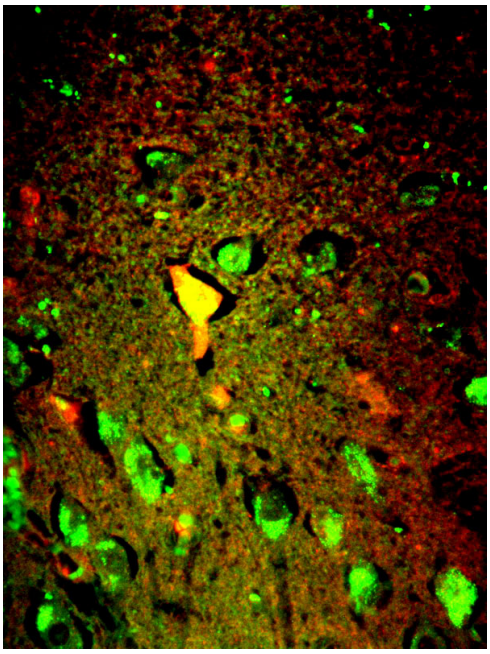


Fig. 2-34 Limited colocalization of FKBP12 (FITC) with ERAB (Cy5) in the HIVE hippocampus. Magnification: 400×.

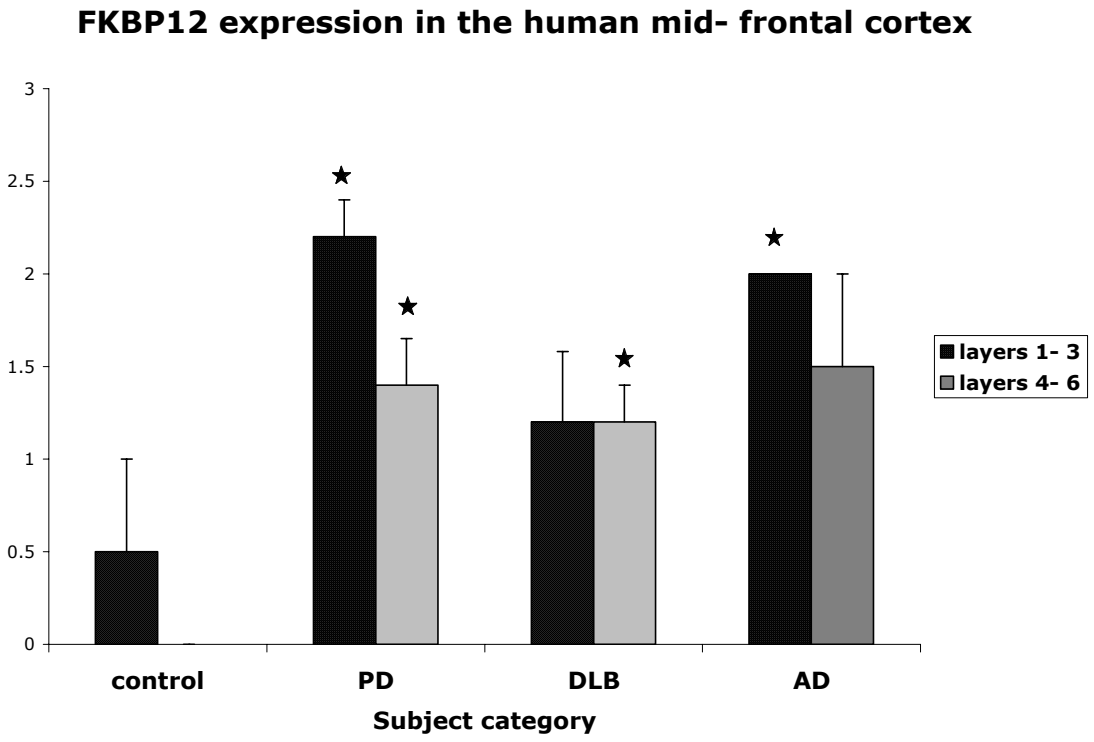
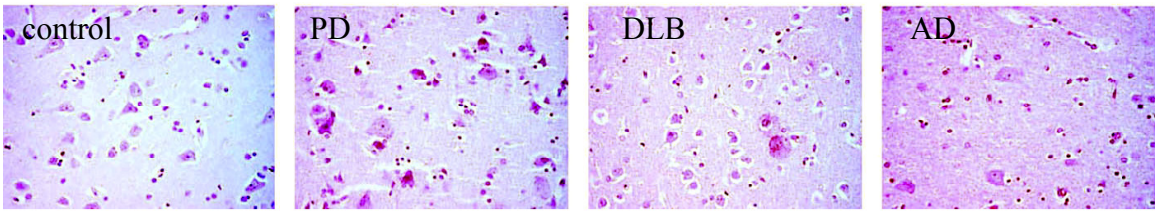


Fig. 2- 35 FKBP12 immunoreactivity levels in mid- frontal cortex of neurodegenerative diseases patients and controls. $^{*}p < 0.05$

A)



B)

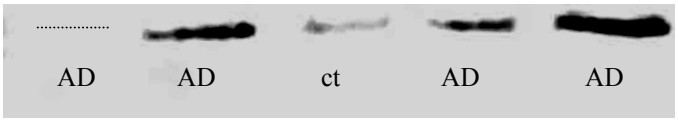


Fig. 2- 36 A: FKBP12 immunostaining in the mid- frontal cortex of neurodegenerative diseases, patients and controls (magnification: 200×) B: Western blot detection of FKBP12 protein in the frontal cortex of AD cases and normal control.

2.3.5. FKBP12 expression in the mid- frontal cortex

Normal subjects

In the frontal cortex of normal subjects, FKBP12 expression is low in the gray matter and undetectable in the white matter (Fig. 2-35, 2-36) .

PD patients

PD cases, although devoid of "classical" markers of cortical pathology, display significantly increased FKBP12 immunoreactivity in the superficial and deep GM (mainly in layers II, III and V; Fig. 2-35, 2-36). Spherical aggregates, positive for FKBP12, are observed occasionally (Fig. 2-37 (c), (d)). FKBP12 often colocalizes with the neuronal (somatic and dendritic) marker MAP-2 (results not shown) and axonal neurofilament immunoreactivity (Fig. 2-37 a- d). Colocalization with NF in the perinuclear area is evident (Fig. 2-37 (b)-(d)). Interestingly, occasional plaques contain an FKBP12- positive core, as well as immunoreactive peripheral cells and processes (Fig. 2-37 (c)). Many cells do not contain significant amounts of this IP, or do so in the soma only (Fig. 2-37 (d)). However, even in this case, cells are surrounded by FKBP12- positive processes and neighboring positive spherical bodies.

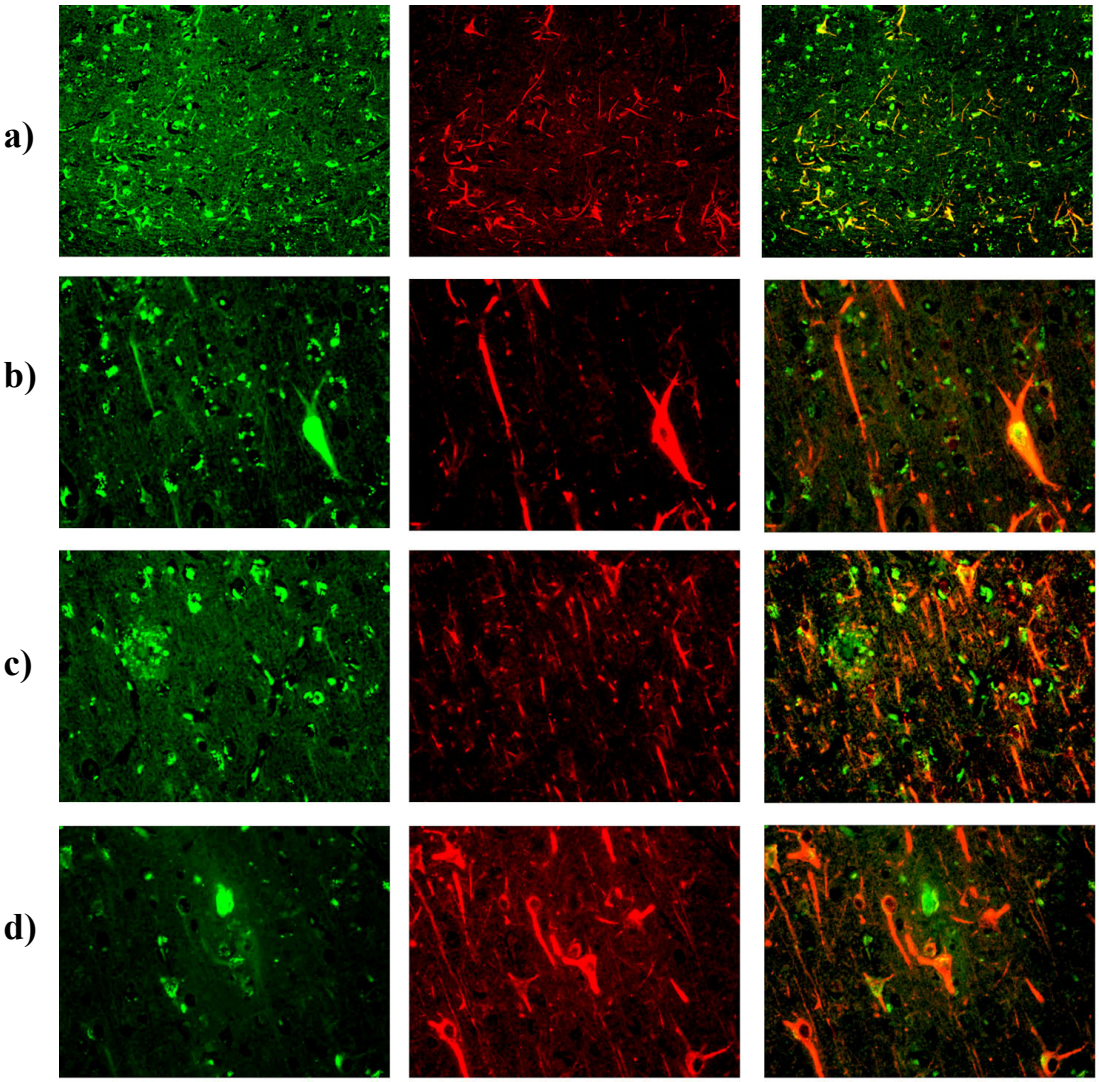


Fig. 2- 37 Double labeling for FKBP12 (FITC) and NF (Cy3) in PD (a- d). Occasional plaques contain an FKBP12- positive core and immunoreactive peripheral cells and processes (c). Many cells do not contain significant amounts of this IP, or do so in the soma only (d). Magnification: 200× (a, c); 400× (b, d)

DLB patients

DLB cases are characterized by significantly elevated FKBP12 immunoreactivity in the deep layers of GM, as well as enhanced immunoreactivity in superficial layers (Fig. 2-35, 2-36). The lowest IP levels are present in the DLB case with well preserved cortical neuronal population and no neuritic plaques. FKBP12 often colocalizes with alpha- synuclein in Lewy bodies and Lewy neurites (results not shown).

AD patients

AD cases are characterized by significantly increased FKBP12 immunoreactivity, especially in superficial cortical layers (Fig. 2-35, 2-36). Western blots (Fig. 2-36 B) indicate that the amount of FKBP12 protein is increased in three of the AD cases and decreased in the one with marked cerebral atrophy and neuronal loss. Immunopositive neuronal cell bodies, processes and neuropil are present predominantly in layers II and III, but also in deeper layers. Higher FKBP12 levels are observed in dystrophic neuronal processes of AD patients, as well as intense immunostaining of blood vessel walls and globular aggregates. Both diffuse IP and IP-containing inclusions are evident**.** Glial immunoreactivity is more frequently encountered than in PD and DLB frontal cortices. The lowest FKBP12 levels are present in the AD case with pronounced cortical atrophy and widespread neuronal loss.

FKBP12 often colocalizes with the neuronal (somatic and dendritic) marker MAP-2, especially in cell bodies, but also in apical dendrites (Fig**.** 2-38). Frequent IP- positive, MAP-2-negative aggregates and perinuclear inclusions are present in the superficial and deep cortical layers (Fig. 2-38 (a)). Conversely, some MAP – labeled cells are not immunoreactive for FKBP12 (Fig. 2-38 (b)). Fig. 3-38 illustrates the presence of numerous strongly FKBP12-positive plaques in the AD mid- frontal cortex. In regions where FKBP12- positive plaques and spherical aggregates are present, axons often bear low amounts of this IP. Intense immunostaining of blood vessel walls is illustrated in Fig. 2-39 (b) and (c). Axons bearing high FKBP12 amounts are observed in sections labeled for NF (Fig. 2-39 (a), (b)). Numerous cell bodies are positive for both markers (Fig. 2-39 (a)- (c)). Thickened blood vessel walls are strongly FKBP12- positive.

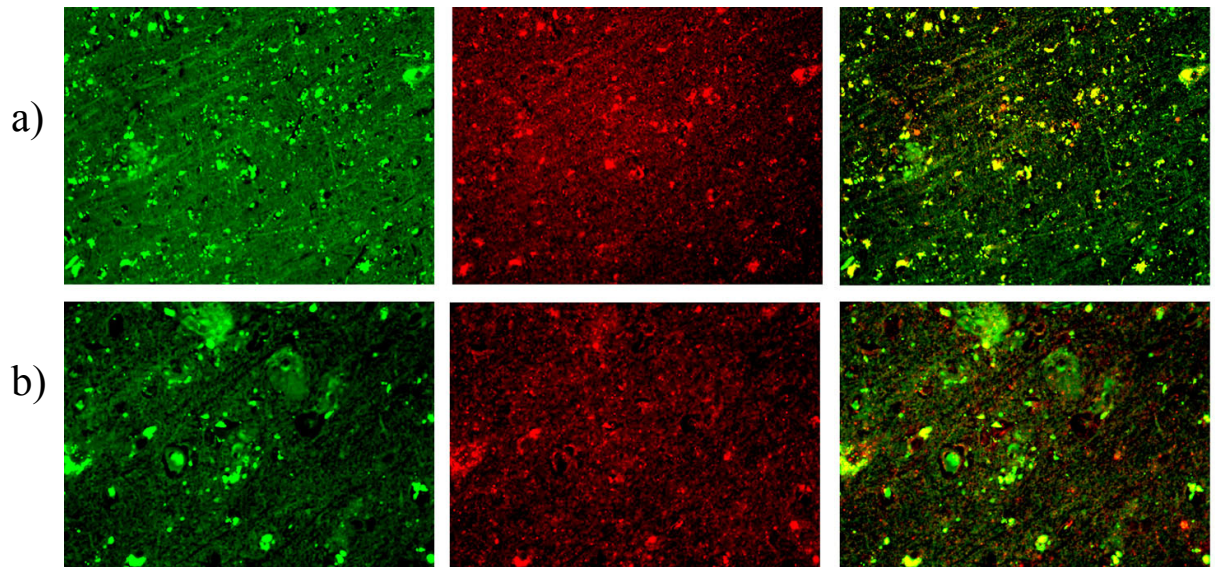


Fig. 2- 38 FKBP12 (FITC) and MAP-2 (Cy3) double labeling in AD frontal cortex. Magnification: 200×.

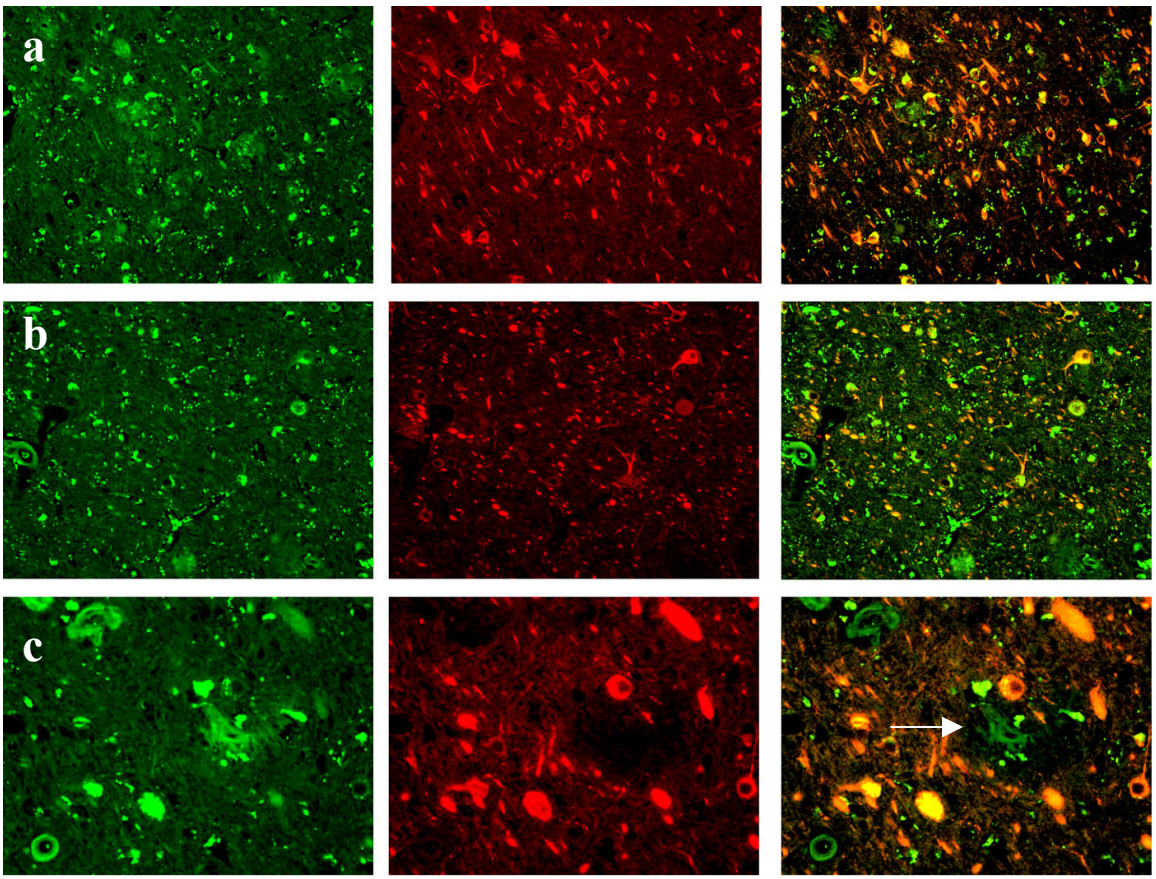


Fig. 2- 39 FKBP12 (FITC) and NF (Cy3) double labeling (a- deep layer; b- superficial layer). Thickened blood vessel walls are strongly FKBP12- positive (arrow in c). Magnification: 200× (a, b), 400× (c).

(2-39 (c)). Interestingly, numerous extracellular areas of increased FKBP12 immunoreactivity and minimal neuronal markers, many with "cotton ball" appearance, appear to be developing plaques (comparison illustrated in Fig. 2-39 (c) vs. 2-38 (b)).

FKBP12 is present in most NFT (flame- shaped or globular) in superficial and deep layers of frontal cortex (Fig. 2- 40). Numerous FKBP12 positive, tau- negative aggregates are present throughout the AD cortical layers. Some plaques have minimal tau reactivity (Fig. 2-40 (a)), despite the strong FKBP12 immunostaining. Tau- positive plaques include a large FKBP12-

positive core and an FKBP12- positive periphery that also stains with the antibody against NFT (dystrophic neurites). Fig. 2-40 (c) illustrates these types of plaques and the extent of AD pathological changes at a lower magnification. In addition, a distinct type of large, non- cored plaques was identified, which are reminiscent of “cotton wool” plaques. These have a diffuse FKBP12 immunoreactivity and no clearly- defined FKBP12- positive core.

Numerous dystrophic processes are positive for both FKBP12 and NFT, predominantly in the region adjacent to the axolemma.

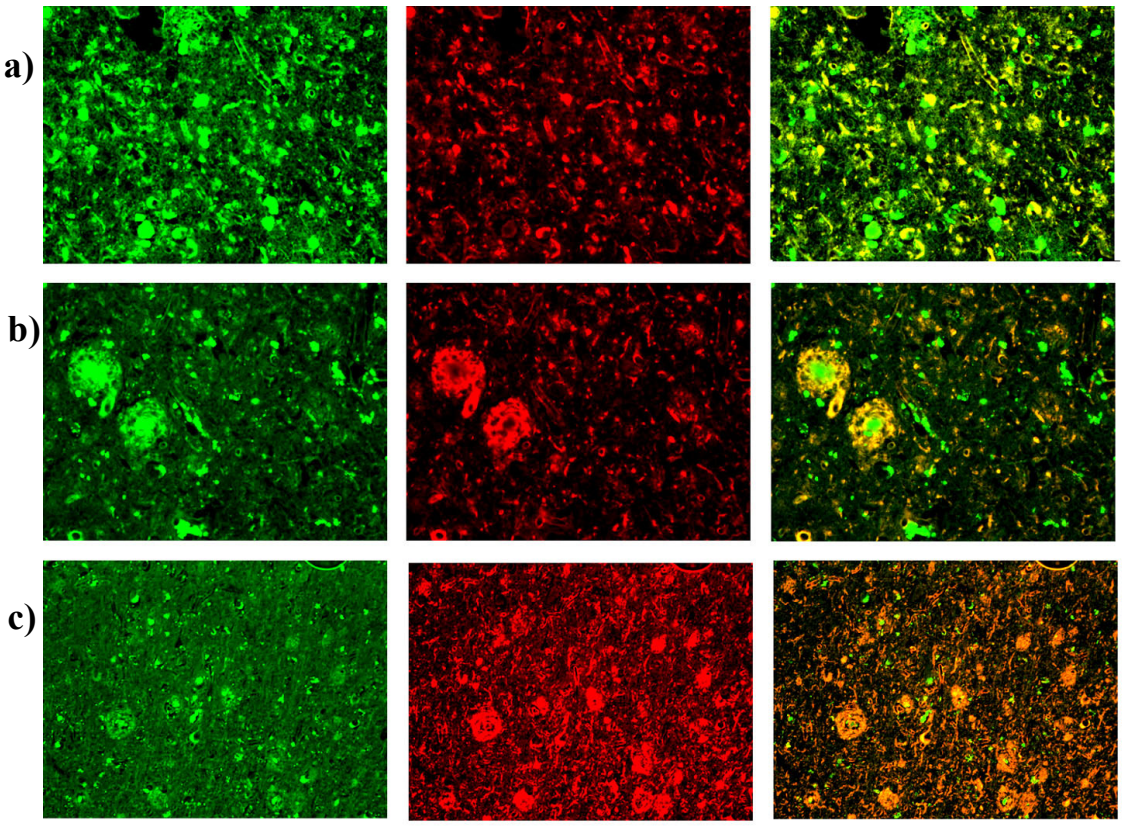


Fig. 2- 40 FKBP12 (FITC) is present in NFT (Cy3) in superficial and deep layers of AD frontal cortex (a). Tau- positive plaques include a large FKBP12- positive core and an FKBP12- positive periphery that also stains with the antibody against NFT (dystrophic neurites) (b). (c) illustrates these types of plaques and the extent of AD cortical pathology. Magnification: 200× (a, b); 400× (c).

HIV- positive patients

Overall, HIV-positive patients display decreased FKBP12 levels in the neurons of cortical GM compared to seronegative controls. (Fig. 2-41, 2-42). Dense immunostaining is present in distrophic cortical neurites. Severe encephalitis is associated with markedly decreased levels in the grey matter.

Increased expression in the WM, especially in non- encephalitic subjects, is observed, mainly due to diffuse extracellular immunoreactivity. In HIVE subjects, FKBP12 is present in astrocytes and microglia, as well as in macrophages (Fig. 2-44). A high percentage of HAM56-positive cells, i.e. rod- shaped, activated cells of the macrophage- microglia lineage, are strongly positive for FKBP12 (Fig. 2-44 (a)). Perivascular macrophages, probably hematogenous, are intensely FKBP12- immunoreactive (b).

Colocalization of FKBP12 with ERAB, albeit not as extensive as in AD, is noted in the superficial and deep cortical layers (Fig.2- 43). This can be due to a lower amount of ER amyloid binding protein or to an intracellular segregation of the two proteins (arrow). The two proteins colocalize, as expected, in cell bodies (b, dashed arrow) and not in axons (devoid of ER) (a, asterisk).

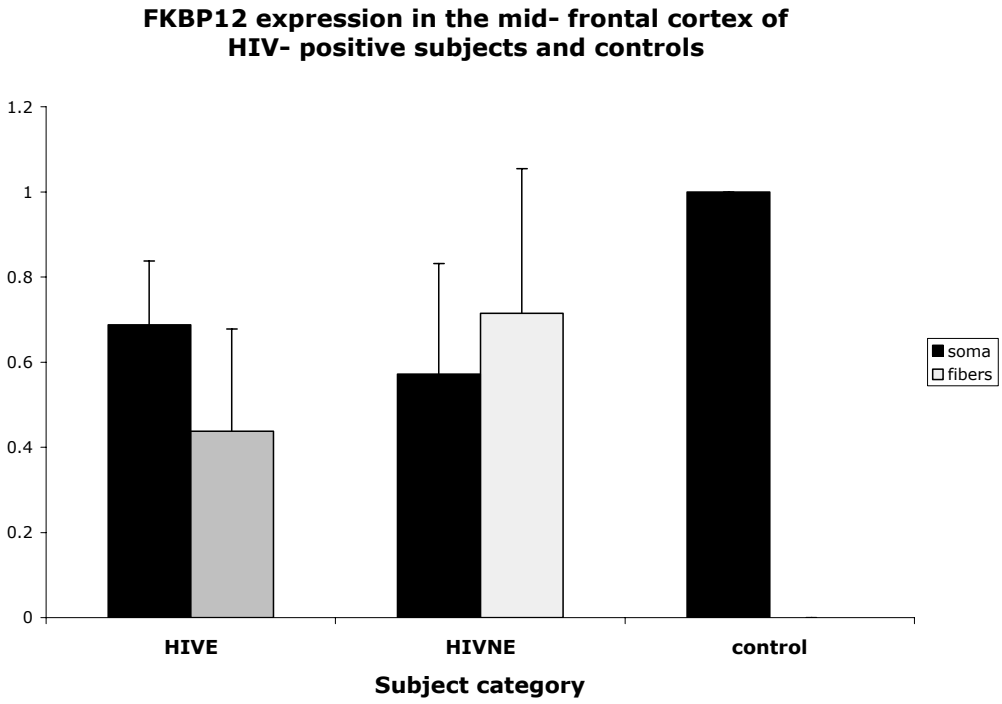


Fig. 2-41 FKBP12 immunoreactivity levels in the frontal cortex of HIV- positive patients and controls.

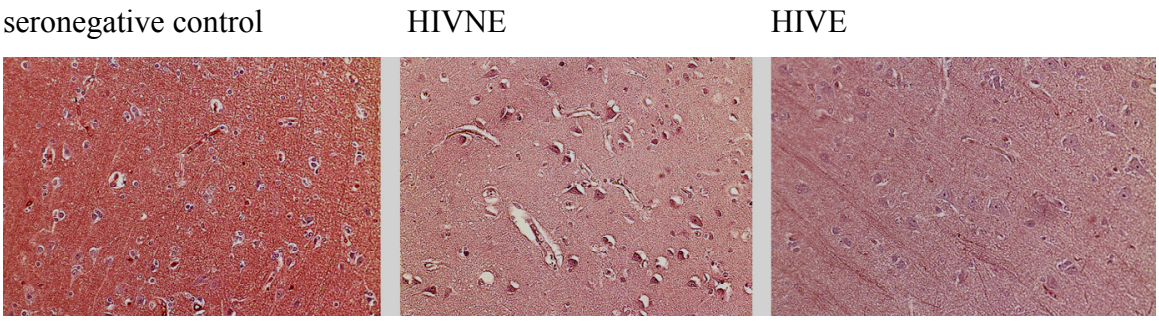


Fig. 2- 42 FKBP12 immunostaining in the frontal cortex of HIV- positive patients and controls. Nova- Red chromogen and haematoxylin counterstaining. Magnification: 200×

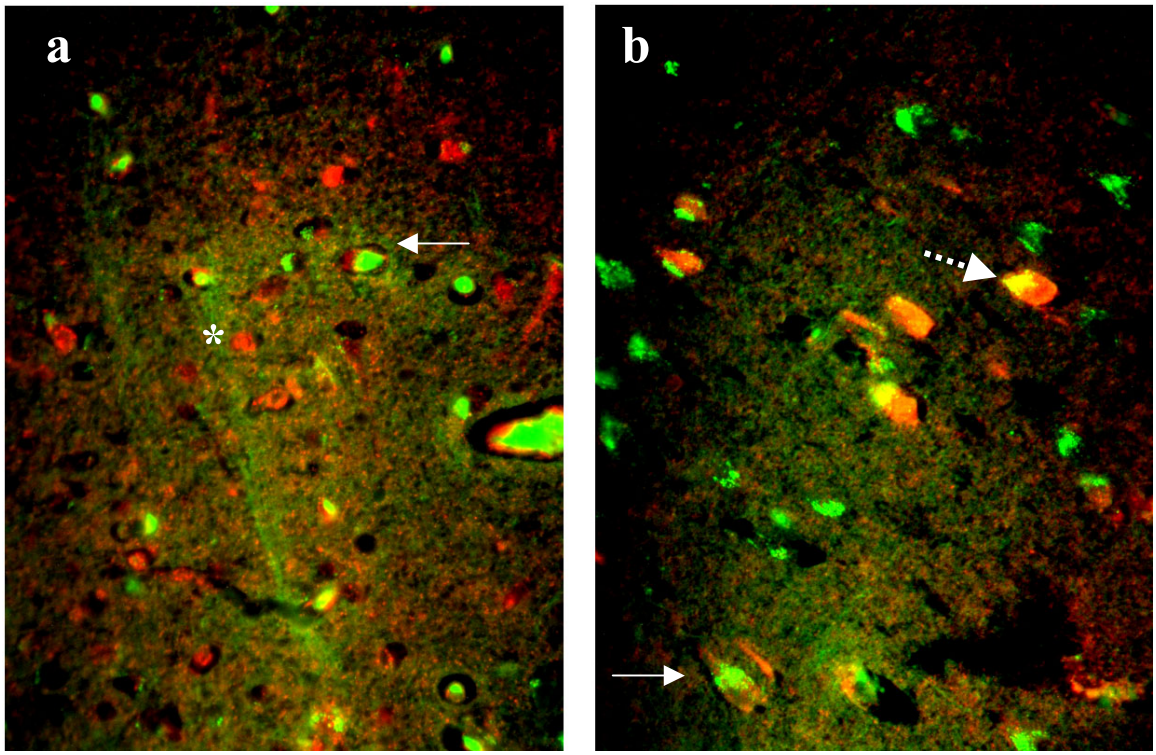


Fig. 2-43 Colocalization of FKBP12 (FITC) with ERAB (Cy5) in the superficial and deep cortical layers of HIVE patient. Magnification: 400×.

