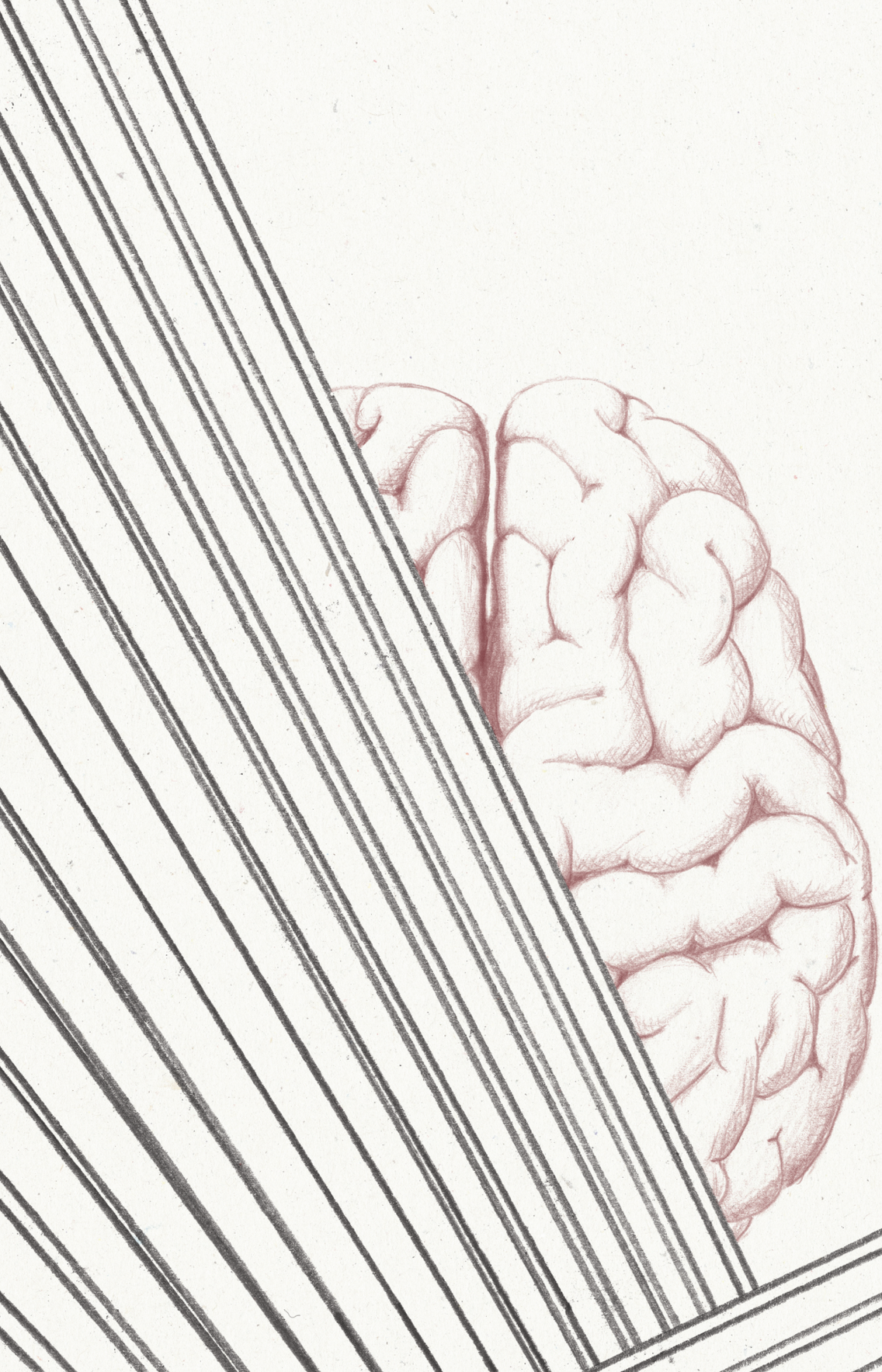
# Novel Insights into Progressive Supranuclear Palsy and Related Disorders

Wang Zheng Chiu



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Novel Insights into

# **Progressive Supranuclear Palsy**

and Related Disorders

Nieuwe inzichten in

**Progressieve supranucleaire verlamming**

en gerelateerde aandoeningen

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# 1

# INTRODUCTION

# 1.1

## General introduction to the thesis

Over 50 years ago a Canadian neurologist named J.C. Richardson identified nine patients with progressive parkinsonism with postural instability, supranuclear gaze palsy, pseudobulbar dysfunction, and cognitive impairment. Together with neurology resident J. Steele and neuropathologist J. Olszewski they proceeded to describe the neuropathological findings of the disease, comprising neurofibrillary tangles, neuronal cell loss and gliosis in the basal ganglia, brainstem and cerebellum and termed this disorder as progressive supranuclear palsy (PSP).[1]

It has since been recognized as the most common form of atypical parkinsonism, comprising about 5% of patients presenting with parkinsonism, with a prevalence of 5 per 100.000. Genetic and clinicopathologic discoveries, such as the recognition of tau-containing inclusions as the pathologic hallmark of PSP, have shed much light on clinical and pathological heterogeneity and have led to an increasing understanding of the pathophysiology of this disorder. Despite these insights, no effective treatment is currently available.

In 2003 a longitudinal study on PSP was started at the Erasmus Medical Center in Rotterdam. Patients were recruited by nationwide referral. Over the years we have included over 260 patients into the study. Previous research from our center has focused on the hereditary aspects of PSP, as well as the clinical presentation, and found 1) familial aggregation in a subset of PSP patients[2] and 2) a subgroup of PSP patients with a predominant frontal presentation, that is, with cognitive dysfunction and behavioural changes, resembling the clinical presentation of the behavioural variant of frontotemporal degeneration (FTD).[3] Overlap between PSP and FTD is further emphasized by the aggregation of tau protein that characterizes these neurodegenerative disorders. Bearing this in mind, PSP can be considered part of the frontotemporal lobar degenerations spectrum.

The main aim of this thesis was to assess clinical, neuropathological and neurochemical aspects within this spectrum, but focused mainly on PSP. Furthermore, we describe a mutation in a novel neurodegenerative disorder.

**Chapter 1** provides a general overview of PSP, including clinical, genetic and pathological aspects. In **chapter 2.1** a comparative study on survival in two large cohorts of PSP and FTD patients is described. The next chapters focus on the clinicoradiological **(chapter 2.2)** and neurochemical **(chapter 3)** aspects of the midcingulate cortex in PSP. The latter includes an analysis of twenty different receptors from seven neurotransmitter systems in 16 post-mortem PSP brains. **Chapter 4** comprises studies of PSP-like disorders. In **chapter 4.1** we describe the various steps to link a mutation to a novel neurodegenerative disorder clinically characterized by dementia and/or parkinsonism. **Chapter 4.