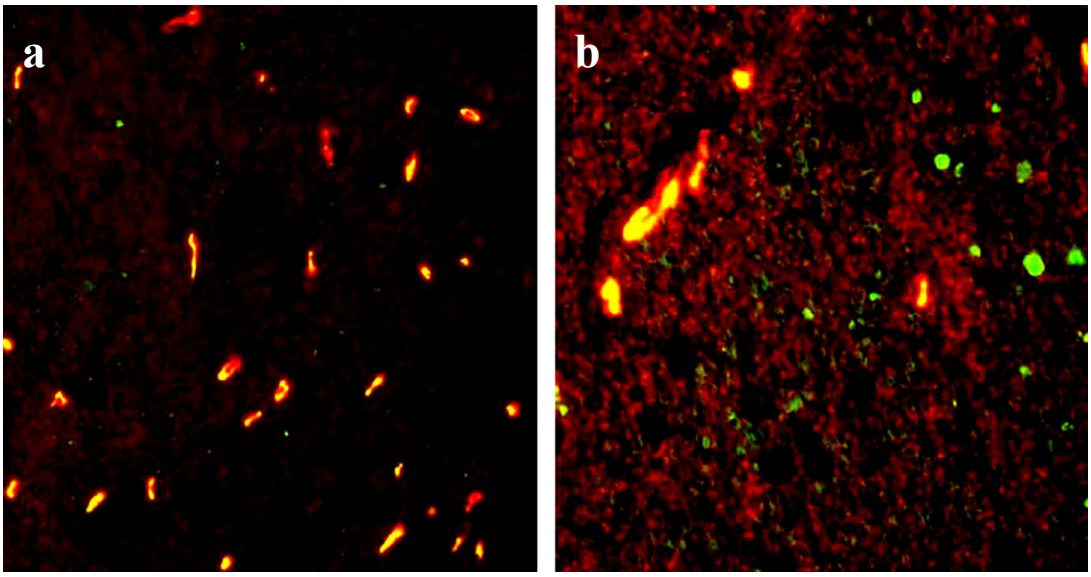


Fig. 2- 44 FKBP12 (FITC) is present in microglia/ macrophages (HAM56- positive, Cy5). Magnification: 200× (a), 400× (b).

2.4. Summary and discussion

This study shows for the first time that the immunophilin FKBP12, a peptidyl- prolyl *cis-trans* isomerase and a receptor for immunosuppressive drugs, is present in the human brain. Among the regions analyzed, high levels of FKBP12 are found in the substantia nigra- deep gray matter axis. This is consistent with previous autoradiographic studies in rodents (Steiner et al., 1992). Interestingly, the post- hoc comparison of average IP levels in the two groups of normal subjects (average age 72 and 40 years, respectively) suggests that FKBP12 levels vary with age. For example, higher IP levels are observed in dopaminergic cell bodies, putamen and pallidum of younger subjects, coupled with lower levels in pars reticularis. Significant expression is also present in the hippocampus, especially in the DG of younger subjects. The mid- frontal cortex of normal aging human brain has low, often undetectable amounts of FKBP12. Higher average cortical levels are present in younger normal subjects.

Under normal conditions, FKBP12 expression is exclusively neuronal. The IP is present in cell bodies, processes and neuropil. It often exhibits a predominantly perinuclear, polarized distribution, suggesting the association with an intracellular organelle, possibly the endoplasmic reticulum. The expression of the immunophilin FKBP12 is altered in the mesencephalon, deep gray matter, hippocampus and mid- frontal cortex of patients with neurodegenerative diseases, including HIV- induced degeneration. In these circumstances, the IP colocalizes with markers of pathology. Disease- specific and region- specific changes in FKBP12 expression are evident.

2.4.1. FKBP12 changes in the substantia nigra

Parkinson's disease

The SNc of PD patients contains lower levels of FKBP12 in fibers of the lateral part and also in cell bodies. In the medial part, levels are similar to controls. Numerous neurons in the SNc bear IP- positive inclusions. FKBP12 is present in distinct non- laminated inclusions and in extracellular globular deposits. In the SNr of PD patients, there is a tendency towards lower FKBP12 expression in the medial part and an increase in the lateral sector. The labeling pattern and the colocalization with synaptophysin and NF suggest that the immunophilin is contained in plexiform terminals of fibers projecting to SNi and abnormal, degenerating axons. In PD (as in DLB) cases, FKBP12 often co-localizes with a- synuclein- positive LB and Lewy threads, although numerous synuclein- negative and FKBP12- negative inclusions are present.

Dementia with Lewy bodies

In DLB patients, unlike in PD, FKBP12 immunoreactivity is enhanced in fibers of medial part of the SNc and the entire SNr, with a statistically significant increase in the lateral SNr. IP levels tend to decrease in cell bodies and fibers of lateral SNc, which is a common DLB and PD feature. Numerous neurons have IP- positive neuronal inclusions. FKBP12 is present in distinct non- laminated inclusions and in extracellular globular deposits. Laser confocal micrographs indicate extensive (stronger than in PD) colocalization of FKBP12 and α- synuclein inside LB and Lewy threads. Intracellular FKBP12 immunostaining is observed mainly around the nucleus. Z- sectioning indicates the punctate perinuclear staining is indeed intracytoplasmic, as opposed to belonging to axo- somatic synapses. In dystrophic neurites, the IP colocalizes with α- synuclein mostly in the region adjacent to the axolemma.

Alzheimer's disease

The increase in FKBP12 levels in AD patients is observed predominantly across the ventral part of the SN, especially medial part of the SNc and the entire pars reticularis. This pattern is similar to the one observed in DLB. FKBP12 levels tend to be lower in fibers of lateral pars compacta (as they are in PD and DLB patients). This could indicate a common underlying pathologic process.

Here, too, this IP is contained in plexiform terminals of fibers projecting to SNi and abnormal, degenerating processes. FKBP12 is rarely located in MAP-2 positive processes, i.e. dendrites and occasionally in DAT- positive processes. Most often, FKBP12 is present in synaptophysin- and NF- positive fibers, which indicates its axonal localization.

AD cases with increased FKBP12 levels in the mesencephalon and deep gray matter also exhibit FKBP12- positive nigral aggregates. In these patients, the intracellular IP immunostaining appears diffuse in the medial SNi (as opposed to perinuclear in DRN), while in normal subjects it exhibits a predominantly perinuclear, polarized distribution in both regions. This might be explained by the colocalization of FKBP12 with ERAB (see below), which undergoes a redistribution from the ER to the cytoplasmic membrane when cell is exposed to beta amyloid. Indeed, the IP deposits are sometimes found to line the cell membrane.

HIV- positive patients

Higher levels of FKBP12 are present in the nigral region of HIVE patients compared to non- encephalitic patients and normal controls. HIV- positive patients without overt

encephalopathy have higher FKBP12 levels than normal controls, which could indicate a degenerative process under development at the time of death.

General interpretation and anatomical correlations

Markedly increased FKBP12 levels are noted in the nigral area in DLB and AD cases. In PD, overall FKBP12 expression is decreased (with the exception of lateral SNr). This is in part attributable to the general loss of cell bodies and processes in the area. The relative preservation of the nigral cell population in AD and some DLB patients indicate that high FKBP12 levels can be protective. No increase in this IP in PD nigral neurons can be therefore viewed as promoting degeneration. Interestingly, the increase in FKBP12 in fibers of medial SNc and SNr is a common feature of DLB and AD and might indicate a response to pathological changes in the striatum, as well as a protective response that leads to cell preservation in medial SNc (absent in PD). PD and DLB, characterized by the degeneration of cells in SNc, have in common the decreased IP level in nigral cell bodies. In contrast, a negligible decrease is present in AD. This indicates that FKBP12 (perhaps an inability to upregulate its levels) might play a role in the degenerative process underlying both conditions. In HIVE patients, with subcortical dementia evolving over a relatively short period of time, the IP levels in SN cell bodies are increased compared with non- encephalitic and seronegative controls. This would suggest that the elevated cellular FKBP12 levels occur early in the history of dopaminergic system damage and might serve, at least initially, a protective role. In all four conditions, in various degrees, FKBP12 levels are decreased in processes of lateral pars compacta and increased in lateral pars reticularis. The low FKBP12 levels in the lateral SNc of PD (and probably DLB) patients can be related to the natural history of the disease. The ventrolateral SN neurons and their axons projecting to the dorsal putamen are affected early and severely, therefore in this SN region there is a lower amount of FKBP12. In PD, the increase in FKBP12 is concentrated in the lateral SNr. Afferent projections to the SN arise mainly from the striatum (caudate and putamen) and the external part of globus pallidus. (This part of the globus pallidus exhibits the highest FKBP12 levels in PD patients, although IP levels are elevated in the other groups as well.) Afferent fibers from the dorsal part of the putamen project somatotopically to the lateral parts of the substantia nigra, which relates to the motor circuit system ([30]). These projections are traversing lateral pars reticularis. It is tempting to speculate that the high IP levels here originate in the dorsal putamen and serve to enhance axonal transport in the target SN area. Medial SN receives projections from

the medium- sized neurons of the ventral putamen mainly. In PD patients, the ventral putamen appears to be characterized by the highest FKBP12 levels. The high IP levels in the ventral putamen, however, do not translate in high medial SNr levels. Given the high IP content in this putaminal region, it is possible that it stays in the soma of medium- size neurons and does not reach axonal terminals. FKBP12 levels in medial SN fibers (SNc, SNr) and striatal cell bodies are, however, elevated in DLB and AD, indicating a possible relocation of the IP to axonal terminals.

Retrograde tracing studies in primates revealed that medial SNc contains clusters of neurons projecting both to the putamen (innermost part of SN) and to the caudate. The ventral tier of pars compacta projects to dorsal caudate (striosomes), which displays increased IP levels in PD, but not in DLB. Again, it is possible that a polarization of FKBP12 content occurs through translocation from medial SN to striatum in PD (leading to elevated levels here, see below) and through concentration in cell bodies in DLB (leading to relatively high levels in medial SNc).

. Relatively high levels of FKBP12 are noted in nigral neuronal cell bodies of AD, DLB and HIV patients. In PD patients, in addition to FKBP12- positive neuronal inclusions, there are extracellular globular deposits immunoreactive for FKBP12. AD cases can also exhibit FKBP12-positive intracellular nigral deposits with a diffuse appearance, which contrasts with the predominant perinuclear distribution observed in other regions (e.g., dorsal raphe nucleus) and in normal subjects. In some instances, the IP deposits appear to be associated with the extracellular membrane. Therefore, the increase in IP may be associated with a translocation between cellular compartments, e.g. endoplasmic reticulum, cytoplasm and cell membrane.

The increase in nigral FKBP12 in AD cases correlates with the presence of plaques, tangles and neuropil threads in various regions of the brain and reflects a possible striatal dysfunction. Although the AD cases in this study did not display the classic nigral markers of pathology, the distribution of FKBP12 was studied as a control for PD and also because the occurrence of unconspicuous pathological changes in this region is possible. For example, it was found that the mean area and areal fraction of neuromelanin is lower in Alzheimer's disease (without LB) than controls, but the number and size of the neuronal cell bodies, nuclei and nucleoli does not differ between the two groups (Reyes et al., 2003). The decreased amount of neuromelanin in nigral neuronal cell bodies could result from neurofibrillary degeneration,

retrograde degeneration caused by diffuse plaques, beta amyloid- induced damage of nigral dopaminergic terminals in the striatum and possibly transneuronal degeneration from damage of cell bodies or dendrites of nigral neurons by their plaque- and tangle- laden striatal, neocortical and other subcortical nigral connections. The above types of degeneration could lower the rate of dopamine metabolism and the formation of neuromelanin, one of its by-products. The increased FKBP12 levels in medial pars compacta and entire pars reticularis of AD patients might represent an attempt to compensate for axonal dysfunction occurring in this disease. Likewise, damage to DA neurons is thought to occur in early stages of HIVE. Koutsilieri et al. (2002) found that, in SIV-infected monkeys, the DA content is reduced by 44%. Patients with HIV have decreased levels of cerebrospinal fluid DA, and patients with HAD have a reduction of the DA metabolite homovanillic acid but a relative preservation of other neurotransmitters, suggesting a loss of DA neurons (Lopez et al., 1999). In this case, too, FKBP increase can represent a compensatory response.

FKBP12 appears to be distributed in the plexiform terminals of striatal, pallidal or cortical projections to SNi and increased in abnormal, degenerating nigral processes. Furthermore, double- labeling immunofluorescence studies indicate that FKBP12 is often present in synaptophysin- and NF- positive axonal processes. Medium sized neurons in the striatum of PD patients are strongly FKBP12- positive (see below). FKBP12 often co-localizes with α-synuclein- positive LB and Lewy threads. Overall, these results indicate the presence of this FKBP in the presynaptic compartment.

2.4.2. FKBP12 changes in the deep gray matter

Overall, neurodegenerative processes affecting primarily the dopaminergic system (i.e., PD, DLB and HIVE) are associated with significantly increased FKBP12 levels in the globus pallidus, especially in fiber tracts. DLB and AD patients, in which dementia is a salient feature, show increased FKBP12 levels mainly in pars interna. PD patients shown the strongest increase in FKBP12 in fibers of GPe, therefore the amount of IP in this region could serve as a marker that distinguishes PD from DLB. This part of the globus pallidus projects to the substantia nigra, where FKBP12 levels are also increased. The connections between nigra and pallidus play an important role in the function of the basal ganglia circuitry and their plasticity has been

suggested recently. Positron emission tomography studies (Whone et al., 2003) demonstrated compensatory changes in PD in a less studied midbrain dopamine projection to the basal ganglia, the nigropallidal projection to the internal segment of the GP. Increased (18) F-Dopa uptake in the GPi is seen in early PD, which then is lost in advanced PD. Early PD cases show an absence of significant clinical progression in the face of a continuing loss of nigrostriatal projections. This could indicate a compensatory neuronal plasticity that is likely to involve the nigropallidal dopamine pathway to the GPi. Enhanced function of the dopamine projection to the GPi in the initial stages can serve to maintain a more normal pattern of pallidal output to ventral thalamus and motor cortex in early PD, whereas loss of this adaptive pathway in advanced disease may contribute to the progression of the disease. In the GP, increased FKBP12 might be part of the restructuring that occurs in response to injury and persists at later stages. GPe neurons synapse not only on the subthalamic nucleus neurons, but also on the two major output structures of the basal ganglia, the SNr and the GPi. The increased FKBP12 in GPe at later stages might be part of a long- term plasticity phenomenon ultimately involving GPe and its projections to SN. The late stage analyzed in our study could explain the relatively lower IP levels in GPi.

In addition, the ventral putamen in PD patients has high amounts of FKBP12. This, coupled with observations that FKBP12 colocalizes with synaptophysin in the SNi, suggest that FKBP12 localizes to the presynaptic compartment and is distributed in the plexiform terminals of striatal projections to SN. Less colocalization of FKBP12 with synaptophysin is evident in some patients, especially in AD. This may be due to either decreased synaptophysin levels or to a relocation of the IP protein towards fiber terminals in other regions (e.g., SN). Since the synaptic injury is an early event during AD progression (Masliah et al., 2001), FKBP12 predominance in neuronal perikarya, rather than fiber tracts, in these patients might reflect a loss of IP associated with synaptic vesicle proteins (e.g., in cortico- striatal fibers). Not all FKBP12- positive fibers in the striatum and especially pallidum are synaptophysin- positive, therefore the IP is also present in the postsynaptic compartment. Since the ventrolateral SN neurons and their axons projecting to the dorsal putamen are affected early in the disease and display a lower amount of FKBP12 chaperone, as does the dorsal putamen, it is possible that the increase in IP levels plays a role in maintaining neuronal homeostasis in the medial SN. The increased levels of the chaperone FKBP12 in striatal neurons and their projections to the substantia nigra might indicate a role for this immunophilin in anterograde and retrograde protein transport (see below).

Changes in FKBP12 in HIV patients are particularly intriguing. Symptoms of HIV dementia include psychomotor slowing, apathy and motor disorders similar to the bradykinesia and postural and gait abnormalities observed in late Parkinson's disease. The core symptoms of HIV- associated dementia (HAD) are generally similar to those seen in patients with frontal-striatal dysfunction, the "subcortical dementias" (e.g., Parkinson disease, Huntington disease, progressive supranuclear palsy) (Lopez et al., 1999). Consequently, HIV has been discussed during the last few years as an additional cause for parkinsonism. HIV infection selectively targets the basal ganglia, resulting in loss of dopaminergic neurons (Nath et al., 2000). Damage to DA neurons appears to occur in early stages of the disease. Koutsilieri and collaborators (2002) found that in SIV-infected monkeys the DA content is reduced by 44% within as few as two months of infection. Patients with HIV have decreased levels of cerebrospinal fluid DA, and patients with HAD have a reduction of the DA metabolite homovanillic acid but a relative preservation of other neurotransmitters, suggesting a loss of DA neurons (Lopez et al., 1999). Neuropathologic examinations have shown neuronal loss in the globus pallidus, which is less severe in the neocortex. The markedly increased FKBP12 levels in the globus pallidus and the relatively low neocortical levels indicate a potential role for this IP in the pathogenesis of HIV-induced dopaminergic deficit. The causes of this vulnerability of the DA system to HIV infection are unknown. The high content of FKBP12, that binds the V3 loop of gp120, in the basal ganglia and the presence of the IP in neurons, astrocytes, microglia and macrophages can lead to enhanced viral entry and replication in cells of this region. This hypothesis is supported by the recent detection of HIV-1 DNA in microglia/macrophages, astrocytes and neurons isolated from brain tissue with HIV-1 encephalitis by laser capture microdissection (Trillo- Pazos, 2003). The infection in cells other than macrophage/ microglia is, however, likely to be nonproductive. Understanding the mechanism of viral affinity for basal ganglia is important in developing neuroprotective agents in the treatment of HIV encephalitis and in designing new therapies for HAD-related psychiatric symptoms.

2.4.3. FKBP12 changes in the amygdala

In AD, the primary transmitter deficit is cholinergic and the amygdala receives a major cholinergic projection from the nucleus basalis of Meynert, which may play an important role in the retention of affective conditioning and memory consolidation. In addition to hippocampus and

neocortex, in AD the amyloid beta peptide is deposited in the amygdala. These observations and other (e.g., the amygdaloid atrophy in AD) prompted us to assess FKBP12 expression in this region. Basolateral nucleus of the amygdala receives projections, among others, from the SNc, raphe nuclei, basal forebrain and hippocampal CA1. Low levels of FKBP12 in, for example, hippocampal CA1 could translate in a reduced content of this IP in amygdala projection terminals. They could also reflect a decreased FKBP content in the nucleus basalis- amygdala pathway. In turn, basolateral amygdala sends projections to the caudate, putamen and central amygdala, which can contribute to the overall FKBP12 content in these areas. PHA-L tracer injections into the caudal part of the BLA produced a dense labeling of fibers in the medial surface of the frontal cortex (Kita and Kitai, 1990). In most of the cortical regions, labeled fibers were predominantly distributed in two bands: one in the deep part of layers I and II and the other, heavier band, in layers V and VI. These layers contain enhanced amounts of FKBP12 in AD, which contrast with the low levels present in the amygdala. A relocation of the protein to the axonal terminals can be assumed.

Due to the heterogeneity and multiple intrinsic connections of amygdaloid complex nuclei, a detailed analysis of IP expression in each of them is needed.

FKBP12 runs as a band with an apparent molecular weight of 13- 14 kD in the two AD patients with increased IP levels, which indicates that it might be complexed with another protein ("natural ligand") or modified. The increase in apparent molecular weight could be due to the presence of post-translational modifications such as glycosylation or phosphorylation. Furthermore, proline-proline sequences may alter the protein structure resulting in an altered migration on SDS-PAGE and increasing its apparent size.

The difference between FKBP12 patterns of expression in immunohistochemical versus western blotting studies can be attributed to the fact that blots use extracts from blocks containing heterogeneous subregions of the amygdaloid complex. The observed FKBP12 amount in blot bands does not reflect the differential expression of the protein in fibers and cell bodies of various areas. The analyzed immunostained sections include mainly the corticomedial region of the amygdala and represent a minimal part of the anteroposterior extent of the amygdala complex. In addition, since FKBP12 was shown to be released from mast cells and regulate Ca^{++} signaling in neutrophils, it is possible that it has cytokine- like actions in brain tissue, too. Therefore, the extracellular FKBP12 might be a confounding factor. An intriguing possibility is that, in fact,

high concentrations of FKBP12 are present and released extracellularly in AD, which leads to a cellular depletion (evidenced by histological scoring).

2.4.4. FKBP12 changes in the hippocampal formation

All AD patients exhibit elevated FKBP12 levels in the GM of DG, as well as GM and fibers of CA4/ CA3, but rarely in neurons of subicular and entorhinal areas. From this point of view, AD patients differ from DLB ones, which renders FKBP12 useful in distinguishing types of dementia and related changes in the entorhinal- hippocampal circuitry. IP levels in fibers of DG and in the CA1 region are either similar to control subjects or reduced. Here, there is little colocalization with NF in axonal processes. The low FKBP12 level in DG fibers, in addition to the low entorhinal levels, suggests that, in AD (unlike in DLB), the perforant pathway has reduced FKBP12 levels. CA1 levels might be reduced due to the overall extensive neuronal death occurring in this region, or because of reduced IP transport in the Schaffer collaterals arising from CA3.

FKBP12- positive aggregates and neuritic processes are found in the DG hilus, CA4 subfield and pyramidal cell layer of CA3 area, as well as occasional nuclear immunopositivity. Low or absent staining in fibers of CA1 area is correlated with the presence of severe cell loss and numerous NFT, neuritic threads in this region.

Many NFT in the hippocampus of AD patients are positive for FKBP12. The IP is also strongly expressed in the core of mature NP, as well as in the surrounding radiating cells (microglia or astrocytes), and tau- positive neurites. FKBP12 is abundant in flame- shaped NFT of various hippocampal areas.

In AD patients, amyloid angiopathy leads to thick- walled arterioles that are consistently and strongly positive for FKBP12. In addition to the presence of FKBP12 in the amyloid core of plaques, this suggested the association of this chaperone protein with amyloid aggregates. Indeed, double labeling for FKBP12 and ERAB indicate that FKBP12 co- localizes with amyloid. The colocalization of FKBP12 and the ER protein ERAB is extensive, with the two markers associating in cell bodies and possibly dendrites of DG. FKBP12- positive, ERAB- negative aggregates are present. The two markers also colocalize in the wall of blood vessels or perivascular space. This is consistent with the present view on the pathogenesis of cerebral

amyloid angiopathy (CAA). Observations on human brains suggest that peptides such as amyloid beta are eliminated along the peri-arterial interstitial fluid drainage pathways that are effectively the lymphatics of the brain. In CAA, amyloid beta becomes entrapped in drainage pathways in the walls of cerebral arteries, reflecting a failure of elimination from the brain (for a review, see Weller and Nicoll, 2003). One consequence of failure in clearance of amyloid beta is its accumulation, associated with cognitive decline in AD. Replacement of vascular smooth muscle cells by amyloid beta occurs in severe CAA with weakening of artery walls and increased risk of vessel rupture and intracerebral hemorrhage. The increase in FKBP in the arterial wall and perivascular space of AD patients suggests that this IP might represent an attempt to enhance the traffic and clearing of the accumulating peptide.

The hippocampus proper (CA regions) and DG are three- layered. They both have a superficial molecular cell layer (synaptic) and a deep, polymorphic one. The intermediate stratum is the granular cell layer in DG and the pyramidal one for CA (mix of dendrites, axons and interneurons; similar to neocortical layer VI).

The most prominent source of hippocampal afferents is the entorhinal cortex. The entorhinal cortex itself receives almost all types of sensory information. Granule cells in the dentate gyrus receive the main extrinsic input from the entorhinal cortex via the perforant pathway, ending in the outer and middle thirds of the molecular layer. The entorhinal cortex provides projections via layer II neurons to the dentate/hilar region and CA3, and via layer III neurons directly to CA1 and the subiculum. The colocalization of IP with synaptophysin in fibers of the DG hilus area can reflect the presence of this chaperone protein in axons of the perforant pathway. Cells of the entorhinal cortex were found to contain relatively high amounts of FKBP12 in DLB patients, which translates in increased IP in DG terminals. In AD, where entorhinal FKBP12 levels are low, there is also little FKBP12 in fibers of granular cell layer, reflecting the reduced IP in the perforant pathway in this disease. This renders FKBP12 useful in distinguishing types of dementia and related changes in the entorhinal- hippocampal circuitry.

The pyramidal cells send an axon into the fornix, as well as a Schaffer collateral that projects to CA1 part of the hippocampal formation. In AD and DLB, CA1 FKBP12 levels might be reduced due to the overall extensive neuronal death occurring in this region, or because of reduced IP traffic in the Schaffer collaterals arising from CA3. In HIVE, FKBP12 is increased in the CA1 region, as well as in CA3, reflecting an increased IP content in the Schaffer collaterals.

DG granule cells project via mossy fibres to the hippocampal pyramidal cells. The inner molecular layer contains mossy fibers, with a high FKBP12 content in normal, PD, DLB and HIVE subjects. In AD, mossy fibers display reduced IP. In patients, there was an expansion of the inner molecular layer and relative changes in immunoreactivity that resulted in a clearer delineation of the inner and outer molecular layers.

In our study, the inner molecular layer appears to contain both dendrites and axons and is rich in synaptophysin (consistent with its synaptic nature). In the CA, FKBP12 often colocalizes with synaptophysin. However, little or no colocalization of IP with NF along axonal processes is detected. This suggests that the immunophilin is present predominantly in axonal terminals, where it is associated with synaptic vesicles. The high level of FKBP12 in the CA2/3 region of PD and DLB patients might be in part due to a translocation in the mossy fibers from DG cell bodies rich in FKBP12.

Neuronal cell loss and damage to axonal terminals in CA1 of DLB and AD patients is associated with low FKBP12 levels. In HIVE (subcortical dementia), however, IP content is slightly elevated, which might account for the preservation of neuronal population, despite the impaired synaptic function (Anderson et al., 2003).

Decreased FKBP12 levels are present in a patient with severe hippocampal atrophy and cell loss. The very low degree of hippocampal atrophy and the absence of NFT in one case are associated with an absence of FKBP12 alterations. Numerous NFT and NP in CA regions in DLB associate with high FKBP12 levels in neuronal processes. Conversely, patients with no NFT or NP in the hippocampus or without significant hippocampal atrophy have low levels of IP. One explanation may be that the chaperone protein levels are low in the initial phases of the degenerative process, as well as in terminal ones. During the degeneration process, FKBP12 levels are enhanced in order to improve axonal transport and mitigate protein aggregation . In support of this hypothesis, FKBP12 often colocalizes with synaptophysin and a- synuclein. The presence of FKBP12 in the amyloid core of plaques further indicates an association of this chaperone protein with amyloid aggregates. A correlation between FKBP12 levels and pathological hallmarks or disease is therefore apparent.

The significance of the strong FKBP12 association with ERAB remains to be established. The colocalization of FKBP12 and the ER protein ERAB is extensive, with the two markers associating in cell bodies and of DG, as well as in the molecular cell layer. The intracellular

polypeptide ERAB (endoplasmic reticulum- associated binding protein), a member of the short-chain dehydrogenase/reductase (SDR) family, is known to mediate apoptosis in different cell lines and to be a class II hydroxyacyl-CoA dehydrogenase. It binds amyloid- beta and is expressed in normal tissues, but is overexpressed in neurons affected in Alzheimer's disease. ERAB immunoprecipitates with amyloid-beta, and when cell cultures are exposed to amyloid-beta, ERAB inside the cell is rapidly redistributed to the plasma membrane (Yan et al., 1997). This behavior is paralleled by changes in intracellular localization of FKBP12 in AD patients, from a predominantly perinuclear distribution to a diffuse one. The toxic effect of amyloid-beta on these cells is prevented by blocking ERAB and is enhanced by overexpression of ERAB. By interacting with intracellular amyloid beta, ERAB may therefore contribute to the neuronal dysfunction associated with Alzheimer's disease. FKBP12 might play a role in the ERAB-amyloid complex via a potential initial interaction with ERAB at the ER or by virtue of its preexisting association with amyloid.

A study by Frackowiak et al. (2001) found no ERAB in amyloid plaques or vascular amyloid. The neuronal expression of ERAB was not correlated with the severity of amyloid load in neuropil. ERAB was expressed in vascular smooth muscle cells in young and old controls and in amyloid-free blood vessels in AD cases, but little or no ERAB was in smooth muscle cells in arteries with amyloid deposits. Cells that accumulated amyloid beta were those with low expression of ERAB and the proteins did not co-localize. This is a puzzling finding, since in our study ERAB colocalizes extensively with FKBP12, which is enhanced in various types of amyloid deposits, including in vascular amyloid.

Further studies employing amyloid, ERAB and FKBP12 triple labeling and analyzing intracellular localizations need to be performed. One possibility is that different types of interactions between these proteins characterize intra- and extracellular amyloid accumulation. It is also possible that the lack of colocalization of ERAB and amyloid inside the cell in AD is due to ERAB sequestration/ transport/ refolding by FKBP12 or similar proteins (e.g., FKBP13, a putative ER folding factor). Our findings of low FKBP12 levels in brain tissue and blood vessels of normal controls and increased levels in AD neurons and blood vessels with amyloid deposits supports this hypothesis. The absence of ERAB in amyloid plaques in AD (despite its presence in plaques of a transgenic mouse model) might indicate that ERAB plays a role in pathogenetic mechanisms mainly at early stages.

He and collaborators (2001) found that in normal tissues ERAB is present in the mitochondria, not in ER. In AD, amyloid or other factors could initially lead to ERAB increased expression or translocation into ER, followed by a decrease in expression or detectability by immunocytochemical methods, in parallel with increased amyloid deposition. This time frame of ERAB expression matches the one of FKBP12**.**

2.4.5. FKBP12 changes in mid- frontal cortex

AD is characterized by neuronal and synaptic loss and by synapto-axonal pathology. According to Masliah et al. (1993), most of the synaptic loss in the neocortex is derived from loss of cortico-cortical associational input into the modules. This hypothesis also predicts that neuritic plaque formation in the neocortical modules could represent an aberrant sprouting reaction of associational fibers responding to abnormal growth stimuli or to local damage. On these bases, it is also proposed that the cellular substrate of AD pathology is synapto-axonal. AD cases are characterized by an increase in FKBP12 levels in neuronal soma and processes of superficial and deep cortical layers, especially in subjects with abundant NFT, NP and NT pathology. The IP colocalizes extensively with tau in NFT and in various types of NP where it is present both in the amyloid core and at the periphery. FKBP12 could, therefore, play a role in sprouting reactions. One argument supporting this hypothesis is FKBP12 increase in regenerating axons (Lyons et al., 1995). A role for FKBP12 in amyloidogenetic processes is also possible. One interesting aspect that supports this hypothesis is the presence of FKBP12 in the amyloid core of plaques and in blood vessel walls that are affected by amyloid angiopathy. An interesting parallel can be made with Hsps that also might be present in AD plaques. In AD brains, for example, the small Hsps alphaB-crystallin and Hsp27 occur at increased levels and colocalize with plaques. In vitro, small Hsps act as molecular chaperones that recognize unfolding peptides and prevent their aggregation. The presence of FKBP12 in AD brains may thus reflect an attempt to prevent amyloid fibril formation and toxicity. Stege and collaborators (1999) reported that alphaB-crystallin does indeed prevent in vitro fibril formation of Abeta(1-40). However, rather than protecting cultured neurons against Abeta(1-40) toxicity, alphaB-crystallin actually increases the toxic effect. This indicates that the interaction of alphaB-crystallin with conformationally altering Abeta(1-40) may keep the latter in a nonfibrillar, yet

highly toxic form. From this point of view, if FKBP12 has a similar effect, inhibition of its activity using FK506 or analogs can be beneficial.

A distinct type of large, non- cored plaques was identified in AD brains, which are reminiscent of “cotton wool” plaques (CWP). These have a diffuse FKBP12 immunoreactivity and no clearly- defined FKBP12 (or amyloid)- positive core. It is possible that they are nascent plaques, that will coalesce around an FKBP12- positive core as the disease progresses. Although initially they were thought to be characteristic to early- onset AD with spastic paraparesis, a recent study also found these ball- like, non- centered plaques in sporadic late-onset AD with severe amyloid angiopathy. The study (Le et al., 2001) also described these plaques as well-circumscribed amyloid deposits infiltrated by ramified microglia and surrounded by dystrophic neurites. We confirm the fact that late- onset AD cases with significant amyloid angiopathy display CWP. These plaques are also positive for FKBP12, which is consistent with the fact that the IP associates with amyloid.

In a study by Dickson and Vickers (2001), plaque-associated dystrophic neurites in preclinical AD cases were immunolabeled for neurofilament proteins whereas, in end-stage cases, these abnormal neurites were variably labelled for tau or neurofilaments. Double labelling demonstrated that the proportion of diffuse, fibrillar and dense-cored plaques that were neuritic was 12, 47 and 82% and 24, 82 and 76% in preclinical and end-stage cases, respectively. Most dystrophic neurites in Alzheimer's disease cases were labeled for either neurofilaments or tau, however, confocal analysis determined that 30% of neurofilament-labelled bulb-like or elongated neurites had a core of tau immunoreactivity. These results indicate that all morphologically defined beta-amyloid plaque variants were present in both early and late stages of Alzheimer's disease. However, progression to clinical dementia was associated with both a shift to a higher proportion of fibrillar plaques that induced local neuritic alterations and a transformation of cytoskeletal proteins within associated abnormal neuronal processes. FKBP12 might be involved in the reported alteration of cytoskeletal proteins in abnormal processes.

The total number of neurons associated with end-stage, dense-core, non-neuritic plaques declined by 70% (per unit plaque area) compared with neuritic plaque forms. This decline, together with the fact that virtually all of those remaining were TUNEL-positive, suggests that neuronal cell damage increases as plaques evolve from diffuse to more complex forms and that

eventually all plaque-associated neurons are lost (Sheng et al., 1998). The presence of FKBP12 in plaque neurons is likely to contribute to these end- stage phenomena.

Two types of dystrophic neurites have been described in neuritic plaques in Alzheimer's disease (AD). Type 1 dystrophic neurites display tau-positive paired helical filaments (PHF) while those of type 2 are swollen and positive for both amyloid precursor protein and Chromogranin A. FKBP12 appears to be present both in tau- positive neurites and at lower levels in swollen ones.

FKBP12 immunoreactivity is increased in the frontal cortex of PD and DLB subjects. PD cases are characterized by enhanced immunoreactivity in the neuropil, neuronal cell bodies and processes of layers II, III and V. FKBP12 is present in both dendritic and axonal processes. *A potential relationship with the dopaminergic deficit in this condition is suggested by the fact that these are the cortical layers receiving the densest dopaminergic input*. In addition, changes in cortical FKBP12 in PD patients might signal pathological processes underlying cognitive deficits in PD. These are accompanied by neural changes that are related to, but distinct from, those changes that underlie motoric deficits in these patients.

All DLB cases display increased IP amounts in neurons throughout the frontal cortical layers with significant enhancement in deep layers. The lowest IP levels are present in the DLB case with well preserved cortical neuronal population and no neuritic plaques, indicating that increased IP levels play a role in the evolution of the pathogenetic process, possibly in relation with protein aggregation. Indeed, FKBP12 often colocalizes with alpha- synuclein in Lewy bodies and Lewy neurites.

The low levels of FKBP12 in the gray matter of HIV- positive patients might occur due to the combined effect of neuronal and synaptic loss. A higher level of FKBP12 in the white matter, especially in non- encephalitic HIV patients, could be indicative of a relocation of the IP, perhaps in the cortico- cortical sector, as well as of macrophage- microglial activation. It was shown that beta-APP accumulation in the white matter of SIV-infected macaques develops during SIV infection in close correlation with levels of viral replication, macrophage infiltration and microglial activation (Mankowski et al., 2002). Since FKBP12 associates tightly with amyloid in a variety of regions, it might accumulate in the white matter in association with the beta- APP (reflecting a protective response or a causative process).

Strong FKBP12 immunoreactivity is present in HAM56- positive, rod- shaped microglia, which suggests that FKBP12 might play a role in viral entry into cells of microglial- macrophage lineage.

2.4.6. Potential roles of FKB12 in the normal and degenerating human brain

Following focal cerebral ischemia in rats, FKBP12 decreases in the ischemic core, but increases in surviving neurons, indicating a role for FKBP12 in the process of neuronal survival after acute injury (Kato et al., 2000). Lyons et al. (1995) demonstrated that crush injury of facial or sciatic nerves in rat leads to markedly increased FKBP12 levels in the respective nerve nuclei and this increase is related to nerve regeneration. Elevated FKBP12 levels in the brain of patients with neurodegenerative diseases might therefore indicate a restorative process in which IP participate.

Our finding of altered FKBP12 levels in the deep gray matter- substantia nigra axis of PD and DLB patients, as well as the association of FKBP12 with α- synuclein, tau and amyloid deposits, might be related to the role of this IP in protein folding and as a scaffold linking- and carrier- protein. FKBP12 might participate in the cellular response to unfolded proteins, thereby preventing aggregation of mutant and damaged proteins, catalyzing protein folding, solubilizing aggregates, promoting ubiquitination and degradation of abnormal proteins. Previous studies reported that cyclophilin A protects cells from death following expression of mutant Cu/ Zn superoxide dismutase (SOD), which is associated with familial amyotrophic lateral sclerosis (Lee et al., 1999). The mutant SOD may lead to increased protein damage and a greater reliance on rotamase activity. Elevated levels of Pin1, a member of the parvulin class of peptidyl- prolyl *cis-trans* isomerases, were found in the NFT- rich cytoplasm of AD- affected neurons compared to healthy neurons (Thorpe et al., 2001). Pin1 is depleted from the nucleus in AD and redirected to the large amounts of hyperphosphorylated tau in NFT. Phosphorylation of tau and other proteins on serine or threonine residues preceding proline seems to precede tangle formation and neurodegeneration in Alzheimer's disease. Notably, these phospho(Ser/Thr)-Pro motifs exist in two distinct conformations, whose conversion in some proteins is catalysed by the Pin1 prolyl isomerase. Pin1 activity can directly restore the conformation and function of phosphorylated tau or it can do so indirectly by promoting its dephosphorylation, which suggests that Pin1 is involved in neurodegeneration. An analogous mechanism may underlie FKBP12 behavior under

pathological conditions. In degenerating neurons, this IP might be relocated to different cellular compartments and binding to its targets can result in a depletion of available soluble FKBP12, ultimately leading to cell death.

Liou et al. (2003) showed that Pin1 expression is inversely correlated with predicted neuronal vulnerability and actual neurofibrillary degeneration in Alzheimer's disease. Pin1 knockout in mice causes progressive age-dependent neuropathy characterized by motor and behavioural deficits, tau hyperphosphorylation, tau filament formation and neuronal degeneration. The present study suggests that the expression of FKBP12 PPIase is also inversely correlated with neuronal vulnerability to neurodegeneration. Thus, PPIases like Pin1 and FKBP12 appear to be pivotal in protecting against age-dependent neurodegeneration and provide insight into the pathogenesis and treatment of Alzheimer's disease and other tauopathies.

FKBP12 might be protective due to its action of binding to and stabilizing IP3 and ryanodine calcium channels (Jayraman et al., 1992; Brillantes et al., 1994; Wagenknecht et al., 1996; Marks, 2000), since an increase in intracellular calcium levels has been linked to neuronal death. The IP also functions to anchor the phosphatase calcineurin to the channels. It was suggested also that rotamase activity may be required to maintain proteins in a correct conformation during their transport in axons (Lee et al., 1999). Cyclophilin, for example, was shown to be transported by slow axonal transport (Yuan et al., 1997), indicating that it maintains proteins in a native state.

In order to survive and function properly, neurons depend on the efficient delivery of proteins from the cell body to neuritic processes. Axons in particular are highly susceptible to transport deficiencies because of the low level of protein synthesis. In this context, it was suggested that defects in protein transport play a critical role in Alzheimer's disease and other neurodegenerative conditions (Morfini et al., 2002). Kinase and phosphatase activities play a key role in regulating fast axonal transport and in protein phosphorylation (e.g., tau). Given the crucial role of axonal transport in neuronal function, a misregulation of transport induced by an imbalance in specific kinase/phosphatase activities within neurons may represent an early and critical step of neuronal pathology. FKBP12 can be an important player in transport regulation by virtue of its intrinsic enzymatic activity and its protein complex- docking properties (including association with the phosphatase calcineurin and potentially FRAP kinase). FKBP

misregulation would have detrimental consequences on neuronal survival and function by impairing IP- mediated transport and the activity of its associated enzymes.

The association of FKBP12 with tau- (itself a microtubule- associated protein) and amyloid lesions, both leading to axonal transport and protein trafficking impairment, indicates a potential role for FKBP12 in counteracting protein misregulation and the compromised axoplasmic flow. In addition, by virtue of its calcineurin- anchoring capacity, increased FKBP levels might represent an attempt to counteract the hyperphosphorylation of paired helical filaments (PHF) tau and the decreased calcineurin activity in AD (Lian et al., 2001).

The presence of FKBP12 in association with synuclein in dystrophic neurites suggests the possibility of the IP being trapped by abnormal protein aggregates. FKBP12 immunopositivity at the center of a strangulated, ballooned portion the neurite depicted in Fig. 2-6 supports this possibility. A trapping of the IP involved in axonal trafficking might lead to a further deterioration of axoplasmic flow. Neuronal cells are extremely sensitive to transport defects because of their highly polarized morphology and large number of specialized microdomains. The finding that FKBP12 associates with amyloid indicates the possibility that it may serve as an anchor to microtubules for specific subcellular fractions containing amyloidogenic fragments. This is consistent with the proposed function of IPs as "adapter proteins" that serve to couple together macromolecules into assemblies. Interestingly, the scaffolding protein AKAP78, which anchors both protein kinase A and calcineurin and targets them to subcellular sites, contains a putative calcineurin- binding domain resembling FKBP12 (Hamilton and Steiner, 1998).

The *FKBP12- calcium receptor- calcineurin* complex is present at the membrane of endoplasmic reticulum. It was found (Richard et al., 1989) that, indeed, numerous enlarged neurites and presynaptic terminals in frontal biopsies from AD patients contain tubulovesicular profiles of endoplasmic reticulum. This can reflect axoplasmic flow perturbations or a compensatory mechanism and can explain the high FKBP12 present in these areas and located presynaptically. The delivery of vesicle-packaged protein from the neuronal soma to the axonal transport system is physiologically coupled to spontaneous fluctuations of intracellular calcium. Vesicle budding from the endoplasmic reticulum (ER) may be a key step regulating anterograde transport and is modulated through events related to the release and reuptake of ER stores of Ca^{++}([16]). FKBP12 may play a role in transporting vesicle- packaged proteins via its

intracellular calcium- regulating capacity. The strong colocalization of FKBP with synaptophysin, a synaptic vesicle protein, further supports a possible role in transporting vesicle components, modulating synaptic vesicle function and neurotransmitter transport.

The ability of various subsets of neurons to mount an efficient rotamase (including FKBP) response might represent a key to their resistance- or selective vulnerability- to death in neurodegenerative diseases. One potential example of FKBP12- induced susceptibility to neuronal damage is the HIV infection of basal ganglia. In this instance, FKBP12 might function as a co- factor for viral entry and chemokine- induced neuronal damage.

3. FKBP expression in a primate model of dopaminergic loss

3.1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease affecting the population over 65. Age is the most important risk factor. By the time patients are diagnosed, approximately 75% of the pathologic substrate of the disease has developed. Clinical manifestations include bradikinesia, tremor, rigidity, gait and postural abnormalities. These disabling symptoms are primarily due to a profound deficit in striatal dopamine content that results from the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) and the loss of their projecting nerve fibers in the striatum. This is paralleled by a decrease in the striatal DA reuptake sites.

Dopamine transporter (DAT) is a protein situated in the presynaptic dopaminergic terminal, regulating the synaptic concentration of dopamine. This dopaminergic cell specific marker aids in elucidating the rate at which dopaminergic cells are lost in PD. Positron emission tomography (PET) can detect the presence of striatal, pallidal, midbrain, and cortical dopamine terminal dysfunction in vivo in PD (reviewed by Brooks, 2003). Significant inverse correlations are evident between disability scores in intermediate or advanced PD patients and the influx constants of ^{18}F-dopa radiotracer.

Several critical aspects of pre- and post-synaptic dopaminergic function can be measured repeatedly in the same subject (Brooks et al., 2003; Pirker, 2003). These aspects include the relative activity of L-dopa-decarboxylase (DDC), the densities (binding potential-BP), of the type 2 vesicular monoamine transporters (VMAT2) and DAT, and the densities of dopamine receptors. It was suggested, although not established, that BP of VMAT2 and DAT measured by PET correlated with the activities or densities of TH, VMAT2 and DAT in experimental PD models. It was also demonstrated that DDC was less affected in PD, especially from pre-clinical to moderate stages, relative to the involvement of DAT and VMAT2 and that DAT BP is reduced on the affected side in patients with hemi-parkinsonism (Brooks et al., 2003; Davis et

al., 2003)

A major goal is to develop neuroprotective treatment strategies that will slow or block the progression of Parkinson's disease. Although dopamine replacement may alleviate the symptoms of the disease, it does not halt the underlying neuronal degeneration. In the past decade, there have been major advances in identifying genetic and molecular causes of parkinsonism and mapping the events involved in nigral cell death. This new understanding of the pathogenesis of the disease now offers novel prospects for therapy based on targeted neuroprotection of vulnerable neurons and effective strategies for their replacement (for a review, see Dunnett and Bjorklund,1999).

Immunophilin ligands are small molecules that have neurotrophic effects in a variety of systems (Snyder et al., 1998; Avramut et al., 2001). They have the advantage of penetrating the blood- brain barrier and exhibiting a good selectivity for damaged cells. FK506 (Tacrolimus) is a potent immunosuppressive drug used in treatment of patients after organ transplantation and in selected autoimmune disorders. FK506 is activated upon binding to members of the immunophilin chaperone proteins designated FK506-binding proteins (FKBPs). It was found that FKBP12 is enriched in neurons throughout the central and peripheral nervous system. Here, levels detected by in situ hybridization are higher than in any peripheral tissue (Steiner et al., 1992).

Several immunophilin ligands have been shown to posses neurotrophic activities (for a review, see Hamilton and Steiner, 1998). Among the newer members of the immunophilin ligand (IPL) family, the non-immunosuppressive drug GPI-1046 shows much promise in promoting regenerative neuritic growth from surviving neurons in various CNS lesions (Costantini et al., 2000; 2001). Another new immunophilin ligand, V-10,367 has been shown to specifically increase the growth of dopaminergic neurons (mostly neurite branching) and protect against MPTP lesioning. One hypothesis is that IPL may have “dopamine-like” activity due to their inhibition of calcineurin activity which increases phosphorylation of dopamine and DARP-32 which in turn will result in inhibition of phophatase-1 and finally in inhibition of neuronal depolarization (Wu et al., 2002). While some of the rodent studies using IPL in parkinsonian models are very encouraging (Steiner et al., 1997; Costantini et al., 2001), a recent report in a large scale primate study questioned the therapeutic potential of these compounds (Eberling et al., 2002). These contradictory results emphasize the need to further characterize the function of

IP in the brain.

In the 1980s, a dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,-tetrahydropyridine (MPTP) was accidentally shown to induce parkinsonism in humans, and subsequently was confirmed to produce an excellent model of parkinsonism in primates. This model shows the most similarities with the human disease in terms of clinical, pathologic and therapeutic features. Primates treated with MPTP develop motor disturbances resembling those seen in idiopathic PD, including bradykinesia, rigidity and postural abnormalities. In addition, MPTP-treated primates are responsive to all commonly used antiparkinsonian agents and display treatment-associated motor complications such as dyskinesia, wearing-off and on-off, which occur during the long-term treatment of the illness (Jenner, 2003). MPTP can increase the expression of alpha-synuclein and potentially lead to formation of Lewy bodies. Recently it was reported that chronic administration of MPTP to baboons produced a-synuclein aggregates in SN and the authors speculate that this chronic model may eventually even result in the formation of Lewy bodies (Kowall et al., 2000).

MPTP lesioning of the DA neurons can be achieved by proximal (intracarotid) or distal (systemic) injection which may result in an acute or chronic disease. In both models, the greatest DA loss is in the SNc. While midbrain dopaminergic cells are the main neuronal population affected by MPTP, it was shown that globus pallidus focal lesions also occur in primates after intracarotid MPTP administration (Zhang et al., 1999). We still do not have a perfect animal model of PD, however, this model has offered excellent opportunities to study the basic mechanisms of parkinsonism and to develop novel therapeutic agents and strategies for improved symptomatic management.

A number of IP have been implicated in cellular processes that appear to underlie PD pathogenetic mechanisms. Although the data are still scarce, several lines of evidence point to a role for IP (CyP and FKBP) in oxidative stress, energy metabolism processes and apoptosis.

1. Neural damage occurs due to excess release of glutamate which acts mainly through N-methyl-D-aspartate (NMDA) receptors. Activation of the NMDA receptor stimulates nitric oxide (NO) production by NO synthase (NOS). NO mediates glutamate neurotoxicity as inhibitors of NOS prevent neuronal death. FK506 binds to FKBP. One target of the FK506/FKBP complex is the calcium/calmodulin-dependent protein phosphatase calcineurin, whose activity is inhibited upon interaction with FK506/FKBP.

FK506 treatment increases phosphorylation level of calcineurin substrates including NOS. As a potent neuroprotective agent in vitro and in vivo, FK506 increases NOS phosphorylation and decreases NO production. NO also activates poly(ADP-ribose) synthetase (PARS), a nuclear enzyme that synthesizes poly(ADP-ribose) from NAD. Prolonged activation of PARS depletes NAD and lowers cellular energy levels (Zhang and Steiner, 1995)

2. Cyclophilin-A participates in the activation of the caspase cascade in neuronal cells, in particular in the form of cascade elicited by excitotoxic stimuli. Neuroprotection by cyclosporin A against excitotoxin-induced apoptosis is, at least in part, due to inhibition of CyPA (Capano et al., 2002). If apoptosis is involved in dopaminergic cell death, the study of IP expression and functions (CyP in particular) would be useful. In addition, it was found that, in vivo, CyPA expression and secretion are increased by oxidative stress and vascular injury. CyPA functions as a secreted redox-sensitive mediator and growth factor for smooth muscle cells (Jin et al., 2000).
3. CyPD is an essential component of the mitochondrial permeability transition pore, which is detrimental via its participation in apoptotic and necrotic cell death. Mitochondrial targeted CyPD might also protect cells from cell death by virtue of its peptidyl prolyl isomerase activity (Lin and Lechleiter, 2002).
4. Immunosuppressive and non-immunosuppressive FKBP ligands reduce H_2O_2-induced cell damage by increasing glutathione levels in NG108-15 cells (Tanaka et al., 2001).
5. The 6-OHDA-induced reduction in dopamine and its metabolites in the striatum of a rodent PD model was significantly normalized by daily administration of the FKBP ligand GPI1046. GPI1046 significantly reduced lipid peroxidation in vivo and increased striatal glutathione (GSH) levels by activating GSH synthesis (Tanaka et al., 2002).
6. The IP FKBP38 binds to and inhibits CaN in the absence of FK506. It also associates with Bcl-2 and Bcl-x(L). Overexpression of FKBP38 blocks apoptosis, whereas functional inhibition of this protein by a dominant-negative mutant or by RNA interference promotes apoptosis. Thus, it was concluded that FKBP38 might function to inhibit apoptosis by anchoring Bcl-2 and Bcl-x(L) to mitochondria (Shirane and Nakayama, 2003). This suggests the possibility of FKBP IP alterations occuring in response to potential MPTP- induced cell death cascades.

The goal of the present study was to establish a primate model of DA loss that can be used to test the hypothesis that changes in FKBP12 expression and distribution play a role in the pathogenesis of PD. In contrast to other experimental models of parkinsonism, the objectives of this study were to examine the similarities and differences between monkeys with acute onset: short-duration parkinsonism and asymptomatic dopaminergic injury. This paradigm mimics the early stages of PD and establishes a relationship between the causative injury and FKBP reaction.

Unilateral intracarotid artery MPTP administration induces a hemiparkinsonian syndrome, with the advantage of allowing the animal to groom and feed itself and having a control side in the same animal (Oiwa et al., 2003). Different concentrations of MPTP were used to induce various degrees of acute lesioning. An acute dosing regimen was chosen, since this leads to the destruction of DA terminals without significant apoptosis and thus better mimics autopsy findings in humans.

We performed: PET imaging for DAT using the ^{11}C-CFT ligand, clinical and behavioral assessments and necropsy studies. The decrease in dopamine uptake in the striatum was evidenced by the diminished C-CFT binding capacity. In addition, we compared the neuropathologic findings in necropsy materials from MPTP monkeys to autopsy materials from PD patients. The results of the autopsy studies indicate that FKBP12 is expressed in the primate brain. The distribution of the IP is similar to human brain. MPTP treatment leads to alterations in FKBP12 levels.

3.2. Materials and methods

3.2.1. Animals

These preliminary studies used one adult rhesus (Macacca mulatta) and two cynomolgus (Macacca fascicularis) monkeys. All animals were housed in a room with a 12 hr light/dark

cycle. All aspects of this study complied with established federal and institutional guidelines for the care and use of laboratory animals.

3.2.2. MPTP administration and neurobehavioral assessment

Each animal was given a single unilateral (left side) intracarotid injection of MPTP (MPTP-HCl; dissolved in sterile normal saline; Sigma) in doses ranging from 0.33 to 0.75 mg/kg, to produce mild parkinsonism (acute paradigm). The monkey parkinsonism clinical rating scale developed by Smith et al. (1993) was used. This scale consists of a 0- to 5- point rating of different parkinsonian features, including tremor, posture, gait, bradykinesia, balance, and gross motor skills. Both tremor and gross motor skills were rated for the right and left forelimb separately. Subscores were added to determine an overall parkinsonian score. This scale is comparable to the human motor Unified Parkinson's Disease Rating Scale (UPDRS).
Movements were recorded with a video camera weekly before and after the MPTP injections. The animals were euthanized 3 months or 1 year after the MPTP injection and brain tissue was collected.
The time frame and outcome for the procedures in each animal is described below.

Animal	MPTP dose (mg/ kg)	Post- lesion survival time (mos)	DAT reduction (%)	Hemiparkinsonism
CA 01-01	0.4	> 12	50	no
CA 02-02	0.7	3	100	yes
CA 02-03	0.6	3		no

3.2.3. PET imaging

This technique allows serial and longitudinal studies to be performed in the same animal and gives the opportunity to follow a single animal over time and to monitor the effects of interventions on disease progression and outcome. To estimate the progression of disease and magnitude of dopaminergic degeneration, we measured the BP of DAT with [^{11}C]CFT ((carbon-11-labeled 2b-carbomethoxy-3b-(4-fluorophenyl) tropane or WIN 35,428), a cocaine analogue.

The kinetics of [^{11}C]CFT in the brain is well-characterized and the method for estimating DAT-BP is now standardized for [^{11}C]CFT without requiring arterial blood samples (Wullner et al., 1994). Furthermore, it was demonstrated that reproducibility of [^{18}F]CFT is in a 1.9 – 4.1% range in patients with PD and in a 1.1 – 4.0% range in normal subjects (Nurmi et al., 2000). This renders [^{11}C]CFT suitable for detecting changes in DAT-BP over progression of experimental PD. PET scans were performed before MPTP administration and 6 weeks after.

Prior to the PET procedure, the experimental animals were sedated with a mixture of ketamine hydrochloride (5 mg/kg) and medetomidine hydrochloride (50 μg/kg) (i.m.). Once sedated, the monkeys were intubated and an intravenous catheter was placed in the right saphenous vein. Under ketamine/medetomidine sedation the monkey was transported to the PET facility, placed in dorsal recumbency on a heating blanket, positioned in the scanner, and placed on isoflurane gas anesthesia. Atropine sulfate (0.02 mg/kg) was administered intravenously at the initiation of isofluorane anesthesia to maintain heart rate. Isoflurane levels were maintained at 0.5 –1.0 % during the entire 1.5 hour PET procedure. Isoflurane was terminated at the end of the PET procedure and the monkey was returned to its home cage for recovery.

A 9 mCi dose of high specific activity [C-11]CFT was administered intravenously as a bolus injection. Brain activity was be determined with a dynamic series of 10 (1-minute) scans followed by 10 (5-minute) emission scans over 60 minutes. DAT binding potential (BP) measurements were be generated using a reference tissue model (RPM) developed and validated at Hammersmith Hospital. The cerebellum was used as the reference tissue input function. Use of RPM obviates the need for invasive arterial catheterization, blood sampling, and metabolite corrections for CFT PET imaging. All 3-D PET images were reconstructed using standard commercial software as 63 transaxial slices (each 2.4 mm thick) with a transaxial spatial resolution of 4.5-5.0 mm FWHM. The left and right hemispheres were sampled separately. ROI analysis on the parametric BP images was performed on the following striatal (sub-) regions: whole striatum, caudate nucleus and putamen. Multiple time graphical analysis for rapidly dissociating radioligand was used to obtain the binding potential of [^{11}C]CFT using the radioactivity in the cerebellum as the input function. The basis function parametric image method was used to reconstruct BP images.

3.2.4. Necropsy and immunohistochemical analysis

Animals were deeply anesthetized using Nembutal (100mg/kg) and then trans-cardially perfused with 4% paraformaldehyde in phosphate buffered saline (pH 7.2). The brain was removed, cut into 1-cm slices and postfixed in 4% paraformaldehyde for 24 hours. Tissue blocks from basal ganglia were then paraffin embedded. Six micron sections were cut on a microtome and mounted onto glass slides (Fisher Scientific, Springfield, NJ) for histologic analysis.

Slides were incubated at 55° C for 15 min and deparaffinized by immersion in Histoclear (3 x 10 min) (National Diagnostics, Atlanta, GA). Sections were then rehydrated in 100% ethanol (2x10 min), followed by 95%, 90% and 70% ethanol (5 min. each), rinsed in H_2O and immersed in 3% H_2O_2/ methanol (30 min) to inactivate endogenous peroxidase activity. After rinsing, antigen unmasking was achieved by placing slides in 0.4 % pepsin (37° C, 10 min) and blocking was performed using 10% normal donkey serum in phosphate buffered saline (PBS) (30 min, room temperature). Primary antibody anti- FKBP12 (rabbit; Alexis Biochemicals) was applied at a concentration of 1: 400 overnight at 4° C. Secondary antibody (biotinylated donkey anti- rabbit, Jackson Immunoresearch Laboratories, West Grove, PA) was then used at a concentration of 1:200 for a 30 min. incubation. Sections were subsequently incubated with an ABC complex (Vector Elite kit, Vector Laboratories, Burlingame, CA) for 30 min, rinsed and subjected to chromogen incubation for 5 min (Vector Nova Red substrate kit, Vector Laboratories), the dehydrated and mounted with Permount. Left (lesioned) and right (unlesioned) sides of the anatomical region were processed simultaneously. Experiments were repeated twice for each region and animal.

3.2.5. Light microscopic scoring and statistical analysis

Each section was scored for staining intensity based on the following system: 0- absent; 1- present, but faint; 2- moderate; 3 - strong. Staining characteristics of cellular localization and overall distribution were recorded. Due to differences in the species used and degrees of lesion, statistical analyses were not employed.

3.3. Results

Following MPTP intracarotid infusion, one animal was rendered parkinsonian (CA 02-02), while the other two (CA01-01 and CA02-03) were clinically normal.

3.3.1. In vivo imaging of dopaminergic loss in the primate MPTP model

Parametric PET images (Fig. 3-1) of ^{11}C-CFT Binding Potential (BP) in the rhesus monkey (CA 01-01) show the striatum at baseline and 6 weeks post-MPTP treatment. Unilateral lesioning of dopamine transporters in the left striatum is evident (50% loss).

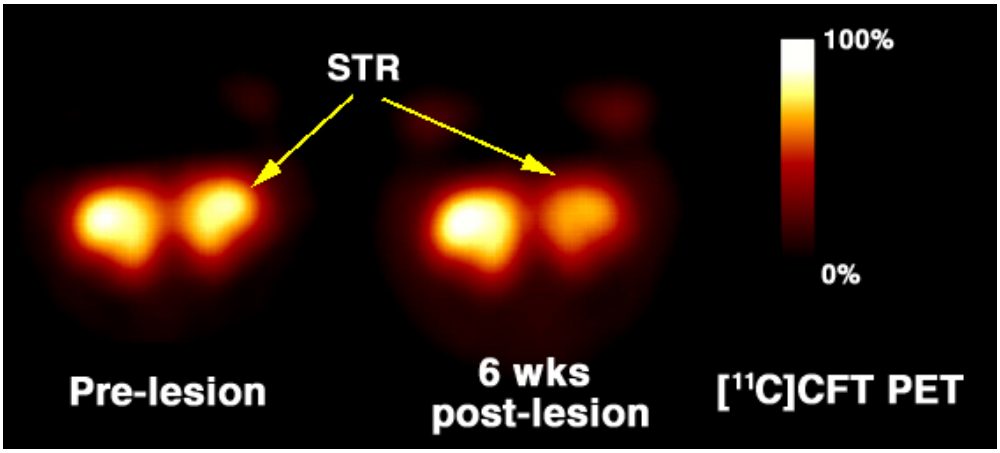


Fig. 3-1: CFT PET imaging in a rhesus monkey pre- and post-unilateral intracarotid injection of a single dose of MPTP (0.4 mg/kg). Although we could measure by PET a 50% loss of DAT, the animal showed no neurologic symptoms.

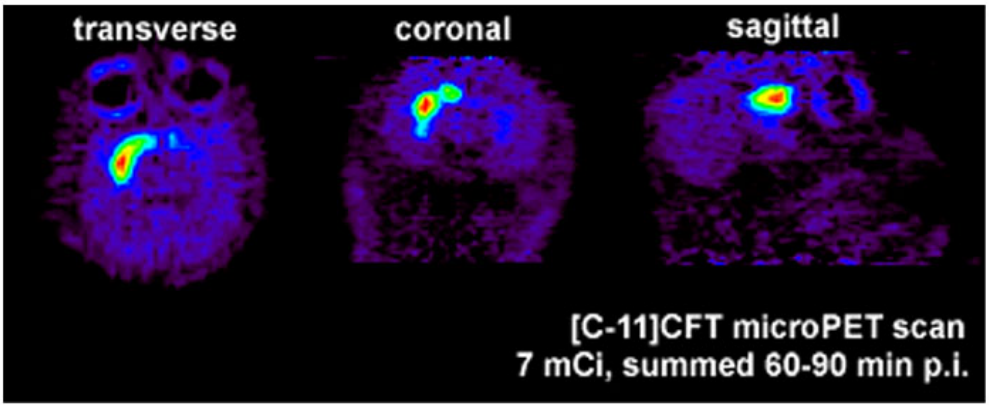


Fig. 3-2: CFT microPET imaging in a cynomolgus monkey post-unilateral intracarotid injection of a single dose of MPTP (0.7 mg/kg). Dopamine transporter binding on the lesioned side is abolished. The animal displayed hemiparkinsonian symptoms followed by tendency to recover.

Micro- PET scanning (Fig. 3-2) reveals the absence of DAT binding in the lesioned striatum of the cynomolgus monkey CA02-02 (100% reduction).

4.3.2. Necropsy studies of FKBP12 expression

FKBP12 immunoreactivity was altered in the SN and deep gray matter in all three MPTP- treated monkeys. The changes are summarized in Table 3-1.

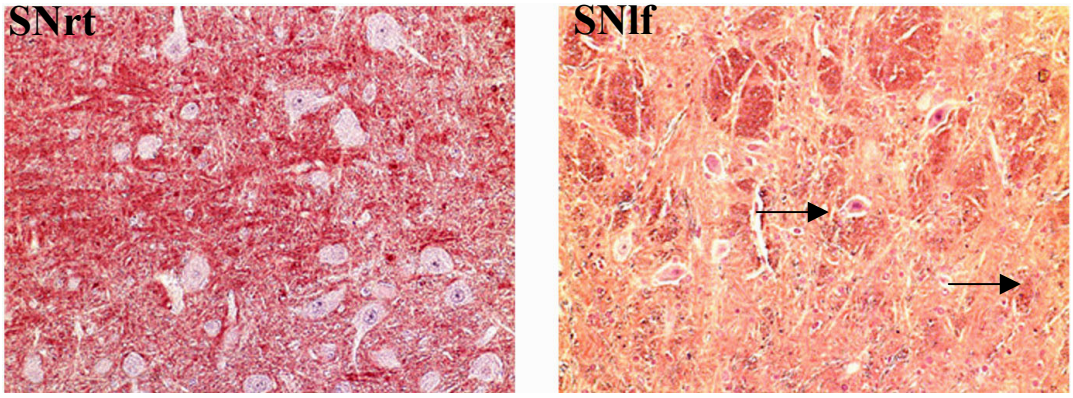
Table 3-1: Changes in FKBP12 immunoreactivity on the lesioned (left) side in MPTP- treated monkeys. "s": neuronal soma; "f": fibers; "m": medial; "l": lateral; SNc: substantia nigra pars compacta; SNr: substantia nigra pars reticularis; Pu: putamen; GP: globus pallidus ("e": pars externa).

Animal	**MPTP (mg/kg)**	**Post-lesion survival time (mos)**	**Hemi-parkin-sonism**	**FKBP12 immunoreactivity change (lesioned side)**	
				SN	**DGM**
CA 01-01	0.4	> 12	no	↑SNc-s ↓SNc-fm SNr-m	↑ GP (s, f)
CA 02-03	0.6	3	no	↑SNc-s ↓SNc-fm, SNr-m	↑ Pu (s, f) Gpe-f
CA 02-02	0.7	3	yes	↑SNc-s, fl ↓SNr (l mainly)	↑ Pu-f ↓GP (f)

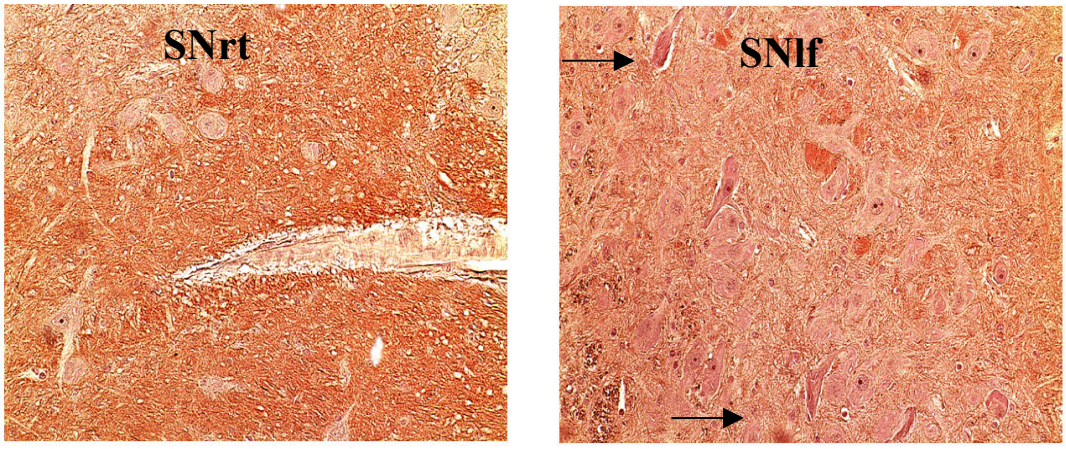
3.3.2. FKB12 changes in the substantia nigra

All animals, regardless of their hemiparkinsonian status or survival time, displayed increased FKBP12 immunoreactivity in the grey matter (GM) of the lesioned SNc (Fig.3-3: A, B, C- arrows).

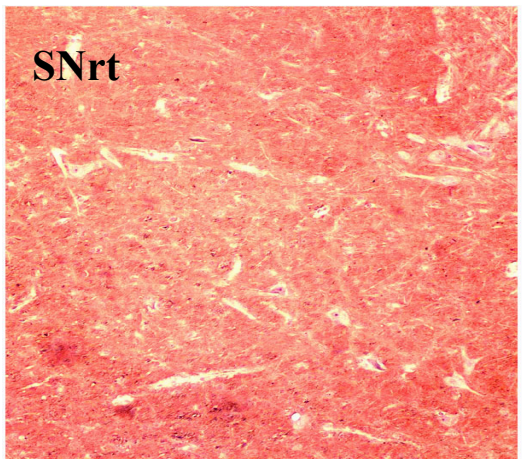
A) CA01-01: SNc

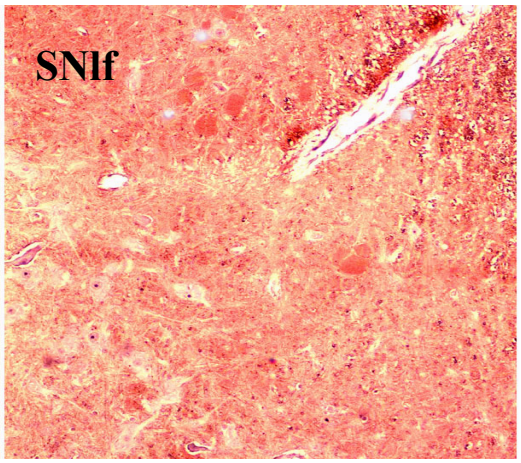


B) CA02-02: SNc (and lateral SNr)

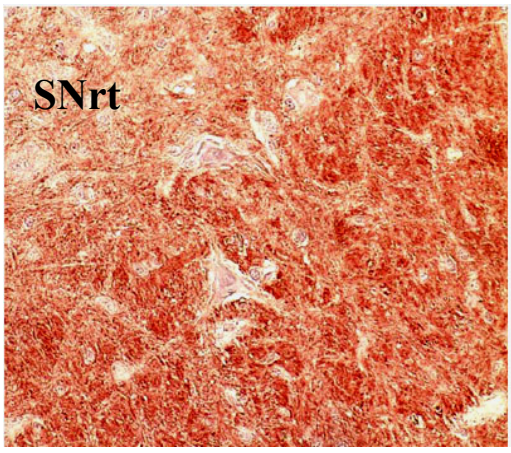


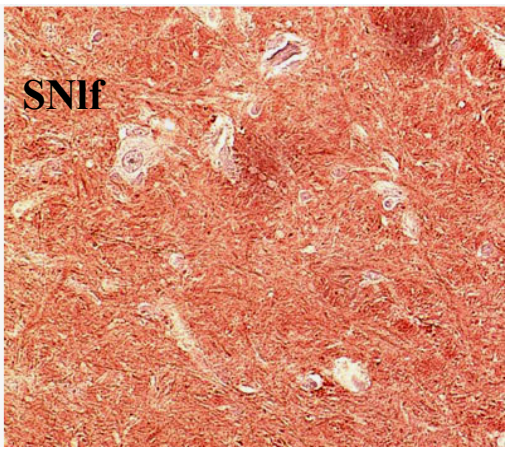
CA02-02:SNr(lateral)





C) CA02-03: SNc





CA02-03: SNr (medial)

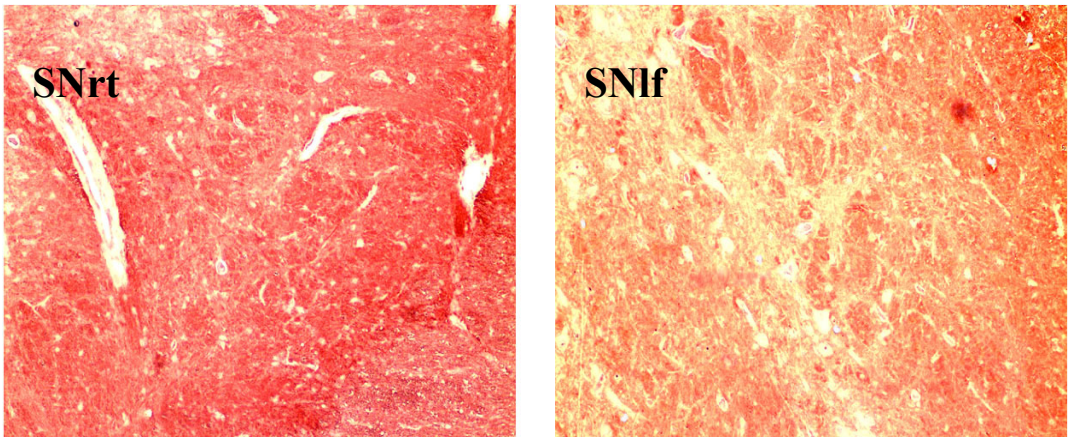


Figure 3-3: Altered expression of FKBP12 in the SN of MPTP- treated primates. Unilateral intra-carotid injection in CA01-01 (A), CA02-02 (B), CA02-03 (C). SNrt: internal control side (right), SNlf: injection side (left). Nova Red chromogen immunostaining and haematoxylin counterstaining. Magnification: 200×.

Non- symptomatic animals had low FKBP12 expression in the WM of medial SNc on the lesioned side (results not shown). The symptomatic animal did not exhibit changes in fibers of this area, however, increased FKBP12 levels were detected in the lateral WM of the lesioned SNc.

In the SNr on the lesion side, the pattern of the change differs, with asymptomatic animals exhibiting low IP levels in the medial SNr and the symptomatic one mainly in the lateral part (Fig. 3-3 B, C).

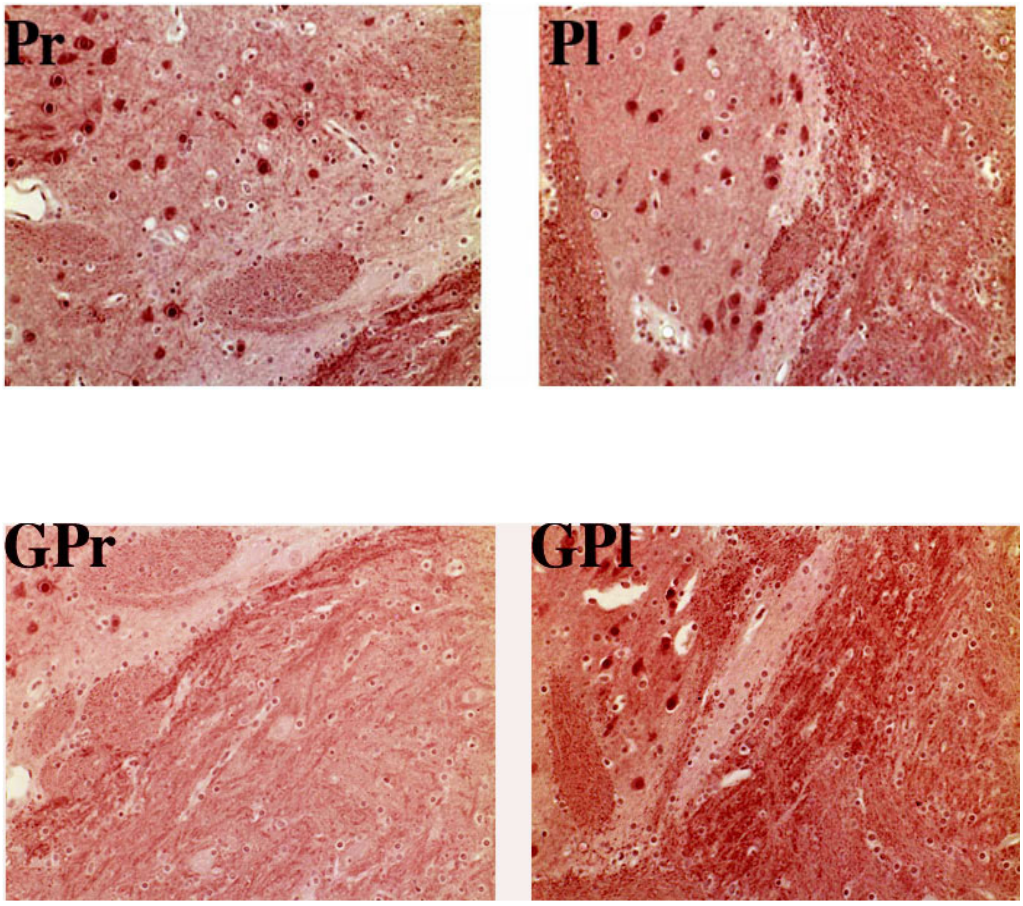
3.3.3. FKBP12 changes in the striato- pallidal system

At early time points post- lesion, there is increased FKBP12 immunoreactivity in the lesioned putamen (Fig. 3-4 (B) and (C)). Putaminal changes are absent in the animal that survived for more than 1 year (Fig. 3-4 (A)).

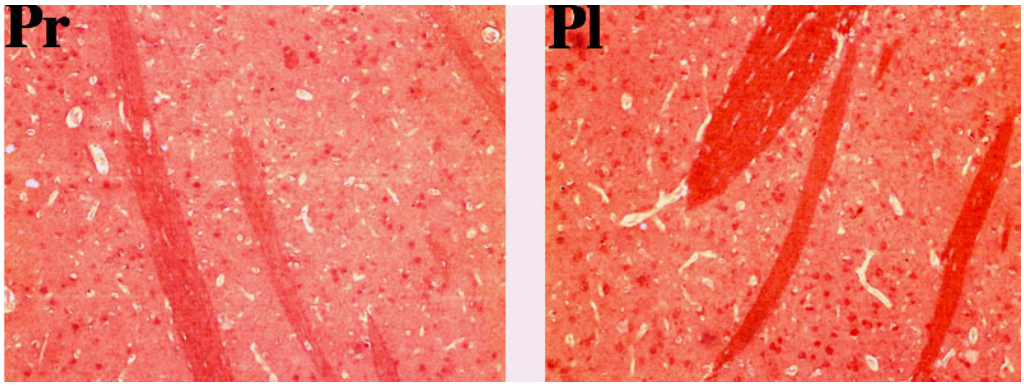
The asymptomatic monkeys displayed increased IP levels in the lesioned GP (both pars interna and pars externa in CA01-01; pars externa WM only in CA02-03), as shown in Fig. 3-4

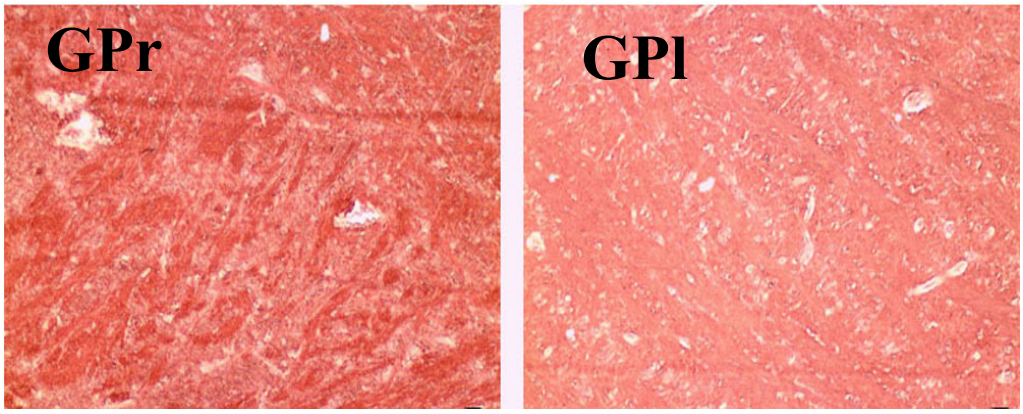
(A) and (C)). The hemiparkinsonian animal exhibited a unilateral decrease in FKBP12 immunoreactivity in the lesioned GP WM (Fig. 3-4 (B)).

A) CA01-01



B) CA02-02





C) CA02-03

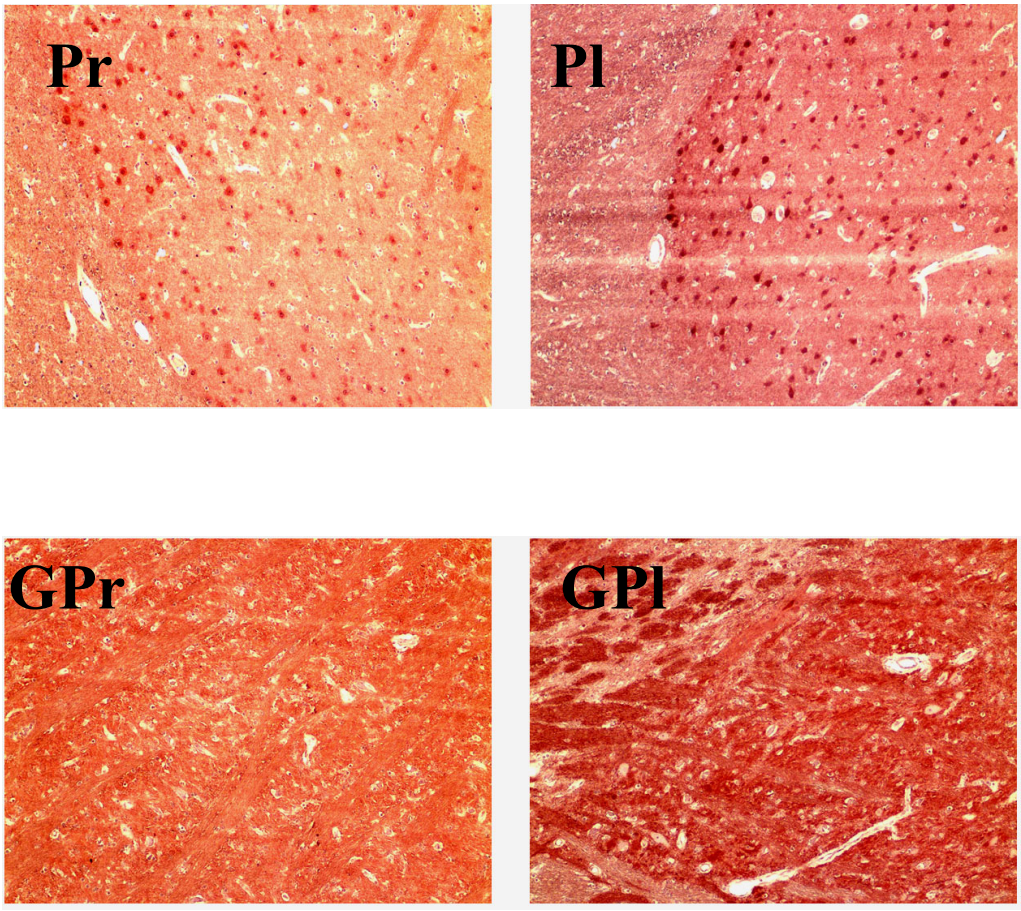


Fig. 3-4: Altered expression of FKBP12 in the striatum and pallidum of MPTP-treated monkeys: Unilateral intra-carotid injection in CA01-01 (A), CA02-02 (B), CA02-03 (C). Pr, GPr: internal control side (right); Pl, GPl: injection side (left). Nova Red chromogen immunostaining and haematoxylin counterstaining. Magnification: 200×.

3.4. Discussion

The results of this study suggest that the presence of symptoms and FKBP content in the SN pars reticularis are correlated with changes in pallidal FKBP12 levels. A preservation of FKBP12 levels in the lateral SN fibers in asymptomatic monkeys was associated with upregulated levels of this IP in the GP. Conversely, decreased levels in the lateral pars reticularis and GP were observed in the symptomatic animal. Taken together, these observations suggest that increased FKBP levels in the pallido- nigral axis play a role in mitigating parkinsonian symptoms.

At early stages, baseline FKBP12 levels in the soma of putaminal neurons were noted in the symptomatic animal. High FKBP12 in the soma and fibers of medium size putaminal neurons were present in association with minimal striatal denervation. These findings suggest a potential protective effect of FKBP12.

The results also question the role of increased FKBP12 expression in nigral DA neuronal cell bodies or putaminal fibers as a primary causative factor in the generation of parkinsonian symptoms. The presence of symptoms associates with decreased FKBP12 levels in fibers of lateral SNr, as well as increased levels in lateral SNc fibers. The asymptomatic animals exhibited lower FKBP12 immunoreactivity in the lesioned medial nigra at early and late time points.

The asymptomatic animals had diminished FKBP12 expression in the WM of medial SNc on the lesioned side, which can be due to a protective redistribution of the IP to a different cellular compartment (cell body or striatal terminals). They exhibited low IP levels in the medial SNr, whereas the symptomatic one shows this change predominantly in the lateral part. A concentration of the protein in the striatal cell bodies originating the SNr fibers can underlie this diminished expression. The displacement of the IP to target- regions of SNr output is also possible.

Overall, the absence of symptoms following MPTP injection associates with decreased FKBP12 expression in fibers of the medial SN (both pars compacta and pars reticularis). This can be due to a retention of the IP in the striatum, to mitigate toxin- induced pathological changes that initially affect this region.

The hemiparkinsonian animal showed increased FKBP12 levels in the lateral WM of the lesioned SNc, which is consistent with a role for this IP in the pathogenetic mechanisms of DA

lesion. This animal only exhibited decreased IP imunoreactivity in the lateral SNr, which might indicate a less efficient compensatory reaction to the toxic aggression. In general, the hemiparkinsonian monkey showed IP changes in fibers of lateral SN (pars compacta and reticularis), which undergoes the most severe degeneration in the experimental MPTP- induced parkinsonism (reviewed by Rinne, 1993) and in PD.

Increased FKBP12 levels in SNc processes might be part of an attempt to increase DA content in this area. Studies in asymptomatic MPTP- treated monkeys suggest that changes in DA and homovanillic acid concentrations in the midbrain DA regions are relatively small and involve central and medial substantia nigra. In this region, we found low levels of FKBP12.

After MPTP treatment, the terminals of the nigrostriatal pathway are affected before the cell bodies. Early studies found that, after MPTP treatment, there is a severe loss of DA in the striatum, both in symptomatic and asymptomatic monkeys (Elsworth et al., 1987). In our study, putaminal increases in FKBP12 occured early following MPTP lesioning, and were not present at later time points. These acute alterations might be caused by the observed initial elevation in FKBP12 levels in the nigral DA neurons and transport to te putamen (hence the increase in putaminal WM). An elevated protein synthesis can also occur in the soma of medium size neurons.

Asymptomatic monkeys displayed increased IP levels in the lesioned pallidum: GPe (WM) at early stages and, later, the entire GP. The presence of parkinsonian symptoms was associated with decreased FKBP12 immunoreactivity in the GP WM on the lesioned side, at least at early stages. Thus, the inability to upregulate FKBP12 levels in the GP is likely to play a role in the pathogenesis of parkinsonism.

One potential model emerging from these data is that, following MPTP lesioning of dopaminergic terminals, axonal transport to the SN (and, potentially, subthalamic nucleus) is impaired. FKBP12 concentrates in the putamen and GP, where it plays a protective role. SN is subsequently affected (fibers and, ultimately, cell bodies). Increased FKBP12 immunoreactivity in DA cell bodies (especially in animals showing a marked deterioration of striatal DA uptake) indicates the possibility that this IP plays a role in mitigating the toxic effects of MPTP.

In MPTP- treated parkinsonian primates, at early stages, the structure whose increase in local cerebral metabolic rate for glucose correlated most closely to the clinical severity of parkinsonism was the external segment of the globus pallidus (Schwartzman et al., 1988). Other

studies of regional brain metabolism found that MPTP-induced parkinsonism is characterised by abnormally increased activity of internal pallidal neurons which project to the thalamus and pedunculopontine nucleus (Crossman, 1987). Our study indicates that changes in pallidal FKBP12 levels also correlate with the presence of symptoms and might be employed in the early diagnosis of dopaminergic damage. By virtue of their protein folding and trafficking functions, IP are likely to participate in the complex metabolic processes underlying these lesions.

In attempting to reconcile the results from studies of animal models of PD with results from postmortem studies of PD brains, one encounters several problems. Both types of studies have a number of associated difficulties that hinder the interpretation of their respective findings. The animal studies described here have been conducted on acute models of parkinsonism, i.e., animals with a rapid onset of DA neuron degeneration, striatal DA depletion and short duration of symptoms. A complicating factor in human studies is that IP expression may be affected by the long-term use of various anti-Parkinson drug therapies or by the variability of the disease itself (i.e., degree of striatal DA depletion). Nevertheless, revealing the FKBP12 expression levels and pattern shortly after dopaminergic system impairment is likely to render valuable information about the role played by these IP in nervous system injury.

Neuronal loss in the substantia nigra of patients with PD does not occur evenly throughout the nucleus: the ventrolateral part of the substantia nigra degenerates more severely, whereas the medial part is relatively preserved (reviewed by Rinne, 1993). This aspect is mimicked by the MPTP- induced DA damage. The toxin induces a significant reduction in neuronal cell density in the substantia nigra, with obvious mediolateral and dorsoventral gradients of neuronal cell loss (Varastet et al., 1994). First, the lateral divisions of the pars compacta are more depleted than the medial divisions. Second, the ventral regions of the pars compacta degenerate more than the dorsal parts. The lesioned ventrolateral SN was the region where we found diminished levels of FKBP12 in the parkinsonian primate. In addition, in the asymptomatic animals, the levels of FKBP12 in lateral SN were preserved.

The pattern of nigral neuronal loss is compatible with the uneven loss of dopamine in the striatum (the putamen being more affected than the caudate nucleus). The predominant loss of ventrolateral nigrostriatal projections in PD, leading to substantial loss of dopamine especially in the putamen, is thought to contribute to the motor symptoms of the patients. The anterolateral "motor" area of the SNr is involved in the development of appendicular parkinsonian motor signs

(Wichmann et al., 2001), which are the predominant feature of the hemiparkinsonism in our study.

The current opinion is that PD is due to a combination of genetic and environmental factors. The striking increase in the risk for PD in the presence of multiple defects in xenobiotic metabolizing pathways is consistent with this hypothesis. It was proposed that abnormalities of several enzyme systems might be required for a neurotoxic "hit" to take place (Langston, 1998). If FKBP belongs to one of these pathways, abnormal levels or function of the IP could translate into an increased vulnerability to toxic injury.

One of the few uncontested risk factors for PD is increasing age. The disease is rare before the age of 40, and the incidence increases at least through the eighth to ninth decades of life. While there has been a great deal of research on the pathophysiology of the disease, the reason why it has a predilection for the aged nervous system remains unknown, although theories abound. One line of thought is that there may be protective factors that decline with aging in certain brain areas, including chaperone proteins (Soti and Csermely, 2002). The decreased FKBP12 immunoreactivity in selected striato-pallidal and nigral regions in parkinsonian monkeys and PD patients can be part of an impaired chaperone system that renders DA neurons vulnerable to various insults and leads to parkinsonism.

Here we describe an animal model that can help understand the symptoms and therapeutic avenues of PD, since different degrees of lesion and severities of parkinsonian symptoms can be mimicked. Excitotoxicity, mitochondrial dysfunction and free radical induced oxidative damage have been implicated in the pathogenesis of several different neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease. Much of the interest in the association of neurodegeneration with mitochondrial dysfunction and oxidative damage emerged from animal studies using mitochondrial toxins. MPP+, the active metabolite of MPTP, inhibits the activity of respiratory chain complex I (NADH-coenzyme Q reductase of the electron transport chain). In patients with PD, a reduced complex I activity was found in substantia nigra, skeletal muscle, and platelets (Pautot et al., 1998). The interruption of oxidative phosphorylation results in decreased levels of ATP. A consequence is partial neuronal depolarization and secondary activation of voltage-dependent NMDA receptors, which may result in excitotoxic neuronal cell death (secondary excitotoxicity). The increase in intracellular Ca^{2+} concentration leads to an

activation of Ca^{2+}- dependent enzymes, including the constitutive neuronal nitric oxide synthase (cnNOS) which produces NO.. NO. may react with the superoxide anion to from peroxynitrite and ultimately hydroxyl radicals. Oxidative stress can also impair other processes implicated in dopaminergic system impairment, including ubiquitination and degradation of proteins directly. Products of oxidative damage can impair the 26S proteasome. Furthermore, impairment of proteasomal function leads to free radical generation and oxidative stress. Because of its role in modulating Ca^{2+} channels, FKBP12 might play a role in pathogenetic processes linked to parkinsonism.

An intriguing possibility is that, in accordance with its role in peripheral nerve regeneration (Lyons et al., 1995), FKBP12 participates in regenerative processes occurring in the CNS, including the lesioned nigra. Indeed, new neurons are generated from stem cells in a few regions of the adult mammalian brain. Recent studies by Zhao et al. (2003) provided compelling evidence for the generation of dopaminergic projection neurons of the type that are lost in Parkinson's disease from stem cells in the adult rodent brain and showed that the rate of neurogenesis is increased after a lesion. The relocation and possibly increased synthesis of FKBP12 in the nigro- striatal axis could serve the generation and growth of new neurons following the parkinsonism- inducing lesion.

Future studies will be performed using a larger group of MPTP- treated primates with stable parkinsonism. Compounds that bind to FKBP and exhibit neurotrophic properties (e.g., the immunosuppressant drug FK506) will be administered to lesioned animals, with or without implanted neuronal grafts.

4. Neurotrophic effects of FKBP ligand FK506 (Tacrolimus) in human fetal brain cultures

4.1. Introduction

FK506 (Tacrolimus) is a potent immunosuppressive drug used in the treatment of patients after organ transplantation and in selected autoimmune disorders (Thomson and Starzl, 1992; Fung and Starzl, 1994; Letko et al., 1999). It has greater potency and lower toxicity than Cyclosporin A. FK506 is activated upon binding to members of the immunophilin (IP) chaperone proteins (Harding et al., 1989; Schreiber and Crabtree, 1992), designated FK506-binding proteins (FKBP). The immunophilin (IP)- drug complex binds to and inhibits the activity of calcineurin, a calcium- activated phosphatase (Friedman and Weissman, 1991; Liu et al., 1991; Thomson et al., 1995). Consequently, limphokine production, T- cell growth and proliferation are inhibited.

The precise cellular functions of IP and their endogenous ligands are still to be established. It was found that FKBP12 is enriched in neurons throughout the central and peripheral nervous system (Steiner et al., 1992; Dawson et al, 1994). Here, levels detected by in situ hybridization are higher than in any other tissue. This finding, coupled with observations that IP are upregulated in response to nerve transection, prompted the study of FK506 effects on neural tissue. The drug was shown to augment neurite outgrowth in PC12 cells, in the presence of NGF, and also in rat and chick sensory ganglia explants (Lyons et al., 1994; Steiner et al., 1997a). It was also demonstrated that FK506 and its analogues increase the number and length of tyrosine hydroxylase- positive neurites in rodent primary dopamine neuronal cultures (Costantini et al., 1998; Costantini and Isacson, 2000). In animal models, the drug and its analogues stimulate axonal re-growth and enhance functional recovery following peripheral nerve and spinal cord injury (Gold et al., 1994, 1995; Madsen et al., 1998; Bavetta et al., 1999) and have protective and regenerative effects in neurotoxic lesions (Steiner et al., 1997b; Costantini et al., 1998). It was shown that, in rodents, the neuroregenerative effects of FK506 are mediated via

binding to FKBP52 (FKBP59) (Gold, 1999; Dawson, 2001). It is possible, however, that neuroprotective effects are still a result of FK506 binding to FKBP12.

So far, the effects of the immunosuppressive drug FK506 on human primary brain cell cultures have not been studied. This is the first analysis of the effects of FK506 on embryonic human neural cells. We employed laser scanning confocal microscopy of second trimester fetal neuro- glial cultures and computer- assisted image analysis to characterize the actions of FK506 and compare them to the effects of brain-derived neurotrophic factor (BDNF) (White et al., 1999).

Second trimester human brain cultures (HBC) contain neuroprogenitor cells that can develop along the neuronal lineage (White et al., 1999). The neuro- glial cells survive and differentiate in vitro as attached cultures, in which they form a complex and abundant network of neuritic processes. The proliferative stage characterizes the first 10 days in vitro, peaking around day 5. By 4 weeks, the cultures contain a balanced population of neurons and astrocytes, approximately 40% each, microglia and undifferentiated cells that do not express any traditional neuro- glial markers. It was previously shown that BDNF treatment induces proliferation and differentiation of the neuroprogenitor cells (Lowenstein and Arsenault, 1996; White et al., 1999).

The major finding of this study is the ability of FK506 to promote human fetal neuronal survival and differentiation. Additionally, FK506 was found to decrease the extension of astrocytic processes. This effect became statistically significant when FK506 was combined with BDNF. Both these actions are desirable in the treatment of neurodegenerative diseases, especially when cell transplants are employed.

The effects of FK506 in this system can reflect the participation of the FK506- FKBP12 complex in signal transduction cascades that are essential for neuronal survival and growth. Furthermore, it is known that the drug blocks the enzymatic activity of FKBP IP, as well as dislocates FKBP12 from the intracellular Ca^{2+} channels it modulates, leading to increased Ca^{2+} flux. Therefore, the findings described here can provide a window into potential roles of human neuronal FKBP.

4.2. Materials and Methods

4.2.1. Cell cultures and treatments

Surgical pathology fetal specimens were collected and processed in accordance with the University of Pittsburgh Human Tissue Committee guidelines. Human telencephalic tissues (18-21 weeks of gestation) obtained from elective abortions were collected and placed in DMEM medium (Life Technologies Inc., Grand Island, NY). The aspiration method of fetal removal did not allow for identification of specific brain structures except the general lobe architecture. Tissue processing followed a modified mouse brain dissociation protocol (Martin and Wiley, 1994). Within 1 h of collection, the brain material was processed for in vitro culture as described previously (White et al, 1999). The single cell suspension was brought to a final density of 10^6 cells/ml and plated on poly-l-ornithine (Sigma)- and laminin (Life Technologies)- coated 12 mm glass coverslips (Fisher, Pittsburgh, PA) on 24-well plates (Becton Dickinson). Throughout the experiments, cultures were incubated in 5% CO_2 at 37° C and fed every other day, starting at day 1, 5, 12, or 21 in culture, with fresh NPMM (Bio Whittaker, San Diego, CA), with or without various concentrations of FK506 (Fujisawa, Japan) and/or BDNF (5 ng/ml, Promega, Madison, WI). Astrocytic cultures were obtained same tissue dissociation procedure. The cell suspension (2×10^6 cells /ml) was placed in 75-cm^2 tissue culture flasks (Costar, Cambridge, MA), incubated in 5% CO at 37 °C and fed with 10% fetal calf serum in DMEM. Upon reaching confluence, the cultures were rinsed, trypsinized and transferred to new flasks. After two passages, pure astrocytic cultures were plated on poly-L-ornithine and laminin-coated coverslips in 24-well plates at a density of 10^6 cells/ ml. Starting at day 5, 7 or 12 post-plating, the medium was supplemented with FK506 (5 nM) and/or BDNF (5 ng/ml). The treatments were administered every other day, for 7 days. The absence of contaminating neurons in astrocytic cultures was verified by immunostaining for the neuronal marker MAP-2. All experiments were performed three times, using separate fetal specimens, except for the NeuN immunostaining analysis, which was performed twice. Each treatment regimen was run in duplicate or triplicate per experiment.

4.2.2. Immunocytochemistry

After 7 days of treatment, cultures were washed with PBS and PBS with 0.05% Tween 20 (Sigma), fixed with 4% paraformaldehyde for 30 min and then incubated for 30 min with PBS

with 0.2% BSA and 0.1% Triton X (Sigma). Cultures were incubated overnight at 4° C with primary antibodies: mouse anti-MAP-2 (1:500, Sternberger Monoclonals Inc., Baltimore, MD), rabbit anti-GFAP (1: 1000, Dako, Capinteria, CA), mouse anti NeuN (1:200, Chemicon, Temecula, CA), mouse anti- SMI312 (1:400; Sternberger), rabbit anti- FKBP12 (1:350; Alexis Biochemicals). After thorough washing with PBS with Tween 20, a secondary antibody was applied for 2 h (FITC- conjugated goat anti – mouse and FITC- conjugated goat anti- rabbit; 1:200, Jackson ImmunoResearch, West Grove, PA). When appropriate, RNase A (10 μg/ml; Sigma) was added during the secondary antibody incubation (20 min), followed by the nuclear stain propidium iodide (5 μg/ml, Sigma). For double labeling experiments, anti- calreticulin (1:100) (RDI, NJ) was applied after ther completion of the primary labeling (FKBP12).

4.2.3. BrdU cell proliferation immunoassay

We used the 5-bromo-2' deoxy-uridine (BrdU) Labeling and Detection Kit from Roche to measure cell proliferation. Cell cultures were exposed to BrdU for 12 hrs. Following fixative incubation and acid DNA denaturing, the BrdU- labeled DNA was detected using a highly specific anti- BrdU mouse monoclonal, fluorescein- labeled antibody. Total DNA was counterstained with propidium iodide (1 μg/ ml). Propidium iodide- stained and BrdU- positive nuclei were counted using the Argon/ Krypton laser confocal microscope (Model 2001, Molecular Dynamics, Sunnyvale, CA), as described below.

4.2.4. Laser confocal microscopy and statistical analysis

For quantitative data, six randomly chosen fields from all areas of the well (center and periphery) were selected for cell counts and marker surface analysis, as described before (White et al, 1999). The Argon/ Krypton laser confocal microscope 40× objective with oil immersion lens (Model 2001, Molecular Dynamics, Sunnyvale, CA) was used. Images of 512 × 512 pixels were collected at a pixel size of 0.5 mm^2. Vertical sections were acquired in order to define vertical borders for the collection of Z- series. The step size between adjacent sections was 0.50 μm. FITC was excited with a wavelength of 488 nm and collected with a 530DF30 filter. Propidium iodide was excited with a wavelength of 568 nm and collected with a 590DFLP filter. Thresholds were set to eliminate any background signal and maintained throughout the immunofluorescent area quantification process. Positive pixels, converted into μm^2 of positivity, were tabulated using ImageSpace software (Molecular Dynamics) over a full Z-series of a given sample (10 steps). Counts consisted of the number of cells, as well as MAP-2 - or GFAP – positive surface

per section. We chose to divide the overall neuronal marker area to the total number of cells because the finding of an increased neuronal marker ratio in this context (a bias against our hypothesis) is all the more significant. The analysis is based on a technique previously used in numerous studies (Masliah et al, 1992, 1997; White et al., 1999; Jordan- Sciutto et al., 2001). This approach ensures a thorough, multi- section analysis of neuritic extension in culture. The measurements from the 10 sections per field were averaged to obtain six measurements per well. Data were pooled from three experiments (except the NeuN – positive nuclei quantification, which was performed twice). All comparisons were performed using one- way analysis of variance (ANOVA), followed by post- hoc Tukey's tests to assess differences between groups (SPSS 10.0.5; * $p < 0.05$).

4.2.5. Tritiated thymidine incorporation assays

The effects of FK506 on human peripheral blood mononuclear cell (PBMC) proliferation were determined on the basis of tritiated thymidine uptake. Heparinized human peripheral blood was obtained from healthy donors. Peripheral blood mononuclear cells (PBMC) were isolated using the Ficoll- Hypaque density- gradient method. Peripheral blood was centrifuged at 2000 rpm and 4°C for 10 min to remove plasma. Blood cells were diluted with PBS, then centrifuged in a Ficoll- Hypaque discontinuous gradient at 1500 rpm for 30 min. The PBMC layers were collected and washed with cold distilled water and 10× Hank's buffered saline solution to remove red blood cells. The cells were resuspended to a concentration of 1×10^{6} cells/ ml in RPMI-1640 medium supplemented with 5% human serum AB. The suspension (100 µl) was deposited into a 96- well flat- bottom plate (Nunc 167008), then PHA and various amounts of FK506 (Tacrolimus) (1- 50 ng/ 100µl), were added to the wells. The plates were incubated in 5% humidified CO_2 air at 37 °C for 3 days. Tritiated thymidine (1 µCi/ well; New England Nuclear, Boston, MA) was added to each well during the last 30 hours of incubation. Cells were subsequently harvested and radioactivity was measured with a scintillation counter.

The results indicated that the drug was immunopharmacologically active and strongly suppressed the proliferation of PHA- activated PBMC (Fig. 4-1).

4.3. Results

After 7 days of treatment initiated at day 12 *in vitro* (DIV 12), 5 ng/ml FK506 (6 nM) induced a significant increase in MAP-2 and cell numbers when compared to all the other treatments (0.5, 1, 10 and 50 ng/ml) and to untreated cultures (Fig. 4-2). We therefore used this concentration for all our subsequent experiments and compared the effects to the action of BDNF and the combination of FK506 and BDNF. Higher concentrations did not appear to exert toxic effects, as the number of cells and MAP-2 surface did not differ significantly from control.

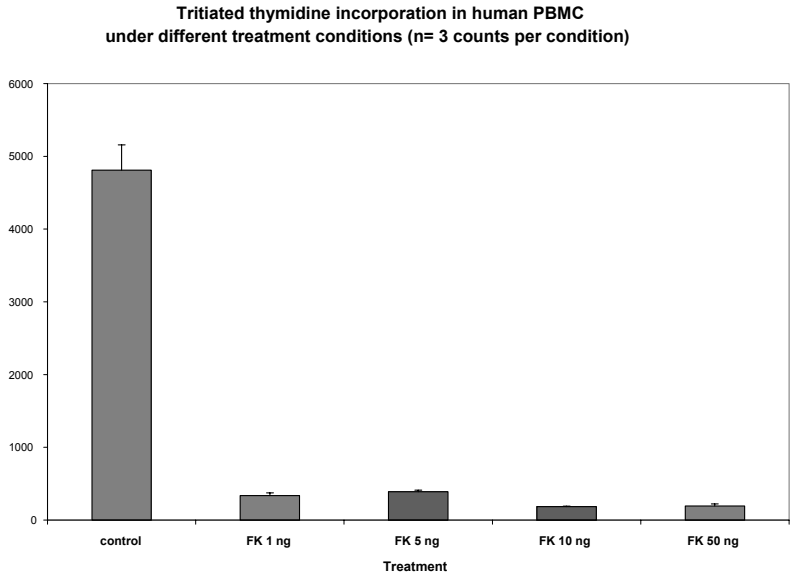


Fig. 4-1 Tritiated thymidine incorporation in FK506- treated vs. control human PBMC

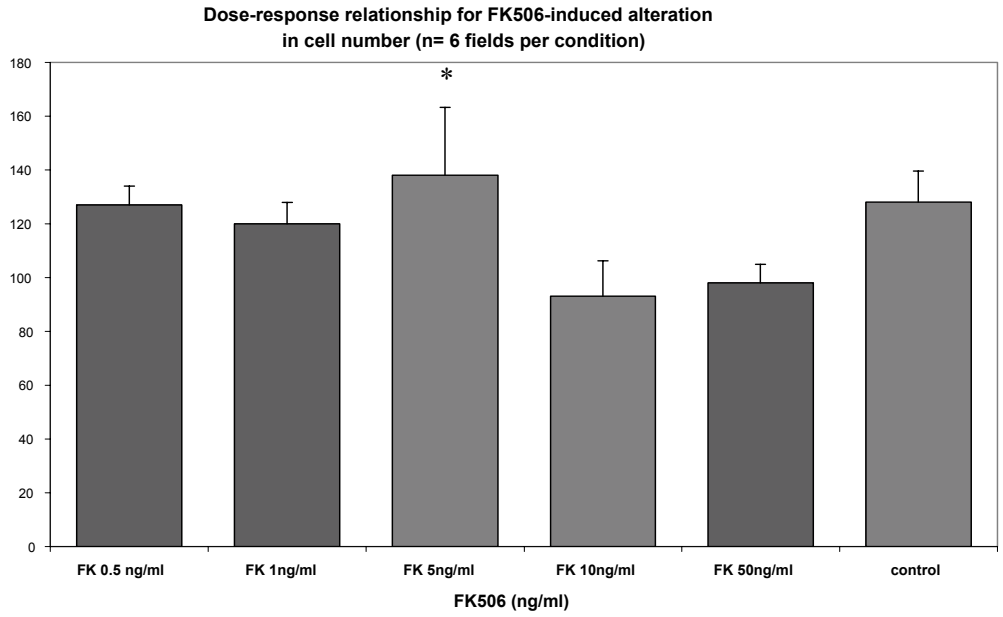


Fig. 4-2 The effect of various FK506 dosages on cell numbers in HBC.

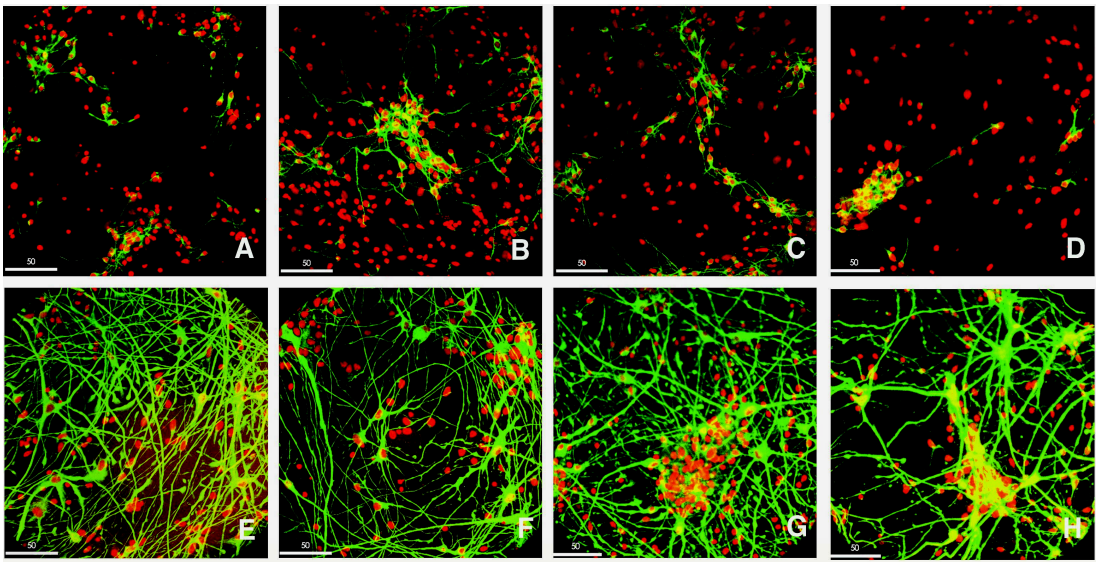


Fig. 4-3 The effects of FK506 (5 ng/ml) (B,F), BDNF (5 ng/ml) (C,G) and FK506 (5 ng/ml) combined with BDNF (5 ng/ml) (D,H) in second trimester HBC. All nuclei are stained with propidium iodide (red). (A–D) Fluorescent immunostaining for neuronal marker MAP-2 (green). (E–H) Fluorescent immunostaining for astrocytic marker GFAP (green). Compared to untreated control (A), cultures treated with FK506 (B) exhibit increased cell numbers (including cellular aggregates, arrow) and expression of MAP-2, as well as longer dendrites. Following BDNF (C) and combined (D) treatments, cell numbers are also increased, but not the MAP-2 surface. Area covered by GFAP is significantly decreased following the combined treatment with FK506 and BDNF (H) when compared to control (E). Thinner, sparser astroglial processes (F) are noted in FK506-treated cultures. Immunofluorescent laser confocal microscope images. Scale bar 50 μm.

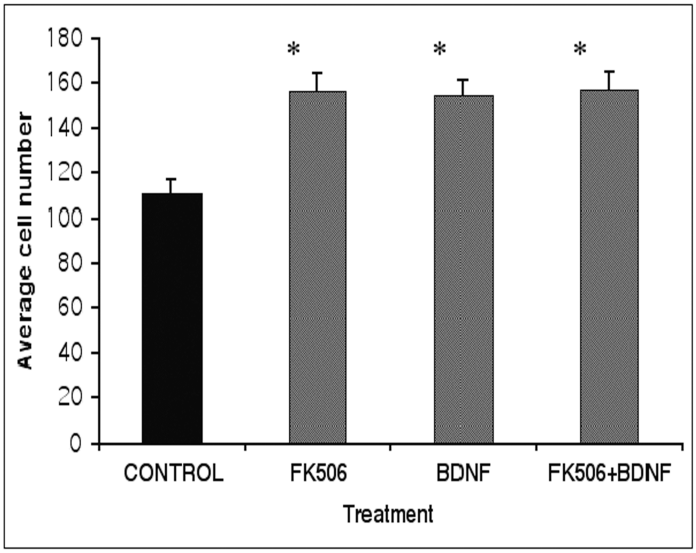


Fig. 4-4 Cell numbers in HBC increase following administration of FK506, BDNF or the combination of FK506 and BDNF (DIC 12–19). Control: n=60; FK506: n=72; BDNF: n=60; FK506+BDNF: n=48. One-way ANOVA: P,0.001. Tukey's test: *P,0.001 vs. control. Bars represent S.E.M.

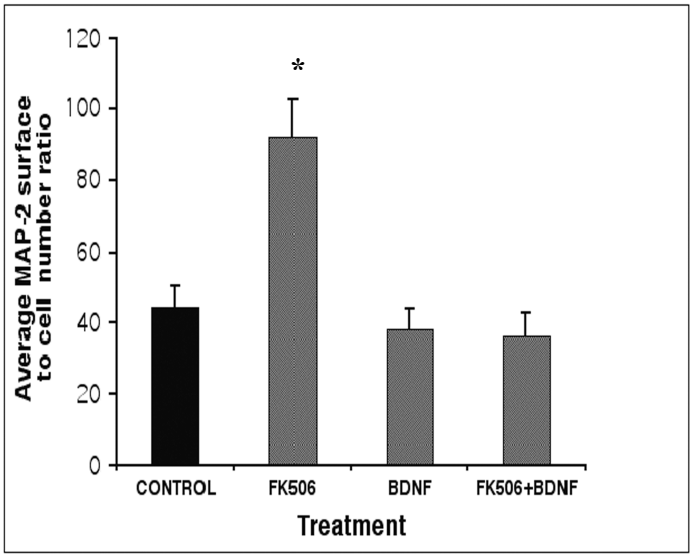


Fig. 4-5 The effect of FK506, BDNF or combined FK506 and BDNF treatments on neuronal growth in HBC. MAP-2 expression increases following FK506 treatment. Control: n=48; FK506: n=66; BDNF: n=42; FK506+BDNF: n=30. One-way ANOVA: P=0.001. Tukey's test: *P< 0.003 vs. control. Bars represent S.E.M.

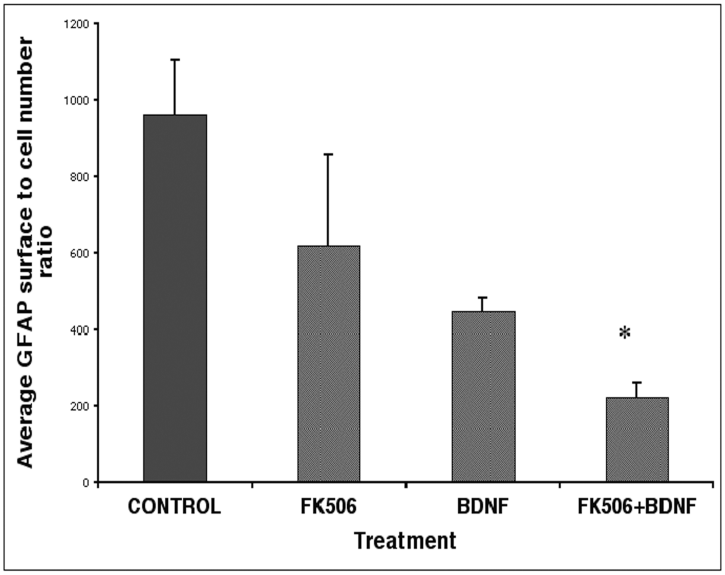


Fig. 4-6 The effect of FK506 and BDNF treatments (DIC 12–19) on astroglial growth in HBC. GFAP markedly decreases after treatment with a combination of FK506 and BDNF. Control: n=33; FK506: n=39; BDNF: n=24; FK506+ BDNF: n=21. One-way ANOVA: P=0.049. Tukey's test: *P=0.04 vs. control. Bars represent S.E.M.

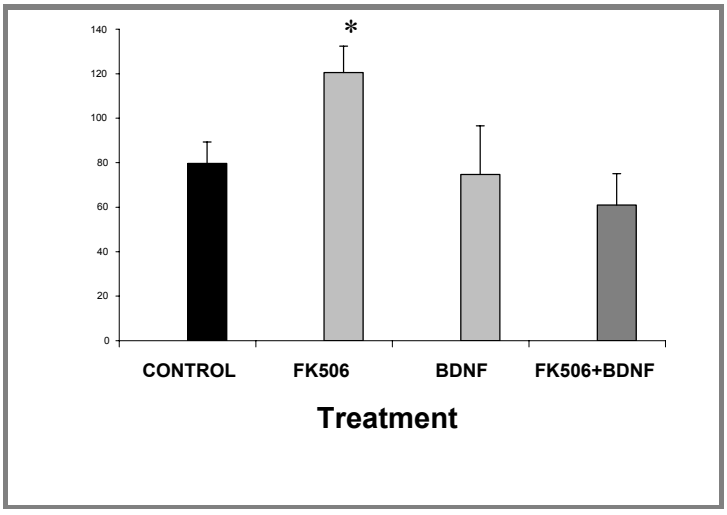


Fig. 4-7. The number of NeuN-labeled neurons in HBC increases following FK506 administration (DIC 12–19). Control: n=30; FK506: n=30; BDNF: n=24; FK506+BDNF: n=24. One-way ANOVA: P=0.022. Tukey's test: *P=0.009 vs. control. Bars represent S.E.M.

We examined the effects of 7-days BDNF (5 ng/ml) treatments on HBC starting at DIV 12. BDNF induced a significant increase in cell numbers ($p < 0.001$), as revealed by propidium iodide staining of nuclei (Fig. 4-3, 4-4). However, the ratio of MAP-2 surface to cell number in BDNF- treated cultures was not significantly different from control (Fig. 4-5). A decrease in the surface occupied by the astrocytic marker GFAP was also noted (Fig. 4-3, 4-6).

FK506 (5 ng/ml) was applied to HBC at DIV 1, 5, 12 and 21. At DIV 1 and 5, after an initial acceleration of neurite sprouting (DIV 2-3) when compared to non-treated samples, the drug-treated cells failed to attach adequately (results not shown). When FK506 was applied every other day, for 7 days, starting at DIV 12, the neurotrophic effects were significant. The drug-treated cultures showed increased numbers of cells when compared to untreated controls (Fig. 4-3, 4-4: $p < 0.001$, 4-9). The effect on cell survival or proliferation was comparable to the one exerted by BDNF and the combination of FK506 and BDNF. We subsequently identified the cell phenotypes influenced by FK506, by employing the neuronal marker MAP-2 (microtubule-associated protein 2) and the astrocytic marker GFAP (glial fibrillary acidic protein). The ratio of marker surface to propidium iodide- stained nuclei was calculated as an average of 10 section planes per field. This technique has been used in the past in numerous studies (Masliah et al., 1992, 1997; White et al., 1999). The significant increase in the area occupied by MAP-2 positive neuronal dendrites (Fig. 4-3) and in the MAP-2 surface/ cell number ratio (Fig. 4-5: $p = 0.003$) suggested that FK506 improves neuronal survival and differentiation in HNC. The neuronal nuclear marker NeuN was consequently employed to confirm that neuronal numbers are indeed increased following FK506 treatments. Quantitative analysis of the NeuN positive nuclei supports this conclusion (Fig. 4-7:$p= 0.01$). The dendritic surface in FK506- treated cultures is significantly greater than in cultures treated with BDNF ($p < 0.001$). As shown in Fig. 4-3, the neuronal processes stained for MAP-2 were longer and thicker in FK506 treated cultures than both in untreated controls and cultures receiving other treatments. The size and number of cellular aggregates present in culture were also increased (Fig. 4-3). In addition, immunocytochemical analysis using the SMI312 marker indicates that FK506 also promotes axonal extension (Fig. 4-8).

The absolute area covered by the astrocytic marker GFAP significantly diminished following FK506 treatment (data not shown) and the glial processes appear slightly sparser and thinner (Fig. 4-3). Although the drug- induced decrease in GFAP surface/ cell number ratio does not reach statistical significance, the drug potentiates the BDNF- induced decrease in astrocytic processes extension (Fig. 4-6: $p = 0.041$). This suggests that FK506 might exert an effect on glial cells, too, by reducing their number or inhibiting the extension of processes. We therefore tested the effects of 7-day FK506 treatments in pure human fetal astrocytic cultures at different time points (DIV 5, 7 and 12). In this system, FK506 did not induce changes in cell numbers or in the growth of GFAP- and S100- positive astrocytic processes.

The combination of BDNF (5 ng/ml) and FK506 (5 ng/ml) did not yield an enhancement of the neurite outgrowth, although it induced a marked increase in cell numbers (Fig. 4-3, 4-4: $p < 0.001$). The MAP-2 immunostaining normalized for the cell number does not exceed control levels (Fig. 4-5). However, a strong effect of reducing the GFAP surface-cell number ratio was observed (Fig. 4-3, 4-6: $p = 0.041$).

Administration of 5ng/ml FK506 to BDNF- pretreated human neuroglial cultures did not reveal significant differences from control.

Combined FK506 and BDNF treatments in astrocytic cultures did not have an effect on cell growth.

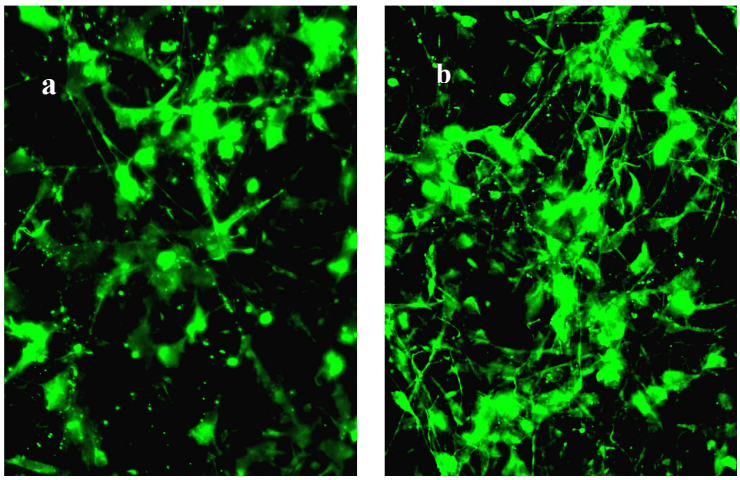


Fig. 4-8: FK506- induced axonal growth as evidenced by SMI312 labeling (FITC). a: untreated control, b: FK506 (5 ng/ml)- treated culture. Magnification: × 400

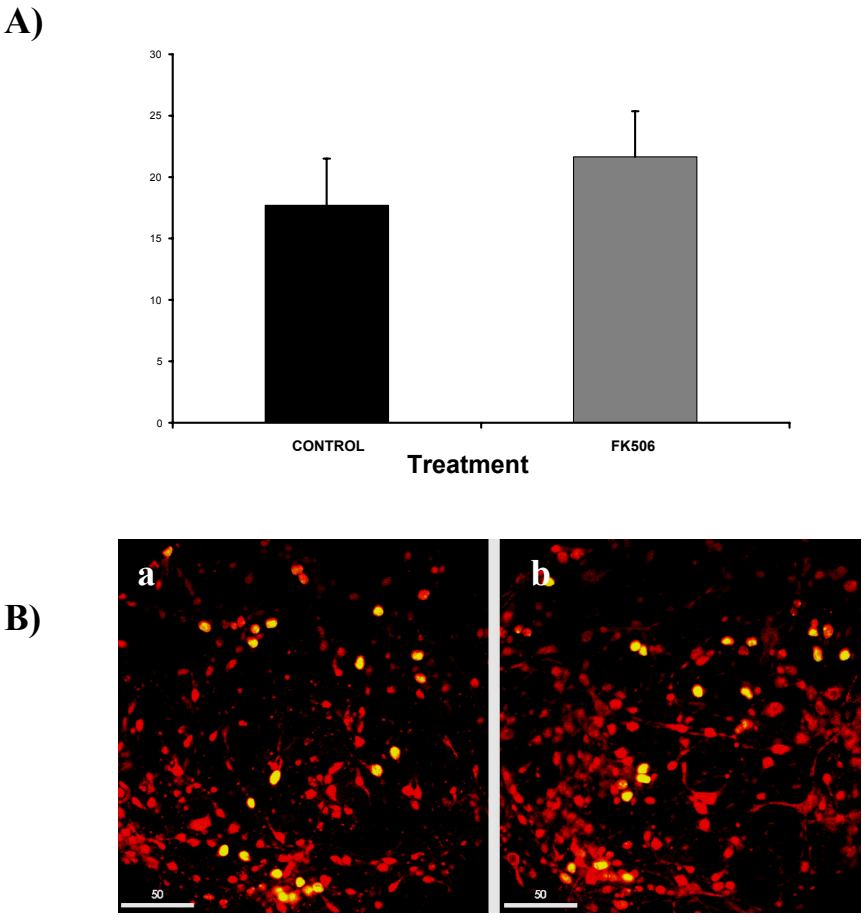


Fig. 4-9 FK506 does not induce a significant increase in the percentage of BrdU- labeled nuclei A) Percentage of BrdU- labeled nuclei in FK506- treated vs. non- treated cultures. Bars represent SEM. B) Laser confocal micrograph of HBC: nuclei are stained with propidium iodide (red); BrdU is green (FITC). Despite the increase in the total number of nuclei, the percentage of proliferating cells is not significantly elevated in FK506- treated cultures (b) compared to controls (a).

To better understand the substrate of FK506- induced increase in cell numbers, BrdU incorporation assays were performed. Despite the increase in the total number of nuclei, the percentage of proliferating cells is not significantly elevated in FK506- treated cultures (Fig. 4-9: A and B).

4.4 Discussion

FK506 increases neuronal numbers and neuritogenesis in human fetal brain cultures

This is the first study showing that the immunosuppressive macrolide FK506 has trophic effects on neurons in human brain primary cultures. The drug potency is remarkable, with only nanomolar amounts increasing cell numbers and neuritic extension. This finding is consistent with previous studies reporting trophic effects of FK506 in vitro in PC12 cells, animal sensory ganglia explants and embryonic dopaminergic cell cultures (Lyons et al., 1994; Costantini and Isacson, 2000) and in various animal models of nervous system injury (Costantini et al., 1998; Gold et al., 1994, 1995; Steiner et al., 1997; Madsen et al., 1998; Winter et al., 1999). Propidium iodide nuclear staining of second trimester brain cultures revealed a striking increase in cell numbers in cultures treated with FK506 (5 ng/ml). This increase was comparable to the one induced by BDNF (5 ng/ml). The combination of FK506 (5 ng/ml) and BDNF (5 ng/ml) also produced a prominent effect on cell survival and or proliferation.

To identify the cell phenotype that is mostly affected by different treatments, the cultures were immunostained for the neuronal marker MAP-2 and the astrocytic marker GFAP. We previously showed that, in this in vitro system, BDNF promotes neuronal growth and differentiation (White et al., 1999). In the present study, MAP-2 immunostaining is not increased following BDNF treatment. However, FK506 significantly increases the MAP-2 surface area per

cell. Since FK506 does not appear to increase cell size, this suggests a strong impact of the drug on neuronal differentiation. An increase in axonal extension was also noted. Neuronal processes appear longer and thicker than in untreated controls. This finding is in concordance with recent observations by Costantini and Isacson (2000), indicating that FK506 enhances neuritic elongation in rat embryonic dopamine neurons. Moreover, the number and size of cellular aggregates increase following FK506 administration. In view of the limitations in the image analysis system (i.e., insufficient resolution for discerning cells that are in close proximity), this can lead to an underestimation of the cell numbers. Therefore, FK506 can in fact induce an even stronger increase in cells growth than assessed. The combination of FK506 and BDNF did not alter the neuronal marker expression. To further ascertain the neuronal phenotype of the cells upon which FK506 acts, we employed the NeuN neuronal nuclear marker immunostaining. Neuronal nuclear counts suggested that FK506 by itself increases the number of neurons present in HBC.

In conclusion, all three treatments (FK506, BDNF and the combination of FK506 and BDNF) increase the number of cells in culture. FK506 increases the number of neurons and also promotes neurite outgrowth. When FK506 and BDNF are administered together, however, there is no significant increase in dendritic extension.

FK506 decreases astrocytic growth in human fetal brain cultures

Interestingly, in conjunction with BDNF, FK506 has a prominent capacity to reduce GFAP expression in individual cells. The astrocytic processes in cultures treated with FK506 and BDNF tend to be more sparse and thin. This effect can be the result of an overall stimulation of cell survival and differentiation, coupled with an inhibition of glial processes extension.

The combination of FK506 and BDNF does not increase neuronal numbers, while significantly enhancing the overall number of cells. Since neurons and astrocytes are the main cellular populations in these mixed cultures, it is reasonable to assume that this treatment regimen can increase astrocytic numbers. From this point of view, the observed decrease in GFAP expression is even more significant and indicates an authentic growth reduction. However, the inhibition of cell growth is not present in pure astrocyte cultures, which suggests a neuronal-mediated mechanism of action (data not shown). The same trend to decrease the growth of astrocytic processes was observed in FK506-treated cultures, although the decrease in GFAP expression

did not reach statistical significance. In conclusion, FK506 by itself is likely to exert its effects through an increase in neuronal differentiation and neuritic extension, associated with a tendency to decrease GFAP expression. This finding is important for the potential clinical uses of FK506, as levels of GFAP are regulated under developmental and pathological conditions. Upregulation of GFAP is a characteristic of the astrogliosis commonly observed in CNS injury (Bignami and Dahl, 1995). GFAP- positive cell counts are necessary to verify the presence or absence of increased astrocytic counts in human brain cultures treated with FK506 and the combination of FK506 and BDNF.

FK506 and BDNF appear to act synergistically to decrease astrocytic processes extension in HNC. FK506 administration in BDNF- pretreated cultures did not lead to significant alterations in neuronal or glial markers. Therefore, it appears that the concurrent action of the two neurotrophic agents is required for the effect to occur. A possible substrate for the FK506-induced neuro- glial effects can be an alteration in the synthesis and/or release of neurotrophic factors in culture.

When the treatments are performed in HBC starting at a late time point, i.e. day 21 in culture, no significant effect on glial cells is observed (results not shown). This suggests that the substrate of decreased astrocytic processes extension in treated cultures is growth inhibition, rather than retraction of processes. On the other hand, demonstrating the growth inhibition by the drug–neurotrophic factor combination is difficult, since FK506 administration at early time points in culture (DIV 1, 5) interferes with cell attachment.

Potential mechanisms for the neurotrophic effects of FK506

In the past decade, several mechanisms have been proposed for the effects of FK506. Apparently, the substrates for neuroregenerative and neuritic growth effects differ from the neuroprotective ones. Recent studies in FKBP12$^{-/-}$ mice (Gold, 1999; Dawson et al., 2001) suggested that the immunophilin FKBP12 is not responsible for the regenerative effects of FK506, but rather FKBP52. The drug binds FKBP52/ hsp90 and exerts its neuroregenerative effects via disruption of steroid receptor complexes and independently of calcineurin (Gold, 1999). It is still possible that FKBP12 plays a role in mediating neuroprotective effects of FK506

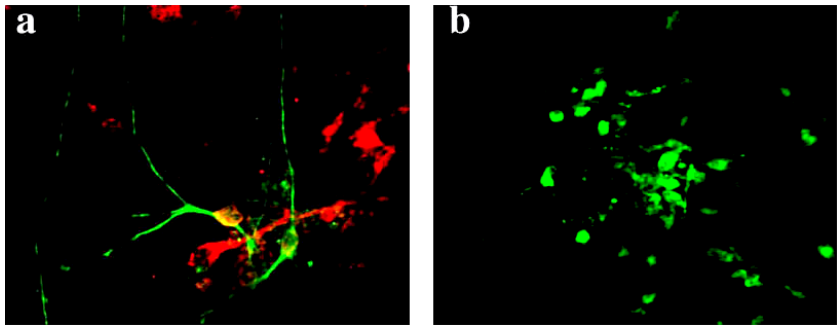


Fig. 4-10 FKBP12 ((a), FITC) and FKBP52 (b) are expressed in human neurons in vitro at DIV20. FKBP12 often colocalizes with calreticulin ((a), Cy5) in neuronal cell bodies. Magnification: ×400

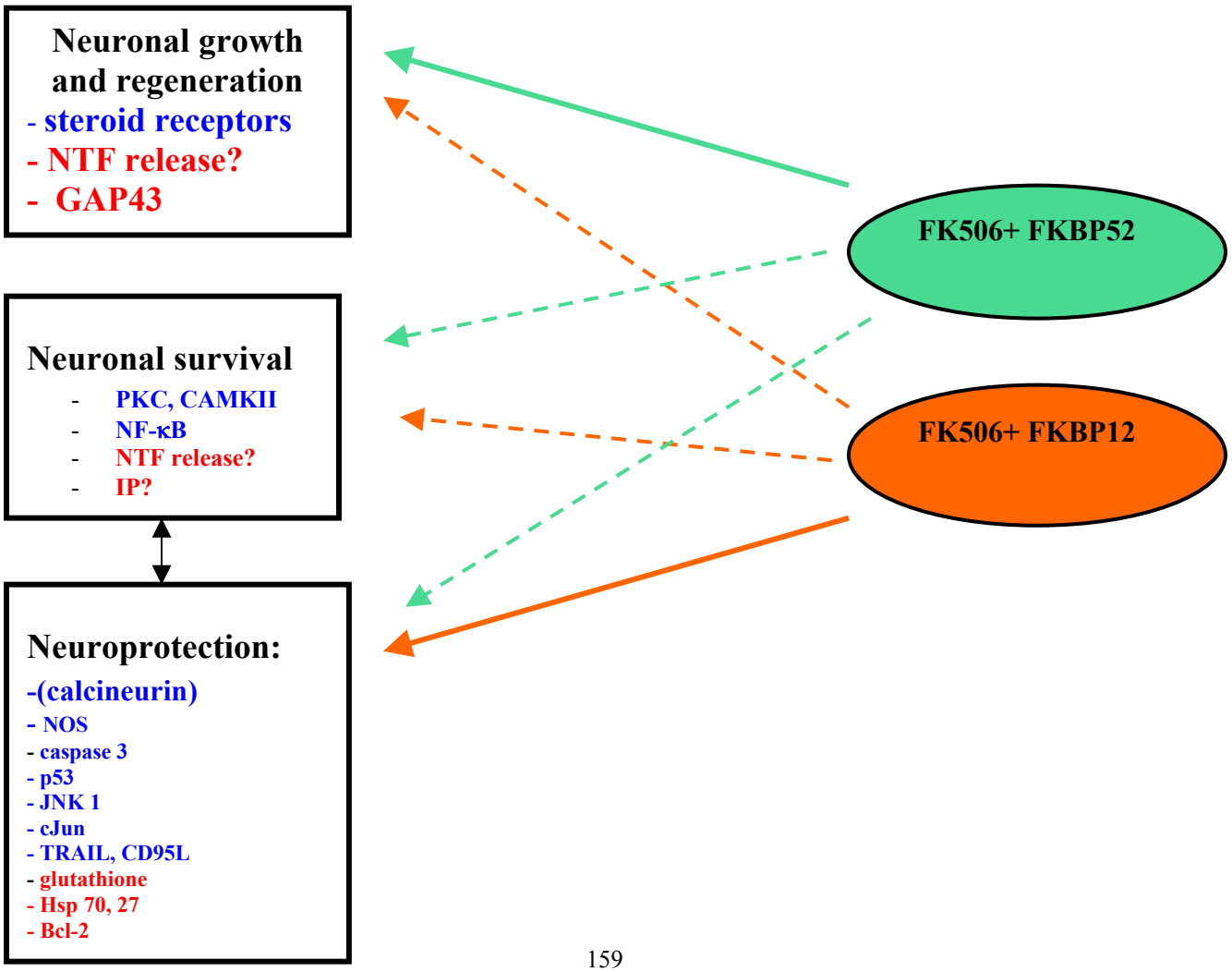


Fig. 4-11 Model of neurotrophic and protective effects of FK506- FKBP complexes. Solid arrows: experimentally demonstrated effects; dashed arrows: hypothetical pathways. Potential effectors shown in blue are inhibited, the ones depicted in red are elevated or induced.

or analogues, and perhaps in human brain cells survival and growth (Fig. 4-11). Both FKBP are expressed in human brain cells at DIV 20. FKBP12 is distributed in neuritic processes, as well as in cell bodies, where it often colocalizes with the endoplasmic reticulum protein calreticulin (Fig. 4-10 (a)). In contrast, FKBP52 is present predominantly in cell bodies, intranuclear or perinuclear (Fig. 4-10 (b)), which is consistent with its association with steroid receptors.

The neurotrophic effects of FK506 in HBC could be mediated through several, potentially overlapping, mechanisms:

1. FK506 – FKBP12/52 complexes acting upon proteins involved in cell survival, growth and differentiation (such as calcineurin, GAP-43, cell cycle proteins, steroid receptors)
2. drug-induced blockage of FKBP12 interaction with intracellular Ca^{2+} receptors
3. altered synthesis or release of neurotrophic factors in culture.
4. altered cytokine levels in culture
5. changes in free radical levels
6. altered neuronal chaperone (including IP) levels or activity

Increased neuronal survival induced by FK506 was demonstrated both following toxic injury and under "physiologic" conditions (Castilho et al., 2000). FK506 has been shown to interfere with the apoptotic pathway in rodent neuronal cells, by inhibiting JNK activity, cytochrome c release, caspase 3 activation, and CD95 ligand expression (reviewed by Klettner and Herdegen, 2003). Moreover, Klettner et al. (2001) showed that FKBP-ligands confer their neuroprotection by rapid de novo synthesis of (functionally) anti-apoptotic proteins. These effects are in part occurring through calcineurin inhibition. In a recent study (Muramoto, 2003), for example, the suppressive effects of FK506 on cell death in thapsigargin- treated SH-SY5Y

cells are caused by the inhibition of calcineurin and subsequent suppression of caspase-3 activation. Several studies argued against calcineurin involvement, based on the trophic effect of non- calcineurin inhibiting FK506 analogues. The ability of these derivatives to induce neuronal growth and protection is, however, questionable. Therefore, calcineurin inhibition cannot be ruled out as a mediator of the anti- apoptotic, pro- survival effects of FK506. Recent studies (Klettner et al., 2001) suggest that the protective properties of FK506 and its non-calcineurin inhibiting derivatives are achieved by a fast induction of heat shock proteins. Our data also suggest that it is cell survival that is increased by FK506, not rate of multiplication, and that the FKBP12 chaperone levels increase in treated human brain cells. To fully rule out the effect on proliferation, further studies are needed that employ different BrdU incubation times and time points in vitro. In addition, blocking studies using rapamycin (which competes with FK506 for FKBP12 binding sites) and non- IPL calcineurin inhibitors would be useful in elucidating the mechanisms of FK506 anti- apoptotic and neuritogenetic actions. Finally, the contribution of FKBP12 can be ascertained *in vitro* using blocking antibodies (less reliable, given their intrinsic neurotrophic effects) or antisense oligonucleotides.

The fact that FK506 promotes neuritic growth, but does not have this effect when combined with BDNF, suggests a possible competition for a common neuronal intracellular signaling pathway. It is tempting to speculate that this pathway, downstream of the BDNF trk B receptor, might be involved in a cross- talk with effectors situated on the FK506- immunophilin signaling stream. Activation and, repectively, repression of transcription factor NF-kappaB activation could be an essential molecular event in the BDNF (Rodriguez- Kern, 2003) and FK506- induced signaling pathways. In drug-hypersensitive PC12m3 cells, FK506 exerts neuritogenetic effects via a novel MAP kinase pathway. The BDNF-dependent activation of NF-kappaB is critically dependent on the upstream activation of p42/p44 MAP kinase signaling. In astrocytes, however, the two neurotrophic agents appear to act synergistically, therefore two different intracellular mechanisms could be involved in neurons and glia. A recent study by Zwadzka and Kaminska (2003) demonstrated a growth-inhibitory effect of FK506 on cortical astrocytes from newborn rats, which confirms our findings of decreased astrocytic growth in human cell cultures. Here, FK506 was found to inhibit Erk and PI-3K/Akt signaling, two crucial pro-survival pathways. The levels of phosphorylated Akt and p42/44 Erk declined a few hours after FK506 addition. Furthermore, in FK506-treated astrocytic cultures, the levels of mRNA

encoding PDGF, bFGF, and CNTF decreased. Downregulation of growth factor expression by FK506 may play a role in the inhibition of mitogenic/ hypertrophic responses. FasL mRNA level was elevated and interaction of FasL with Fas receptor expressed in astrocytes could trigger cell death. Interestingly, BDNF expression increased in a dose-dependent manner in FK506-treated astrocytes. Upregulation of BDNF mRNA and protein level in astrocytes exposed to FK506 may underlie the neuroprotective action of FK506 in mixed (neuronal and glial) cultures. However, in our system, the biosynthesis and release of trophic factors from human neurons in culture might be required for the effects of FK506 to occur, hence the absence of the glial effects in pure astrocytic cultures. Trophic factors could act upon neurons via an autocrine/ paracrine mechanism to promote growth and differentiation, and perhaps also influence glial growth. This mechanism of survival and growth increase might function in conjunction with an FK506-induced decrease in proinflammatory cytokines and increase in glutathione levels. Both glutathione and glutathione peroxidase are involved in the reduction of hydrogen peroxide and organic peroxides.

The mechanisms that regulate growth cone behavior and axon branching include dynamic changes in intracellular calcium signaling, in addition to the reorganization of the actin and microtubule cytoskeleton and the effects of axon guidance molecules (reviewed by Dent et al., 2003). However, the incidence, frequencies, and amplitudes of calcium transients are inversely related to rates of axon outgrowth (Tang et al., 2003). FK506 dissociates FKBP12 from the intracellular ER Ca^{2+} receptors, where the IP exerts a stabilizing effect. The drug can induce increased intracellular Ca^{2+} levels that would slow axonal growth. The versatility of the cellular calcium signaling has to be achieved within the context of calcium being highly toxic. When exceeding its normal spatial and temporal boundaries, it can result in cell death through both necrosis and apoptosis. Anti-apoptotic regulators such as Bcl-2 may act by reducing the flow of Ca^{2+} through the ER/mitochondrial couple (reviewed by Berridge, 2002). Therefore, it is possible that, in HBC, FK506 does not induce increased intracellular Ca^{2+} levels, therefore other mechanisms are likely to come into play in FK506- enhanced cell survival and neuritogenesis.

Interestingly, our preliminary observations in FK506- treated HBC suggest that FKBP12 expression in neurons in vitro can be altered following FK506 (5 ng/ ml) administration for 7

days. (Fig 4-12). In light of the potential role of FKBP12 in the pathogenesis of neurodegeneration (see Chapter 3), it is conceivable that this mechanism might play a role in generating the observed neuroprotective effects of FK506 in vivo in animal models of DA degeneration. The induction of a chaperone response by immunophilin ligands might become a useful target for neuroregeneration and neuroprotection.

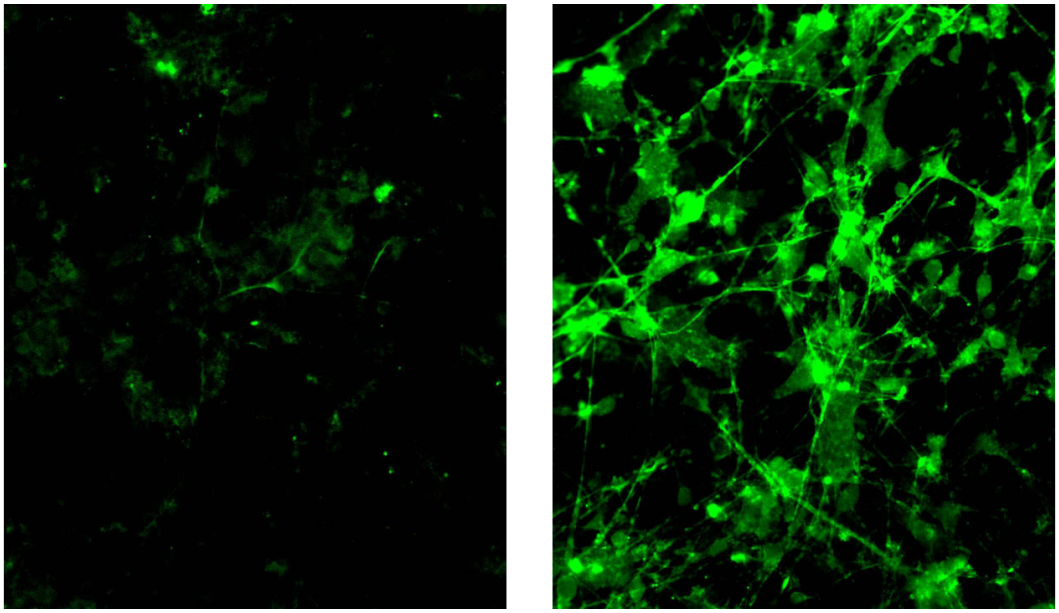


Fig. 4-12 Increased FKBP12 expression in FK506 (5 ng/ml)- treated HBC in cell bodies and neurites. (a)- control, (b)- FK506- treated culture. Magnification: × 400.

Given the polymorphism of IPL effects, it is difficult to establish the exact mechanisms of their actions in neural cells in vitro and in vivo. A meta- analysis of studies available to date is hindered by the heterogeneity of drug dosages and experimental paradigma. However, we do know that IPL bind to the respective FKBP and CyP IP receptors and inhibit their rotamase activity, as well as engage downstream effectors. The model we propose (Fig. 4-11) takes into consideration a multitude of studies conducted in various systems, as well as our own data and hypotheses (reviewed by Snyder, 1998; Hamilton, 1999; Gold, 1999; Klettner, 2003; Avramut and Achim, 2001 and 2003). While FKBP52 participates predominantly in neuronal regeneration

and neuritic outgrowth, it is possible that FKBP12 plays a role mainly in survival processes and neuroprotective pathways. Regardless of the identity of the effectors downstream of the FK506-FKBP complexes, which might be common to a multitude of pathways, ascertaining the presence and functions of brain IPs is necessary, if we are to employ these compounds in the pharmacologic and transplantation therapy of neurodegenerative diseases.

Conclusions and significance

Our findings have several implications. First, they support an immediately available candidate for therapy in neurodegenerative diseases. FK506, already FDA-approved for human use, is a small molecule with ready access to the nervous system. It was shown to act primarily on lesioned neurons, while sparing the healthy ones (Snyder et al., 1998). This is not true for "traditional" neurotrophic peptides, which have less specific effects. Second, as the drug selectively promotes neuronal population growth while potentially inhibiting astrocytosis, it can specifically target neuronal regeneration in patients with neural cell transplants. Potential limitations for the use of tacrolimus include its toxicity. Neurologic adverse effects (including confusion, seizures, tremors, headache) are mostly associated with intravenous administration and generally subside with oral use or dosage reduction (Letko et al., 1999). Our findings may provide an insight into the effects of FK506 on the CNS of transplant patients. Finally, as it is the case for a number of immunosuppressive agents, the effects of this immunophilin ligand may help elucidate intracellular signaling mechanisms mediated by immunophilins, an area of research still in its infancy. If FK506 and its analogues are to be used in the treatment of neurodegenerative diseases, a knowledge of the presence and distribution of their IP receptors in the human brain is necessary. We showed that FKBP12, the archetypal member of the FKBP family, is present in the human brain (see Chapter 3). Moreover, it is implicated in the pathogenesis of neurodegenerative processes. It is possible that the isomerase activity of FKBP contributes to their function in vivo in the human brain. If this is the case, treatments of patients with FK506 and analogues should be carefully considered, mainly from the point of view of adequate dosage. Studies in rodents with cerebral ischemia (Brecht, 2003) have shown that FKBP enzymatic activity in the infarct area was increased and could be blocked dose dependently by FK506, despite the lack of drug- induced changes in FKBP expression levels. Therefore, it is reasonable to believe that high dosages of FK506 (or analogues), while

stimulating neuronal regeneration, might block rotamase activity that is needed in the protection of these cells against various injuries. This aspect is also significant since IPL can be designed based on the structure of FK506 that can selectively bind to FKBP12 or FKBP52 and modulate the activity of these IP to induce neuronal growth and regeneration.

5. General conclusions

5.1. Summary of findings

This is the first study showing that the immunosuppressive macrolide FK506 has trophic effects on neurons in human brain primary cultures (HBC). Our observations in FK506- treated HBC suggest that FKBP12 expression in neurons in vitro can be altered following FK506 administration. In light of the potential role of FKBP12 in the pathogenesis of neurodegeneration, it is possible that this mechanism plays a role in generating the observed neuroprotective effects of FK506 in vivo in animal models of DA degeneration. The induction of a chaperone response by immunophilin ligands might become a useful target for neuroregeneration and neuroprotection.

We have also shown for the first time that the immunophilin FKBP12, a peptidyl- prolyl *cis- trans* isomerase and a receptor for immunosuppressive drugs, is present in the human brain. Among the regions analyzed, high levels of FKBP12 are found in the substantia nigra- deep gray matter axis. This is consistent with previous autoradiographic studies in rodents. Significant expression is also present in the hippocampus. The mid- frontal cortex of normal aging human brain has low, often undetectable amounts of FKBP12.

In normal subjects, FKBP12 expression is exclusively neuronal. The IP is present in cell bodies, processes and neuropil. It often exhibits a predominantly perinuclear, polarized distribution, suggesting the association with an intracellular organelle, possibly the endoplasmic reticulum. The expression of the immunophilin FKBP12 is altered in the mesencephalon, deep gray matter, hippocampus and mid- frontal cortex of patients with neurodegenerative diseases,

including HIV- induced degeneration. In degenerating brains, FKBP12 associates with α-synuclein, tau and amyloid lesions. Disease- specific and region- specific changes in FKBP12 expression are evident. Our extensive study suggests that FKBP12 is present at higher levels in neuronal circuits that are spared in the degeneration process (e.g., in the hippocampus). Cellular FKBP12 expression is often inversely correlated with predicted neuronal vulnerability and actual degeneration in several brain regions and diseases (e.g, in the substantia nigra, SN). Thus, FKBP12 can be an important factor in protecting against age-dependent neurodegeneration, providing insight into the pathogenesis and treatment of PD and AD. However, high FKBP12 levels can also be present in degenerating neurons. In this case, the IP doesn't have a diffuse cytoplasmic distribution, but associates with LB and tangles. This further suggests a potential role for FKBP12 in mitigating the protein aggregation processes.

The ability of various subsets of neurons to mount an efficient rotamase (including FKBP) response might represent a key to their resistance- or selective vulnerability- to death in neurodegenerative diseases. In HIVE, there is a high content of FKBP in the basal ganglia, which are a preferential target for virus- induced neuronal damage. In this instance, FKBP12 might function as a co- factor for viral entry and chemokine- induced neuronal damage.

In accordance to previous findings on other chaperone proteins, we envision a dual role for FKBP12: on one hand, it mitigates misfolded proteins, aggregation and fibrillization; on the other hand, in the process, high levels of toxic protein species can be maintained, leading to further neuronal injury.

Relatively high levels of FKBP12 are noted in the nigral neuronal cell bodies of AD, DLB and HIV patients. In PD patients, in addition to FKBP12- positive neuronal inclusions, there are extracellular globular deposits immunoreactive for FKBP12. In AD, intracellular FKBP12 has a diffuse appearance, which contrasts with the predominant perinuclear distribution observed in other regions (e.g., dorsal raphe nucleus) and in normal subjects. Therefore, the increase in IP may be associated with a translocation between cellular compartments.

5.2. Relevance for the diagnosis and treatment of neurodegenerative diseases

Neurotrophic IPL "represent the first instance of agents that make damaged nerves grow back with restored function." (S. Snyder, 1998). They are orally active small molecules,

therefore their clinical use should not be limited by the drug delivery problems associated with protein and peptide growth factors. An additional important distinction from many neurotrophic polypeptides is that the IPL do not appear to induce aberrant sprouting of neuronal processes when administered to normal animals. In our study, FK506 selectively promotes neuronal population growth while potentially inhibiting astrocytosis, therefore it can specifically target neuronal regeneration in patients with neural cell transplants. These properties render IPL usable in a range of neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, traumatic injuries and stroke. However, the use of FK506 and its analogs in the treatment of neurodegenerative disorders would require the presence of IP receptors, i.e. FKBP, in the human brain. Moreover, since the drug inhibits the potentially important enzymatic activity of the IP, an assessment of FKBP levels and activities in areas of pathology is important.

We showed that FKBP12, the archetypal member of the FKBP family, is present in the human brain. Moreover, its levels are altered in the brain of patients with PD, DLB, AD and HIVE, suggesting that it is implicated in the pathogenesis of neurodegenerative processes. It is possible that the isomerase activity of FKBP contributes to their function in vivo in the human brain. If this is the case, treatments of patients with FK506 and analogues should be carefully considered, mainly from the point of view of adequate dosage. Studies in rodents with cerebral ischemia (Brecht, 2003) have shown that FKBP enzymatic activity in the infarct area was increased and could be blocked dose dependently by FK506, despite the lack of drug- induced changes in FKBP expression levels. Therefore, it is reasonable to believe that high dosages of FK506 (or analogues), while stimulating neuronal regeneration, might block rotamase activity that is needed in the protection of these cells against various injuries. This aspect is also significant since IPL can be designed based on the structure of FK506 that can selectively bind to FKBP12 or FKBP52 and modulate the activity of these IP to induce neuronal growth and regeneration.

The actual process of neuronal degeneration is not well understood, regardless of the disease under study. Where does the actual process of cell damage or death begin? Is cell death rapid or slow? Are neurons nonfunctional long before they die? If so, this would have therapeutic implications. And where does the actual process of cell damage or death begin? Is the *primum movens* occurring in a particular intracellular organelle such as the mitochondria or endoplasmic reticulum, or is it in the nerve terminal, with a subsequent dying-back phenomenon? Whatever

the process that leads to the demise of neurons, one needs to identify features that are common to the populations affected and the defense mechanisms they might employ.

Altered levels of FKBP12 chaperone in areas of degeneration suggest that it can become a novel marker of pathology. Although the precise role of this immunophilin in the normal and degenerating brain awaits further clarification, it is likely that FKBP play a role in neuroprotection against abnormal protein aggregation, as well as participate in axonal transport and synaptic vesicle assembly. Is the IP trying to fight aggregation in, for example, LB? Does it ultimately get "trapped" there? Is it functional? Augmented expression does not necessarily mean enhanced function. The significance of the presence of FKBP12 in characteristic neuropathological lesions (LB, neurofibrillary tangles, amyloid plaques) needs to be further explored. An intriguing possibility is that, indeed, the elevated IP expression contributes to the delay or prevention of misfolding and aggregation, which would bring a new factor in explaining the adult onset of the disease. The highest FKBP levels are often noted in AD and DLB, with an even later onset than PD.

The association of FKBP12 and FKBP52 with neuronal markers needs to be thoroughly explored. Knowledge of the subcellular distribution of these IP (i.e., the potential association with ER, calcineurin, cytoskeletal elements and synaptic vesicles) will provide essential information to help identify potential cellular roles of FKBP in the normal and diseased human brain.

Increased levels of proinflammatory cytokines, cytokine receptors and caspase activities, and reduced levels of neurotrophins were found in the nigrostriatal region in PD patients, and in MPTP- and 6OHDA-produced parkinsonian animals (Nagatsu et al., 2000). This suggests the existence of an increased immune reactivity and programmed cell death (apoptosis) of neuronal or glial cells. The presence of such proapoptotic environment in the substantia nigra in PD that may induce increased vulnerability of neuronal or glial cells towards a variety of neurotoxic factors. The causative linkage among the altered IP levels, increased levels of proinflammatory cytokines, decreased levels of neurotrophins, candidate parkinsonism-producing neurotoxins and the genetic susceptibility to toxic factors, needs further investigation. The increased cytokine levels, decreased neurotrophin ones, and the possible immune response in the nigrostriatal region in PD might justify the employment of new neuroprotective therapy including immunosuppressive or immunophilin-binding drugs

such as FK506. However, the FK506 family of drugs inhibits the enzymatic activity of their FKBP receptors. Therefore, until the role of FKBP in the degenerative process is clarified, this type of therapeutic agents should be considered with caution. If the rotamase activity of FKBP underlies their neuronal actions, therapeutic attempts using rotamase activity- inhibiting IP ligands administration in neurodegenerative diseases patients need to be carefully designed.

5.3. Future studies

Neurotrophic effects of IPL

FK506 effects in HBC are currently being compared to rapamycin. The goal is to ultimately use these compounds in vivo, in the primate model of PD, to increase the survival of the nigral neurons or the integration of human neuronal grafts implanted into the putamen. Several previous studies justify this approach, although the issue of immunosuppression in brain tissue transplantation is still controversial.

The role of FKBP in the brain

Due to the novelty of the findings regarding FKBP expression in the human brain, results will need to be replicated by using brain tissue from an increased number of human autopsy cases.

FKBP expression in vivo and in vitro at the protein and RNA levels will be analyzed in various systems. We will perform in vitro studies employing various injury paradigms and toxic treatments in cells overexpressing FKBP12 and in brain cultures from FKBP12 knockout mice. These in vitro studies will provide valuable information on the function of this IP.

Our analysis of FKBP12 levels in vivo in parkinsonian primates with various degrees of lesion is ongoing and can reveal the time frame of FKBP changes. Tract tracing, neurochemical identification of pathways containing FKBP12 and ultrastructural analyses will also be performed. The ability of MPTP to generate parkinsonian symptoms in FKBP12 knockout mice and the resulting phenotype will be analyzed. The expression of FKBP12 in other animal models (e.g., transgenic mice expressing mutant A53T human α-synuclein) will also be assessed.

The same series of autopsy and in vitro experiments will be used to ascertain the contribution of the IP FKBP52 to the degeneration process. Our preliminary data (Avramut and

Achim, 2003) indicate that this IP is also expressed in the human brain and that its pattern of expression is different from FKBP12.

BIBLIOGRAPHY

6. Bibliography

1. Almenar-Queralt A, Goldstein LS. Linkers, packages and pathways: new concepts in axonal transport. *Curr Opin Neurobiol*. 2001; 11(5): 550-7.

2. Anderson ER, Boyle J, Zink WE, Persidsky Y, Gendelman HE, Xiong H. Hippocampal synaptic dysfunction in a murine model of human immunodeficiency virus type 1 encephalitis. *Neuroscience.* 2003; 118(2):359-69.

3. Andreeva L, Heads R, Green CJ. Cyclophilins and their possible role in the stress response. *Int J Exp Pathol.* 1999; 80(6): 305-15.

4. Arie JP, Sassoon N, Betton JM. Chaperone function of FkpA, a heat shock prolyl isomerase, in the periplasm of Escherichia coli. *Mol Microbiol*. 2001; 39(1): 199-210.

5. Armstrong RA. Is the clustering of neurofibrillary tangles in Alzheimer's patients related to the cells of origin of specific cortico-cortical projections? *Neurosci Lett.* 1993;160(1):57-60.

6. Arriagada PV, Growdon JH, Hedley-Whyte ET, Hyman BT. Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology.* 1992;42(3 Pt 1):631-639.

7. Asai A, Qiu J, Narita Y, Chi S, Saito N, Shinoura N, Hamada H, Kuchino Y, Kirino T. High level calcineurin activity predisposes neuronal cells to apoptosis. *J Biol Chem.* 1999; 274(48):34450-8.

8. Auluck PK, Chan HY, Trojanowski JQ, Lee VM, Bonini NM. Chaperone suppression of alpha-synuclein toxicity in a Drosophila model for Parkinson's disease. *Science.* 2002 ;295(5556):865-8.

9. Avramut M, Achim CL. Immunophilins and their ligands: insights into survival and growth of human neurons. Physiology and Behavior 2002(77): 463- 468.

10. Avramut, M, Achim CL. Immunophilins in nervous system degeneration and regeneration. Curr. Top. Med. Chem. 2003; 3(12):1376-82.

11. Bang H, Muller W, Hans M, Brune K, Swandulla D. Activation of Ca2+ signaling in neutrophils by the mast cell- released immunophilin FKBP12. *Proc. Natl. Acad. Sci. USA* 1995; 92:3435-3438.

12. Bassing CH, Shou W, Muir S, Heitman J, Matzuk MM, Wang XF. FKBP12 is not required for the modulation of transforming growth factor beta receptor I signaling activity in embryonic fibroblasts and thymocytes. Cell Growth Differ. 1998; 9(3):223-8.

13. Bavetta, S., P.J. Hamlyn, G. Burnstock, A.R. Liebermann, Anderson, P.N. The effects of FK506 on dorsal column axons following spinal cord injury in adult rats: Neuroprotection and local regeneration. *Experimental Neurology* 1999; 158: 382-393.

14. Beal MF. Mitochondria, oxidative damage, and inflammation in Parkinson's disease. *Ann N Y Acad Sci.* 2003 ;991:120-31.

15. Bennett PC, Singaretnam LG, Zhao WQ, Lawen A, Ng KT. Peptidyl-prolyl-cis/trans-isomerase activity may be necessary for memory formation. *FEBS Lett*. 1998 Jul;431(3):386-90.

16. Bennett PC, Moutsoulas P, Lawen A, Perini E, Ng KT. Novel effects on memory observed following unilateral intracranial administration of okadaic acid, cyclosporin A, FK506 and [MeVal4]CyA. *Brain Res*. 2003; 988(1-2):56-68.

17. Berger J.R., Arendt, G. HIV dementia: the role of the basal ganglia and dopaminergic systems. *J Psychopharmacol*, 2000; 14(3): p. 214-21.

18. Berridge MJ. The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium* 2002; 32(5-6): 235-249.

19. Betarbet R, Sherer TB, Greenamyre JT. Animal models of Parkinson's disease. *Bioessays*. 2002;24(4):308-18.

20. Bierer BE, Mattila PS, Standaert RF, Herzenberg LA, Burakoff SJ, Crabtree G, Schreiber SL. Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. *Proc Natl Acad Sci U S A*. 1990;87(23):9231-5.

21. Bierer BE. Cyclosporin A, FK506, and rapamycin: binding to immunophilins and biological action. *Chem Immunol*. 1994; 59128-55.

22. Bondareff W, Harrington C, Wischik CM, Hauser DL, Roth M. Immunohistochemical staging of neurofibrillary degeneration in Alzheimer's disease. *J Neuropathol Exp Neurol.* 1994;53(2):158-64.

23. Bose S, Weikl T, Bugl H, Buchner J. Chaperone function of hsp90- associated proteins. Science 1996; 274: 1715- 1718.

24. Bossard MJ, Bergsma DJ, Brandt M, Livi GP, Eng W-K, Johnson RK, Levy MA. Catalytic and ligand binding properties of the FK506 binding protein FKBP12: effects of the single amino acid substitution of Tyr82 to Leu. *Biochem. J*. 1994; 297: 365- 372.

25. Bouras C, Hof PR, Morrison JH. Neurofibrillary tangle densities in the hippocampal formation in a non-demented population define subgroups of patients with differential early pathologic changes. *Neurosci Lett*. 1993 ;153(2):131-5.

26. Braak H, Braak E, Grundke-Iqbal I, Iqbal K. Occurrence of neuropil threads in the senile human brain and in Alzheimer's disease: a third location of paired helical filaments outside of neurofibrillary tangles and neuritic plaques. *Neurosci Lett*. 1986 ;65(3):351-5.

27. Braak H, Braak E. Demonstration of amyloid deposits and neurofibrillary changes in whole brain sections. *Brain Pathol*. 1991;1(3):213-6.

28. Brecht S, Schwarze K, Waetzig V, Christner C, Heiland S, Fischer G, Sartor K, Herdegen T. Changes in peptidyl-prolyl cis/trans isomerase activity and FK506 binding protein expression following neuroprotection by FK506 in the ischemic rat brain. *Neuroscience* 2003; 120(4):1037-48.

29. Brillantes A-M, Ondrias K, Scott A, Kobrinsky E, Ondriasova E, Moschella MC, Jayaraman T, Landers M, Ehrlich BE, Marks AR. Stabilization of calcium release channel (ryanodine receptor) function by FK506- binding protein. *Cell* 1994; 77: 513-523.

30. Brooks DJ. PET studies on the function of dopamine in health and Parkinson's disease. *Ann N Y Acad Sci.* 2003;991:22-35.

31. Brooks DJ, Frey KA, Marek KL, Oakes D, Paty D, Prentice R, Shults CW, Stoessl AJ. Assessment of neuroimaging techniques as biomarkers of the progression of Parkinson's disease. *Exp Neurol*. 2003;184 Suppl 1:68-79.

32. Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS, Schreiber SL. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature*. 1994;369(6483):756-8.

33. Bruce AJ, Boling W, Kindy MS, Peschon J, Kraemer PJ, Carpenter MK, Holtsberg FW, Mattson MP. Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. *Nat Med*. 1996; 2(7):788-94.

34. Bruening W.; Roy, J.; Giasson, B.; Figlewicz, A.;Mushynski, W. E.;Durham, H. D. Up-regulation of protein chaperone preserves viability of cells expressiong toxic Cu/Zn-superoxide dismutase mutants associated with amyotrophic lateral sclerosis. *J. Neurochem.* 1999; *72* (2), 693- 699.

35. Buki A, Okonkwo DO, Povlishock JT. Postinjury cyclosporin A administration limits axonal damage and disconnection in traumatic brain injury. J Neurotrauma. 1999;16(6):511-21.

36. Burnett PE, Barrow RK, Cohen NA, Snyder SH, Sabatini DM. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc Natl Acad Sci U S A*. 1998;95(4):1432-7.

37. Cameron AM, Steiner JP, Sabatini DM, Kaplin AI, Walensky LD, Snyder SH. Immunophilin fk506- binding protein associated with inositol 1,4,5- triphosphate receptor modulates calcium flux. *Proc. Natl. Acad. Sci. USA* 1995; 92: 1784-1788.

38. Capano M, Virji S, Crompton M. Cyclophilin-A is involved in excitotoxin-induced caspase activation in rat neuronal B50 cells. *Biochem J*. 2002; 363(Pt 1):29-36.

39. Castilho RF, Hansson O, Brundin P. FK506 and cyclosporin A enhance the survival of cultured and grafted rat embryonic dopamine neurons. *Exp Neurol*. 2000; 164(1): 94-101.

40. Chambraud B, Radanyi C, Camonis JH, Shazand K, Rajkowski K, Baulieu EE. FAP48, a new protein that forms specific complexes with both immunophilins FKBP59 and FKBP12. Prevention by the immunosuppressant drugs FK506 and rapamycin. *J Biol Chem.* 1996; 271(51):32923-9.

41. Chase TN, Oh JD, Blanchet PJ. Neostriatal mechanisms in Parkinson's disease. *Neurology*. 1998;51(2 Suppl 2):S30-5.

42. Chiesi A, Vella S, Dally LG, Pedersen C, Danner S, Johnson AM, Schwander S, Goebel FD, Glauser M, Antunes F, et al. Epidemiology of AIDS dementia complex in Europe. AIDS in Europe Study Group. *J Acquir Immune Defic Syndr Hum Retrovirol*. 1996 ;11(1):39-44.

43. Chiu MI, Katz H, Berlin V. RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. *Proc Natl Acad Sci U S A*. 1994;91(26):12574-8.

44. Chuang JZ, Zhou H, Zhu M, Li SH, Li XJ, Sung CH. Characterization of a brain-enriched chaperone, MRJ, that inhibits Huntingtin aggregation and toxicity independently. *J Biol Chem.* 2002;277(22):19831-8.

45. Cleveland D. W.;Liu, J. Oxidation versus aggregation- how do SOD1 mutants cause ALS? *Nat. Medicine* **2000**; *1*,1320-1321.

46. Clipstone NA, Crabtree GR. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature*. 1992; 357(6380):695-7.

47. Collins JH. Sequence analysis of the ryanodine receptor: possible association with a 12K, 506- binding immunophilin/ protein kinase C inhibitor. *Biochem. Biophys. Res. Comun*. 1991; 178:1288- 1290.

48. Costantini, L.C., P. Chaturvedi, D.M. Armistead, P.G. McCaffrey, T.W. Deacon and O. Isacson. A novel immunophilin ligand: distinct branching effects on dopaminergic neurons in culture and neurotrophic actions after oral administration in an animal model of Parkinson's disease. *Neurobiol. Dis.* 1998; 5: 97-106.

49. Costantini LC, Isacson O. Immunophilin ligands and GDNF enhance neurite branching or elongation from developing dopamine neurons in culture. *Exp Neurol*. 2000; 164(1): 60-70.

50. Costantini LC, Cole D, Chaturvedi P, Isacson O. Immunophilin ligands can prevent progressive dopaminergic degeneration in animal models of Parkinson's disease. *Eur J Neurosci.* 2001; 13(6): 1085-92.

51. Cotman CW. The beta-amyloid peptide, peptide self-assembly, and the emergence of biological activities. A new principle in peptide function and the induction of neuropathology. *Ann N Y Acad Sci.* 1997 ;814:1-16.

52. Crossman AR, Clarke CE, Boyce S, Robertson RG, Sambrook MA. MPTP-induced parkinsonism in the monkey: neurochemical pathology, complications of treatment and pathophysiological mechanisms. *Can J Neurol Sci*. 1987 ;14(3 Suppl):428-35.

53. Csermely P. Chaperone overload is a possible contributor to 'civilization diseases'. *Trends Genet.* 2001;17(12):701-4.

54. D'Andrea MR, Ilyin S, Plata-Salaman CR. Abnormal patterns of microtubule-associated protein-2 (MAP-2) immunolabeling in neuronal nuclei and Lewy bodies in Parkinson's disease substantia nigra brain tissues. *Neurosci Lett*. 2001;306(3):137-40.

55. Davis MR, Votaw JR, Bremner JD, Byas-Smith MG, Faber TL, Voll RJ, Hoffman JM, Grafton ST, Kilts CD, Goodman MM. Initial human PET imaging studies with the dopamine transporter ligand 18F-FECNT. *J Nucl Med*. 2003 Jun;44(6):855-61.

56. Dawson, T.M., J.P. Steiner, W.E. Lyons, M. Fotuhi, M. Blue, and S.H. Snyder. The immunophilins FK506 binding protein and cyclophilin are discretely localized in the brain: relationship to calcineurin. *Neuroscience* 1994; 62: 569-580.

57. DebBurman SK, Raymond GJ, Caughey B, Lindquist S. Chaperone-supervised conversion of prion protein to its protease-resistant form. *Proc Natl Acad Sci U S A*. 1997;94(25):13938-43.

58. Dickson DW, Crystal H, Mattiace LA, Kress Y, Schwagerl A, Ksiezak-Reding H, Davies P, Yen SH. Diffuse Lewy body disease: light and electron microscopic immunocytochemistry of senile plaques. *Acta Neuropathol (Berl).* 1989;78(6):572-84.

59. Dickson, D.W., et al., Microglia and cytokines in neurological disease, with special reference to AIDS and Alzheimer's disease. *Glia*, 1993; 7(1): p. 75-83.

60. Dickson TC, Vickers JC. The morphological phenotype of beta-amyloid plaques and associated neuritic changes in Alzheimer's disease. *Neuroscience*. 2001;105(1):99-107

61. DuBell WH, Lederer WJ, Rogers TB. K(+) currents responsible for repolarization in mouse ventricle and their modulation by FK-506 and rapamycin. *Am J Physiol Heart Circ Physiol.* 2000; 278(3):H886-97.

62. Dunnett SB, Bjorklund A. Prospects for new restorative and neuroprotective treatments in Parkinson's disease. *Nature*. 1999;399(6738 Suppl):A32-9.

63. Eberling JL, Pivirotto P, Bringas J, Steiner JP, Kordower JH, Chu Y, Emborg ME, Bankiewicz KS. The immunophilin ligand GPI-1046 does not have neuroregenerative effects in MPTP-treated monkeys. *Exp Neurol.* 2002;178(2):236-42.

64. Elsworth JD, Deutch AY, Redmond DE Jr, Sladek JR Jr, Roth RH. Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on catecholamines and metabolites in primate brain and CSF. *Brain Res*. 1987 ;415(2):293-9.

65. Endrich, M. M. , Gehring, H. The V3 loop of human immunodeficiency virus type-1 envelope protein is a high-affinity ligand for immunophilins present in human blood. *Eur .J Biochem.* 1998; 252(3):441-6.

66. Everall, I.P., et al. Cortical synaptic density is reduced in mild to moderate human immunodeficiency virus neurocognitive disorder. HNRC Group. HIV Neurobehavioral Research Center. *Brain Pathol*, 1999. 9(2): p. 209-17.

67. Ferrand-Drake M, Friberg H, Wieloch T. Mitochondrial permeability transition induced DNA-fragmentation in the rat hippocampus following hypoglycemia. *Neuroscience.* 1999; 90(4):1325-38.

68. Fischer G, Bang H, Mech C. Detection of enzyme catalysts for cis- trans isomerization of peptide bonds using proline containing peptides as substrates. *Biomed. Biochim. Acta* 1984; 43 : 1101-1112.

69. Frackowiak J, Mazur-Kolecka B, Kaczmarski W, Dickson D. Deposition of Alzheimer's vascular amyloid-beta is associated with decreased expression of brain L-3-hydroxyacyl-coenzyme A dehydrogenase (ERAB). *Brain Res*. 2001;907(1-2):44-53.

70. Franke EK, Yuan HE, Luban J. Specific incorporation of cyclophilin A into HIV-1 virions. *Nature* 1994; 372(6504): p. 359-62.

71. Franke EK, Luban, J. Inhibition of HIV-1 replication by cyclosporine A or related compounds correlates with the ability to disrupt the Gag-cyclophilin A interaction. *Virology*, 1996; 222(1): p. 279-82.

72. Freskgard P-O, Bergenhem N, Jonsson B-H, Svensson M, Carlson U. Isomerase and chaperone activity of prolyl isomerase in the folding of carbonic anhydrase. *Science* 1992; 258: 466- 468.

73. Friedman, J. and I. Weissman. Two cytoplasmic candidates for immunophilin action are revealed by affinity for a new cyclophilin: one in the presence and one in the absence of CsA. *Cell* 1991; 66: 799-806.

73. Fung JJ, Starzl TE. FK506 in solid organ transplantation. *Transplant Proc* 1994; 26: 3017- 3020.

74. Galat A. Peptidylproline cis- trans isomerases: immunophilins. Eur. J. Biochem. 1993; 216: 689-707.

75. Galat A. Peptidylprolyl cis/trans isomerases (immunophilins): biological diversity targets—functions. *Curr Top Med Chem*. 2003;3(12):1315-47.

76. Galigniana MD, Radanyi C, Renoir JM, Housley PR, Pratt WB. Evidence that the peptidylprolyl isomerase domain of the hsp90-binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus. *J Biol Chem*. 2001; 276(18):14884-9.

77. Garden GA. Microglia in human immunodeficiency virus-associated neurodegeneration. *Glia.* 2002;40(2):240-51.

78. Goedert M. Tau protein and the neurofibrillary pathology of Alzheimer's disease. *Trends Neurosci.* 1993;16(11):460-5.

79. Goedert M. Alpha-synuclein and neurodegenerative diseases. *Nat Rev Neurosci*. 2001;2(7):492-50.

80. Gold, B.G., T. Storm-Dickerson, and D.R. Austin. The immunosuppressant FK506 increases functional recovery and nerve regeneration following peripheral nerve injury. *Restor. Neurol. Neurosci*. 1994; 6: 287-296.

81. Gold, B.G., Katoh, K., Storm-Dickerson, T. The immunosuppressant FK506 increases the rate of axonal regeneration in rat sciatic nerve. *J. Neurosci.* 1995;15: 7509-7516.

82. Gold, B.G., V. Densmore, W. Shou, M.M. Matzuk, Gordon, H.S. Immunophilin FK506-binding protein 52 (not FK506- binding protein 12) mediates the neurotrophic action of FK506. *J. Pharmacol. Exp. Ther*. 1999; 289: 1202-1210.

83. Gothel SF, Marahiel MA. Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. *Cell Mol Life Sci* 1999; 55(3): 423-36.

84. Gray F, Chretien F, Vallat-Decouvelaere AV, Scaravilli F. The changing pattern of HIV neuropathology in the HAART era. *J Neuropathol Exp Neurol*. 2003;62(5):429-40.

85. Greenberg SM, Koo EH, Selkoe DJ, Qiu WQ, Kosik KS. Secreted beta-amyloid precursor protein stimulates mitogen-activated protein kinase and enhances tau phosphorylation. *Proc Natl Acad Sci U S A*. 1994;91(15):7104-8.

86. Guo X, Dawson VL, Dawson TM. (2001). Neuroimmunophilin ligands exert neuroregeneration and neuroprotection in midbrain dopaminergic neurons. *Eur J Neurosci.* 13(9):1683-93.

87. Hamilton GS, Steiner JP. Immunophilins: beyond immunosuppression. *J Med Chem.* 1998; 41(26): 5119-43.

88. Hammerschlag R. Is the intrasomal phase of fast axonal transport driven by oscillations of intracellular calcium? *Neurochemical Research* 1994; 19(11): 1431- 7.

89. Handschumacher RE, Harding MW, Rice J, Drugge RJ, Speicher DW. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* 1984; 226: 544- 546.

90. Harding, M. W, A. Galat, D. E. Uehling, Schreiber, S.L. A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. *Nature* 1989; 341: 758-760.

91. Hartl FU. Molecular chaperones in cellular protein folding. *Nature*. 1996;381(6583):571-9.

92. He XY, Merz G, Yang YZ, Mehta P, Schulz H, Yang SY. Characterization and localization of human type10 17beta-hydroxysteroid dehydrogenase. *Eur J Biochem*. 2001;268(18):4899-907.

93. Higuchi M, Lee VM, Trojanowski JQ. Tau and axonopathy in neurodegenerative disorders. *Neuromolecular Med*. 2002;2(2):131-50.

94. Hollander GA, Bierer BE, Burakoff SJ. Molecular and biological actions of cyclosporin A and FK506 on T cell development and function. *Transfus Sci* 1994; 15(3): 207-20.

95. Hsu VL, Handschumacher RE, Armitage IA. Peptidyl-prolyl cis- trans isomerase activity of cyclophilin studied by one- dimensional H nuclear magnetic resonance spectroscopy. *J. Am. Chem. Soc*. 1990; 112: 6745- 6747.

96. Isacson O, Riche D, Hantraye P, Sofroniew MV, Maziere M. A primate model of Huntington's disease: cross-species implantation of striatal precursor cells to the excitotoxically lesioned baboon caudate-putamen. *Exp Brain Res*. 1989;75(1):213-20.

97. Ishii T, Kim YI, Tatsuma T, Kawano K, Kai T, Kobayashi M. Immunodepressants ameliorate normothermic ischemia injury to the rat liver by down-regulating tumor necrosis factor, not by alleviation of lipid peroxidative injury. *Transpl Int.* 1994;7 Suppl 1:S507-11.

98. Itoh K., Mehraein P, Weis S. Neuronal damage of the substantia nigra in HIV-1 infected brains. *Acta Neuropathol (Berl)*, 2000; 99(4): p. 376-84.

99. Jayraman T, Brillantes A-M ,Timerman AP, Fleischer S, Erdjument- Bromage H, Tempst P, Marks AR. FK506 binding protein associated with the calcium release channel (ryanodine receptor). *J Biol Chem*. 1992; 267: 9474- 9477.

100. Jefferies HB, Reinhard C, Kozma SC, Thomas G. Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. *Proc Natl Acad Sci U S A.* 1994; 91(10):4441-5.

101. Jellinger KA. et al., Neuropathology and general autopsy findings in AIDS during the last 15 years. *Acta Neuropathol (Berl)*, 2000; 100(2): p. 213-20.

102. Jellinger KA. Recent developments in the pathology of Parkinson's disease. *J Neural Transm* Suppl. 2002;(62):347-76.

103. Jenner P. The contribution of the MPTP-treated primate model to the development of new treatment strategies for Parkinson's disease. *Parkinsonism Relat Disord.* 2003 Jan;9(3):131-7.

104. Jin ZG, Melaragno MG, Liao DF, Yan C, Haendeler J, Suh YA, Lambeth JD, Berk BC. Cyclophilin A is a secreted growth factor induced by oxidative stress. *Circ Res*. 2000; 87(9):789-96.

105. Johnston JA, Ward CL, Kopito RR. Aggresomes: a cellular response to misfolded proteins. *J Cell Biol.* 1998;143(7):1883-98.

106. Johnston J. A.; Dalton, M. J.; Gurney, M. E.;Kopito, R. R. Formation of high molecular weight complexes of mutant Cu, Zn superoxide dismutase in a mouse model for familial amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. USA* 2000, *97*,12571-12676.

107. Kaibori M, Inoue T, Tu W, Oda M, Kwon AH, Kamiyama Y, Okumura T. FK506, but not cyclosporin A, prevents mitochondrial dysfunction during hypoxia in rat hepatocytes. *Life Sci*. 2001; 69(1):17-26.

108. Kato H, Oikawa T, Otsuka K, Takahashi A, Itoyama Y. Postischemic changes in the immunophilin FKBP12 in the rat brain. Brain Res Mol Brain Res. 2000; 84(1-2): 58-66.

109. Kawano K, Kim YI, Goto S, Ono M, Kobayashi M. A protective effect of FK506 in ischemically injured rat livers. *Transplantation.* 1991; 52(1):143-5.

110. Kern, G., Kern, D., Schmid F. X., Fischer, G. Reassessment of the putative chaperone function of prolyl- cis/ trans- isomerases. *FEBS Lett*. 1994; (348): 145-148.

111. Khaleghpour K, Pyronnet S, Gingras AC, Sonenberg N. Translational homeostasis: eukaryotic translation initiation factor 4E control of 4E-binding protein 1 and p70 S6 kinase activities. *Mol Cell Biol*. 1999;19(6):4302-10.

112. Kita H, Kitai ST. Amygdaloid projections to the frontal cortex and the striatum in the rat. *J. Comp Neurol*. 1990;298(1):40-9.

113. Klettner A, Baumgrass R, Zhang Y, Fischer G, Burger E, Herdegen T, Mielke K. The neuroprotective actions of FK506 binding protein ligands: neuronal survival is triggered by de novo RNA synthesis, but is independent of inhibition of JNK and calcineurin. *Brain Res Mol Brain Res.* 2001; 97(1):21-31.

114. Klettner A, Herdegen T. The immunophilin-ligands FK506 and V-10,367 mediate neuroprotection by the heat shock response. *Br J Pharmacol*. 2003;138(5):1004-12.

115. Klettner A, Herdegen T. FK506 and its analogs - therapeutic potential for neurological disorders. *Curr Drug Target CNS Neurol Disord*. 2003;2(3):153-62.

116. Korbo, L. and M. West, No loss of hippocampal neurons in AIDS patients. *Acta Neuropathol (Berl)*, 2000. 99(5): p. 529-33.

117. Koutsilieri, E., Sopper ,S., Scheller, C., ter Meulen, V., Riederer ,P. Parkinsonism in HIV dementia. *J Neural Transm*. 2002; 109(5-6):767-75.

118. Kowall NW, Hantraye P, Brouillet E, Beal MF, McKee AC, Ferrante RJ. MPTP induces alpha-synuclein aggregation in the substantia nigra of baboons. *Neuroreport*. 2000 Jan 17;11(1):211-3.

119. Lang K, Schmid FX, Fischer G. Catalysis of protein folding by prolyl isomerases. *Nature* 1987; 329: 268-270.

120. Langford TD., et al., Severe, demyelinating leukoencephalopathy in AIDS patients on antiretroviral therapy. *AIDS* 2002; 16(7): p. 1019-29.

121. Langston JW. Epidemiology versus genetics in Parkinson's disease: progress in resolving an age-old debate. *Ann Neurol*. 1998 ;44(3 Suppl 1):S45-52.

122. Le TV, Crook R, Hardy J, Dickson DW. Cotton wool plaques in non-familial late-onset Alzheimer disease. *J Neuropathol Exp Neurol*. 2001;60(11):1051-61.

123. Le Bihan S, Marsaud V, Mercier-Bodard C, Baulieu EE, Mader S, White JH, Renoir JM. Calcium/calmodulin kinase inhibitors and immunosuppressant macrolides rapamycin and FK506 inhibit progestin- and glucocorticosteroid receptor-mediated transcription in human breast cancer T47D cells. *Mol Endocrinol*. 1998;12(7):986-1001.

124. Lee JP, Palfrey HC, Bindokas VP, Ghadge GD, Ma L, Miller RJ, Roos RP. The role of immunophilins in mutant superoxide dismutase-linked familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* 1999; 96(6): 3251-6.

125. Lee FJ, Liu F, Pristupa ZB, Niznik HB. Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis. *FASEB J*. 2001;15(6):916-26.

126. Letko E., Bhol, V. Pinar, C. S. Foster, Razzaque. Tacrolimus (FK 506). *Ann. Allergy Asthma Immunol*. 1999; 83: 179-190.

127. Lian Q, Ladner CJ, Magnuson D, Lee JM. 2001. Selective changes of calcineurin (protein phosphatase 2B) activity in Alzheimer's disease cerebral cortex. *Exp Neurol*. 167(1):158- 65.

128. Lin DT, Lechleiter JD. Mitochondrial argeted cyclophilin D protects cells from cell death by peptidyl prolyl isomerization. *J Biol Chem*. 2002;277(34):31134-41.

129. Liou YC, Sun A, Ryo A, Zhou XZ, Yu ZX, Huang HK, Uchida T, Bronson R, Bing G, Li X, Hunter T, Lu KP. 2003. Role of the prolyl isomerase Pin1 in protecting against age-dependent neurodegeneration. *Nature* 31;424(6948):556-61

130. Liu, J., J. D. Farmer, W. S. Lane, J. Friedman, I. Weissman, and S.L. Schreiber. 1991. Calcineurin is a common target of cyclophilin- cyclosporin A and FKBP- FK506 complexes. *Cell* 66: 807-815.

131. Lopez OL, Smith G, Meltzer CC, Becker JT. Dopamine systems in human immunodeficiency virus-associated dementia. *Neuropsychiatry Neuropsychol Behav Neurol.* 1999; 12(3):184-92.

132. Lowenstein DH, Arsenault L. The effects of growth factors on the survival and differentiation of cultured dentate gyrus neurons. *J Neurosci*. 1996; 16(5):1759-69.

133. Lyons, W.E., E. B. George, T. M. Dawson, J. P. Steiner, and S. H. Snyder. 1994. Immunosuppressant FK506 promotes neurite outgrowth in cultures of PC12 cells and sensory ganglia. *Proc. Natl. Acad. Sci. USA* **91**: 3191-3195.

134. Lyons WE, Steiner JP, Snyder SH, Dawson TM. Neuronal regeneration enhances the expression of the immunophilin FKBP-12. *J Neurosci*. 1995; 15(4): 2985-94.

135. Macario AJ, Conway de Macario E. Stress and molecular chaperones in disease. *Int J Clin Lab Res.* 2000;30(2):49-66.

136. Macario AJ, Conway De Macario E. The molecular chaperone system and other anti-stress mechanisms in archaea. *Front Biosci.* 2001;6:D262-83.

137. Mankowski JL, Queen SE, Tarwater PM, Fox KJ, Perry VH. 1998. Accumulation of beta- amyloid precursor protein in axons correlates with CNS expression of SIV gp41. *J Neuropathol Exp Neurol*. 61(1):85-90.

138. Marks AR. Cardiac intracellular calcium release channels: role in heart failure. *Circ Res*. 2000; 87(1): 8-11.

139. Madsen, J.R., P. MacDonald, N. Irwin, D. E. Goldberg, G.-L. Yao, K.F. Meiri, I.J. Rimm, P.E. Stieg and L.I. Benowitz. 1998. Tacrolimus (FK506) increases neuronal expression of GAP-43 and improves functional recovery after spinal cord injury in rats. *Exp. Neurol*. 154: 673-683.

140. Manabe Y., Warita H., Murakami T.; Shiote, M.; Hayashi, T.; Omori, N.; Nagano, I.; Shoji, M.;Abe, K. Early decrease of the immunophilin FKBP 52 in the spinal cord of a transgenic model for amyotrophic lateral sclerosis. *Brain Res.* **2002**; *935*,124-128.

141. Mann DM. The site and nature of the primary lesions in Alzheimer's disease. *Prog Clin Biol Res*. 1989;317:303-11.

142. Martin, F.C. and C.A. Wiley. 1994. An alternative method for obtaining high-viability cell suspensions from neonatal mouse brain. *J. Neurosci. Methods* **55**: 99-104.

143. Marty, S., P. Carroll, A. Cellerino, E. Castren, V. Staiger, H. Thoenen, and D. Lindholm. 1996. Brain-derived neurotrophic factor promotes the differentiation of various hippocampal non-pyramidal neurons, including Cajal-Retzius cells, in organotypic slice cultures. *J. Neurosci*. **16**: 675-687.

144. Masliah E, Mallory M, Hansen L, Alford M, Albright T, DeTeresa R, Terry R, Baudier J, Saitoh T. Patterns of aberrant sprouting in Alzheimer's disease. *Neuron*. 1991;6(5):729-39.

145. Masliah E, Ellisman M, Carragher B, Mallory M, Young S, Hansen L, DeTeresa R, Terry RD. Three-dimensional analysis of the relationship between synaptic pathology and neuropil threads in Alzheimer disease. *J Neuropathol Exp Neurol*. 1992 ;51(4):404-14.

146. Masliah E, Mallory M, Hansen L, Alford M, DeTeresa R, Terry R. An antibody against phosphorylated neurofilaments identifies a subset of damaged association axons in Alzheimer's disease. *Am J Pathol*. 1993;142(3):871-82.

147. Masliah E, Miller A, Terry RD The synaptic organization of the neocortex in Alzheimer's disease. *Med Hypotheses*. 1993; 41(4):334-40.

148. Masliah E., et al. Amyloid precursor proteins protect neurons of transgenic mice against acute and chronic excitotoxic injuries in vivo. *Neuroscience*, 1997; 78(1): 135-46.

149. Masliah E, Mallory M, Alford M, DeTeresa R, Hansen LA, McKeel DW, Morris JC. (2001) Altered expression of synaptic proteins occurs early during progression of Alzheimer's disease. *Neurology* 2001; 56(1): 127-9.

150. McArthur JC. Neurologic manifestations of AIDS. *Medicine (Baltimore)*. 1987;66(6):407-37.

151. McKee AC, Kosik KS, Kowall NW. Neuritic pathology and dementia in Alzheimer's disease. *Ann Neurol*. 1991;30(2):156-65.

152. McKeith I, Galasko D, Kosaka K, Perry E, Dickson D, Hansen L, Salmon D, Lowe J, Mirra S, Byrne E, Lennox G, Quinn N, Edwardson J, Ince P, Bergeron C, Burns A, BL M, Lovestone S, Collerton D, Jansen E, Ballard C, de Vos R, Wilcock G, Jellinger K, Perry R. Consensus guidelines for the clinical and pathologic diagnosis of dementia with Lewy bodies (DLB): report of the consortium on DLB international workshop. Neurology. 1996; 47(5): 1113- 24.

153. McLean PJ, Kawamata H, Shariff S, Hewett J, Sharma N, Ueda K, Breakefield XO, Hyman BT. TorsinA and heat shock proteins act as molecular chaperones: suppression of alpha-synuclein aggregation. *J Neurochem*. 2002; 83(4):846-54.

154. Michnik SW, Rosen MK, Wandless TJ, Karplus M, Schreiber SL. Solution structure of FKBP, a rotamase enzyme and receptor for FK506 and rapamycin. *Science* 1991; 252: 836-839.

155. Migheli A, Attanasio A, Pezzulo T, Gullotta F, Giordana MT, Schiffer D. Age-related ubiquitin deposits in dystrophic neuritis: an immunoelectron microscopic study. *Neuropathol Appl Neurobiol*. 1992; 18(1): 3-11.

156. Minagar A, Shapshak P, Fujimura R, Ownby R, Heyes M, Eisdorfer C. The role of macrophage/microglia and astrocytes in the pathogenesis of three neurologic disorders: HIV-associated dementia, Alzheimer disease, and multiple sclerosis. *J Neurol Sci*. 2002;202(1-2):13-23.

157. Moore DJ, Dawson VL, Dawson TM. Role for the ubiquitin-proteasome system in Parkinson's disease and other neurodegenerative brain amyloidoses. *Neuromolecular Med.* 2003;4(1-2):95-108.

158. Morfini G, Pigino G, Beffert U, Busciglio J, Brady ST. 2002. Fast axonal transport misregulation and Alzheimer's disease. *Neuromolecular Med*. 2(2):89-99.

159. Morioka M, Fukunaga K, Kai Y, Todaka T, Yano S, Hamada J, Miyamoto E, Ushio Y. Intravenously injected FK506 failed to inhibit hippocampal calcineurin. *Biochem Biophys Res Commun.* 200;286(4):802-6.

160. Mucke L., et al., Protection against HIV-1 gp120-induced brain damage by neuronal expression of human amyloid precursor protein. *J Exp Med*, 1995; 181(4): p. 1551-6.

161. Mukaetova-Ladinska EB, Hurt J, Honer WG, Harrington CR, Wischik CM. Loss of synaptic but not cytoskeletal proteins in the cerebellum of chronic schizophrenics. *Neurosci Lett*. 2002;317(3):161-5.

162. Muramoto M, Yamazaki T, Nishimura S, Kita Y.Detailed in vitro pharmacological analysis of FK506 – induced neuroprotection. *Neuropharmacology*. 2003; 45(3):394-403.

163. Nath A, Anderson C, Jones M, Maragos W, Booze R, Mactutus C, Bell J, Hauser KF, Mattson M. Neurotoxicity and dysfunction of dopaminergic systems associated with AIDS dementia. *J Psychopharmacol*. 2000; 14(3):222-7.

164. Navia MA. Protein-drug complexes important for immunoregulation and organ transplantation. *Curr Opin Struct Biol*. 1996;6(6):838-47.

165. Ngosuwan J, Wang NM, Fung KL, Chirico WJ. Roles of cytosolic Hsp70 and Hsp40 molecular chaperones in post-translational translocation of presecretory proteins into the endoplasmic reticulum. *J Biol Chem*. 2003;278(9):7034-42.

166. Nurmi E, Bergman J, Eskola O, Solin O, Hinkka SM, Sonninen P, Rinne JO. Reproducibility and effect of levodopa on dopamine transporter function measurements: a [18F]CFT PET study. *J Cereb Blood Flow Metab.* 2000;20(11):1604-9.

167. Oiwa Y, Eberling JL, Nagy D, Pivirotto P, Emborg ME, Bankiewicz KS. Overlesioned hemiparkinsonian non human primate model: correlation between clinical, neurochemical and histochemical changes. *Front Biosci*. 2003;8:a155-66.

168. Panasenko OO, Kim MV, Marston SB, Gusev NB. Interaction of the small heat shock protein with molecular mass 25 kDa (hsp25) with actin. *Eur J Biochem*. 2003; 270(5):892-901.

169. Parker EM, Monopoli A, Ongini E, Lozza G, Babij CM. Rapamycin, but not FK506 and GPI-1046, increases neurite outgrowth in PC12 cells by inhibiting cell cycle progression. *Neuropharmacology*. 2000;39(10):1913-9.

170. Pautot V, Macaigne C, Chariot P. [Mitochondrial DNA and Parkinson disease. Methodologic review]. *Arch Anat Cytol Pathol*. 1998;46(4):261-8.

171. Perry G, Cras P, Siedlak SL, Tabaton M, Kawai M. Beta protein immunoreactivity is found in the majority of neurofibrillary tangles of Alzheimer's disease. *Am J Pathol.* 1992 ;140(2):283-90.

172. Pezzotti, P., et al., The spectrum of AIDS-defining diseases: temporal trends in Italy prior to the use of highly active anti-retroviral therapies, 1982-1996. *Int J Epidemiol*, 1999. 28(5): p. 975-81.

173. Pirker W. Correlation of dopamine transporter imaging with parkinsonian motor handicap: how close is it? *Mov Disord*. 2003;18 Suppl 7:S43-51.

174. Pratt WB. Interaction of hsp90 with steroid receptors: organizing some diverse observations and presenting the newest concepts. *Mol Cell Endocrinol*. 1990;74(1):C69-76.

175. Price, R.W., et al. Neurological outcomes in late HIV infection: adverse impact of neurological impairment on survival and protective effect of antiviral therapy. AIDS Clinical Trial Group and Neurological AIDS Research Consortium study team. *AIDS*, 1999. 13(13): p. 1677-85.

176. Ratajczak T, Ward BK, Minchin RF. Immunophilin chaperones in steroid receptor signalling. *Curr Top Med Chem*. 2003;3(12):1348-57.

177. Renkawek K, Stege GJ, Bosman GJ. Dementia, gliosis and expression of the small heat shock proteins hsp27 and alpha B-crystallin in Parkinson's disease. *Neuroreport.* 1999;10(11):2273-6.

178. Richard S, Brion JP, Couck AM, Flament- Durant J. Accumulation of smooth endoplasmic reticulum in Alzheimer's disease: new morphological evidence of axoplasmic flow disturbances. *Journal of Submicroscopic Cytology and Pathology* 1989; 21(3): 461- 467.

179. Rinne JO. Nigral degeneration in Parkinson's disease. *Mov Disord*. 1993; 8 Suppl 1:S31-5.

180. Rodriguez- Kern A, Gegelashvili M, Schousboe A, Zhang J, Sung L, Gegelashvili G. Beta- amyloid and brain- derived neurotrophic factor, BDNF, up- regulate the expression of glutamate transporter GLT-1/EAAT2 via different signaling pathways utilizing transcription factor NF-kappaB. Neurochem. Int. 2003; 43(4-5): 363-70.

181. Rosenstiel P, Schramm P, Isenmann S, Brecht S, Eickmeier C, Burger E, Herdegen T, Sievers J, Lucius R. Differential effects of immunophilin-ligands (FK506 and V-10,367) on survival and regeneration of rat retinal ganglion cells in vitro and after optic nerve crush in vivo. *J Neurotrauma*. 2003; 20(3):297-307.

182. Sakamoto H, Mezaki Y, Shikimi H, Ukena K, Tsutsui K. Dendritic growth and spine formation in response to estrogen in the developing Purkinje cell. *Endocrinology*. 2003; 144(10):4466-77.

183. Samuel W, Terry RD, DeTeresa R, Butters N, Masliah E. Clinical correlates of cortical and nucleus basalis pathology in Alzheimer dementia. *Arch Neurol.* 1994;51(8):772-8.

184. Saphire AC, Bobardt MD, Gallay PA. Cyclophilin a plays distinct roles in human immunodeficiency virus type 1 entry and postentry events, as revealed by spinoculation. *J Virol,* 2002; 76(9): p. 4671-7.

185. Scammell JG, Hubler TR, Denny WB, Valentine DL. Organization of the human FK506-binding immunophilin FKBP52 protein gene (FKBP4). *Genomics*. 2003; 81(6):640-3.

186. Schiene C, Fischer G. Enzymes that catalyse the restructuring of proteins. *Curr Opin Struct Biol.* 2000; 10(1): 40-5.

187. Schmid F X, Mayr L, Mucke M, Schonbrunner E. R. Prolyl isomerases: the role in protein folding. *Adv Protein Chem* 1993; (44): 25-66.

188. Schmidt ML, Lee VM, Trojanowski JQ. Comparative epitope analysis of neuronal cytoskeletal proteins in Alzheimer's disease senile plaque neurites and neuropil threads. *Lab Invest*. 1991;64(3):352-7.

189. Schreiber SL. Chemistry and biology of the immunophilins and their immunosuppressive ligands. *Science*. 1991; 251(4991): 283-7.

190. Schreiber, S.L. and G. R. Crabtree. 1992. The mechanism of action of cyclosporin A and FK 506. *Immunol Today* **13**: 136-142.

191. Schreier MH. Immunophilins and immunosuppression. *Clin Exp Rheumatol*. 1993:11.

192. Schwartzman RJ, Alexander GM, Ferraro TN, Grothusen JR, Stahl SM. Cerebral metabolism of parkinsonian primates 21 days after MPTP. *Exp Neurol*. 1988;102(3):307-13.

193. Sharkey J, Butcher SP. Immunophilins mediate the neuroprotective effects of FK506 in focal cerebral ischaemia. *Nature.* 1994;371(6495):336-9.

194. Sheng JG, Zhou XQ, Mrak RE, Griffin WS. Progressive neuronal injury associated with amyloid plaque formation in Alzheimer disease. *J Neuropathol Exp Neurol*. 1998 Jul;57(7):714-7.

195. Sherry B, et al., Identification of cyclophilin as a proinflammatory secretory product of lipopolysaccharide-activated macrophages. *Proc Natl Acad Sci U S A*, 1992; 89(8): 3511-5.

196. Sherry B., et al., Role of cyclophilin A in the uptake of HIV-1 by macrophages and T lymphocytes. *Proc Natl Acad Sci U S A*, 1998; 95(4): p. 1758-63.

197. Shirane M, Nakayama KI. Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. Nat Cell Biol. 2003;5(1):28-37.

198. Shohami E, Ginis I, Hallenbeck JM. Dual role of tumor necrosis factor alpha in brain injury. Cytokine Growth Factor Rev. 1999;10(2):119-30.

199. Shou W, Aghdasi B, Armstrong DL, Guo Q, Bao S, Charng MJ, Mathews LM, Schneider MD, Hamilton SL, Matzuk MM. Cardiac defects and altered ryanodine receptor function in mice lacking FKBP12. Nature. 1998; 391(6666):489-92.

200. Silverstein AM, Galigniana MD, Kanelakis KC, Radanyi C, Renoir JM, Pratt WB. Different regions of the immunophilin FKBP52 determine its association with the glucocorticoid receptor, hsp90, and cytoplasmic dynein. *J Biol Chem.* 1999; 274(52):36980-6.

201. Smith RD, Zhang Z, Kurlan R, McDermott M, Gash DM.1993. Developing a stable bilateral model of parkinsonism in rhesus monkeys. *Neuroscience* 52(1): p. 7-16.

202. Snyder, S.H., M.M. Lai, and P.E. Burnett. 1998. Immunophilins in the nervous system. *Neuron* **21**: 283-294.

203. Soti C, Csermely P. Aging and molecular chaperones. *Exp Gerontol*. 2003;38(10):1037-40.

204. Sousa N, Almeida OF. Corticosteroids: sculptors of the hippocampal formation. *Rev Neurosci.* 2002;13(1):59-84.

205. Stege GJ, Bosman GJ. The biochemistry of Alzheimer's disease. *Drugs Aging*. 1999;14(6):437-46.

206. Steiner, J.P., T. M. Dawson, M. Fotuhi, C. E. Glatt, A. M. Snowman, N. Cohen and S. H. Snyder. 1992. High brain densities of the immunophilin FKBP colocalized with calcineurin. *Nature* **358**: 584-587.

207. Steiner JP, Dawson TM, Fotuhi M, Snyder SH. Immunophilin regulation of neurotransmitter release. Mol Med. 1996; 2(3):325-33.

208. Steiner JP., Connoly MA, Valentine HL, Hamilton GS, Dawson TM, Hester L, Snyder SH. Neurotrophic actions of nonimmunosuppressive analogues of immunosuppressive drugs FK506, rapamycin and cyclosporin A. *Nature Med*.;3: 421-428.

209. Steiner JP., Hamilton GS, Ross DT, Valentine HL, Guo H, Connolly MA, Liang S, Ramsey C, Li JH, Huang W, Howorth P, Soni R, Fuller M, Sauer H, Nowotnik AC, Suzdak PD. Neurotrophic immunophilin ligands stimulate structural and functional recovery in neurodegenerative animal models. *Proc. Natl. Acad. Sci. USA* 94: 2019-2024.

210. Takahashi M, Kanuka H, Fujiwara H, Koyama A, Hasegawa M, Miura M, Iwatsubo T. Phosphorylation of alpha-synuclein characteristic of synucleinopathy lesions is recapitulated in alpha-synuclein transgenic Drosophila. *Neurosci Lett*. 2003 ;336(3):155-8.

211. Takahashi N, Hayano T, Suzuki M. Peptidyl- prolyl cis- trans isomerase is the cyclosporine A- binding protein cyclophilin. *Nature* 1989; 337: 473- 475.

212. Takamatsu H, Tsukada H, Noda A, Kakiuchi T, Nishiyama S, Nishimura S, Umemura K. FK506 attenuates early ischemic neuronal death in a monkey model of stroke. *J Nucl Med*. 2001; 42(12):1833-40.

213. Tanaka K, Fujita N, Yoshioka M, Ogawa N. Immunosuppressive and non-immunosuppressive immunophilin ligands improve H(2)O(2)-induced cell damage by increasing glutathione levels in NG108-15 cells. *Brain Res*. 2001;889(1-2):225-8.

214. Tanaka K, Yoshioka M, Miyazaki I, Fujita N, Ogawa N. GPI1046 prevents dopaminergic dysfunction by activating glutathione system in the mouse striatum. *Neurosci Lett*. 2002; 321(1-2):45-8.

215. Tang F, Dent EW, Kalil K. Spontaneous calcium transients in developing cortical neurons regulate axon outgrowth. *J. Neurosci*. 2003; 23(3): 927-936.

216. Terry RD. Neuropathological changes in Alzheimer disease. *Prog Brain Res.* 1994;101:383-90.

217. Thomson, A.W. and T. E. Starzl. FK506 and autoimmune disease: perspective and prospects. *Autoimmunity* 1992; 12: 303-313.

218. Thomson, A.W., C. A. Bonham, and A. Zeevi. Mode of action of tacrolimus (FK506): molecular and cellular mechanisms. *Therapeutic Drug Monitoring* 1995; 17: 584-591.

219. Thorpe JR, Morley SJ, Rulten SL. Utilizing the peptidyl- prolyl cis- trans isomerase Pin1 as a probe of its phosphorylated target proteins: examples of binding to nuclear proteins in a human kidney cell line and to Tau in Alzheimer's diseased brain. *J. Histochem. Cytochem.* 2001; 49(1): 97- 107.

220. Timerman AP, Wiederrecht G, Marcy A, Fleischer S. Characterization of an exchange reaction between soluble FKBP12 and the FKBP- ryanodine receptor complex. Modulation by FKBP mutants deficient in peptidyl- prolyl isomerase activity. J. Biol. Chem.. 1995; 270: 2451- 2459.

221. Trillo-Pazos G, Diamanturos A, Rislove L, Menza T, Chao W, Belem P, Sadiq S, Morgello S, Sharer L, Volsky DJ. Detection of HIV-1 DNA in microglia/macrophages, astrocytes and neurons isolated from brain tissue with HIV-1 encephalitis by laser capture microdissection. *Brain Pathol*. 2003; 13(2):144-54.

222. Trojanowski JQ, Schmidt ML, Shin RW, Bramblett GT, Rao D, Lee VM. Altered tau and neurofilament proteins in neuro-degenerative diseases: diagnostic implications for Alzheimer's disease and Lewy body dementias. *Brain Pathol*. 1993;3(1):45-54.

223. Tropschug, M., Wachter, E., Mayer, S., Schonbrunner, E.R., Schmid, F. X. isolation and sequence of an FK506- binding protein from N. crassa which catalyzes protein folding. *Nature* 1990; 343: 674- 677.

224. Uchino H, Minamikawa-Tachino R, Kristian T, Perkins G, Narazaki M, Siesjo BK, Shibasaki F. Differential neuroprotection by cyclosporin A and FK506 following ischemia corresponds with differing abilities to inhibit calcineurin and the mitochondrial permeability transition. *Neurobiol Dis.* 2002;10(3):219-33.

225. Varastet M, Riche D, Maziere M, Hantraye P. Chronic MPTP treatment reproduces in baboons the differential vulnerability of mesencephalic dopaminergic neurons observed in Parkinson's disease. *Neuroscience.* 1994;63(1):47-56.

226. Vickers JC, Delacourte A, Morrison JH. Progressive transformation of the cytoskeleton associated with normal aging and Alzheimer's disease. *Brain Res.* 1992 ;594(2):273-8.

227. Wagenknecht T, Grassucci R, Berkowitz J, Wiederrecht GJ, Xin HB, Fleischer S. Cryoelectron microscopy resolves FK506-binding protein sites on the skeletal muscle ryanodine receptor. *Biophys J*. 1996; 70(4): 1709-15.

228. Wakita H, Tomimoto H, Akiguchi I, Kimura J. Protective effect of cyclosporin A on white matter changes in the rat brain after chronic cerebral hypoperfusion. *Stroke*. 1995; 26(8):1415-22.

229. Wakita H, Tomimoto H, Akiguchi I, Kimura J. Dose-dependent, protective effect of FK506 against white matter changes in the rat brain after chronic cerebral ischemia. *Brain Res*. 1998;792(1):105-13.

230. Wang TW, Donahoe PK, Zervos AS. Specific interaction of type I receptors of the TGFb family with the immunophilin FKBP12. *Science*1994; 265: 674-676.

231. Weller RO, Nicoll JA. Cerebral amyloid angiopathy: pathogenesis and effects on the ageing and Alzheimer brain. *Neurol Res*. 2003; 25(6):611-6.

232. White, M. G., R. R. Hammond, V. J. Sanders, E. A. Bonaroti, A. P. Mehta, G. Wang, C. A. Wiley, and C. L. Achim. Neuron- enriched second trimester human cultures: growth factor response and in vivo graft survival. *Cell Transpl*. 1999; 8: 59-73.

233. Whone AL, Moore RY, Piccini PP, Brooks DJ. Plasticity of the nigropallidal pathway in Parkinson's disease. *Ann Neurol*. 2003;53(2):206-13.

234. Wichmann T, Kliem MA, DeLong MR. Antiparkinsonian and behavioral effects of inactivation of the substantia nigra pars reticulata in hemiparkinsonian primates. *Exp Neurol*. 2001;167(2):410-24.

235. Wickner S, Maurizi MR, Gottesman S. Posttranslational quality control: folding, refolding, and degrading proteins. *Science*. 1999;286(5446):1888-93.

236. Winter, C., J. Schenkel, E. Burger, C. Eickmeier, M. Zimmerman, and T. Herdegen. 1999. The immunophilin ligand FK506, but not GPI-1046, protects against neuronal death and inhibits c-Jun expression in the substantia nigra pars compacta following transection of the rat medial forebrain bundle. *Neuroscience* **95**: 753-762.

237. Wu YQ, Wilkinson DE, Limburg D, Li JH, Sauer H, Ross D, Liang S, Spicer D, Valentine H, Fuller M, Guo H, Howorth P, Soni R, Chen Y, Steiner JP, Hamilton GS. Synthesis of ketone analogues of prolyl and pipecolyl ester FKBP12 ligands. *J Med Chem*. 2002;45(16):3558-68.

238. Wullner, U., et al., Dopamine terminal loss and onset of motor symptoms in MPTP-treated monkeys: a positron emission tomography study with 11C-CFT. *Exp Neurol*, 1994; 126(2): 305-9.

239. Yamaguchi H, Nakazato Y, Shoji M, Okamoto K, Ihara Y, Morimatsu M, Hirai S. Secondary deposition of beta amyloid within extracellular neurofibrillary tangles in Alzheimer- type dementia. *Am J Pathol* 1991; 183(3): 699-705.

240. Yamasaki Y, Suzuki T, Yamaya H, Matsuura N, Onodera H, Kogure K. Possible involvement of interleukin-1 in ischemic brain edema formation. *Neurosci Lett*. 1992; 142(1):45-7.

241. Yan SD, Fu J, Soto C, Chen X, Zhu H, Al-Mohanna F, Collison K, Zhu A, Stern E, Saido T, Tohyama M, Ogawa S, Roher A, Stern D. An intracellular protein that binds amyloid-beta peptide and mediates neurotoxicity in Alzheimer's disease. *Nature.* 1997;389(6652):689-95.

242. Yan H, Willis AC, Harding JJ. Gamma III-crystallin is the primary target of glycation in the bovine lens incubated under physiological conditions. *Biochem J.* 2003;374(Pt 3):677-85.

243. Yoo BC, Kim SH, Cairns N, Fountoulakis M, Lubec G. Deranged expression of molecular chaperones in brains of patients with Alzheimer's disease. *Biochem Biophys Res Commun*. 2001;280(1):249-58.

244. Yuan A, Mills R, Bamburg J, Bray J. Axonal transport and distribution of cyclophilin A in chicken neurones. *Brain Res*. 1997; 771: 203- 212.

245. Yun HY, Gonzalez-Zulueta M, Dawson VL, Dawson TM. Nitric oxide mediates N-methyl-D-aspartate receptor-induced activation of p21ras. *Proc Natl Acad Sci U S A*. 1998; 95(10):5773-8.

246. Zander K, Sherman MP, Tessmer U, Bruns K, Wray V, Prechtel AT, Schubert E, Henklein P, Luban J, Neidleman J, Greene WC, Schubert U. Cyclophilin A interacts with HIV-1 Vpr and is required for its functional expression. *J Biol Chem*. 2003;278(44):43202-13.

247. Zhang J, Steiner JP. Nitric oxide synthase, immunophilins and poly(ADP-ribose) synthetase: novel targets for the development of neuroprotective drugs. *Neurol Res*. 1995;17(4):285-8.

248. Zhang C, Steiner JP, Hamilton GS, Hicks TP, Poulter MO. Regeneration of dopaminergic function in 6-hydroxydopamine-lesioned rats by neuroimmunophilin ligand treatment. *J Neurosci*. 2001;21(15):RC156.

249. Zhang Z, Zhang M, Ai Y, Avison C, Gash DM. MPTP-Induced pallidal lesions in rhesus monkeys. *Exp Neurol*. 1999;155(1):140-9.

250. Zhao M, Momma S, Delfani K, Carlen M, Cassidy RM, Johansson CB, Brismar H, Shupliakov O, Frisen J, Janson AM. Evidence for neurogenesis in the adult mammalian substantia nigra. *Proc Natl Acad Sci U S A*. 2003;100(13):7925-30.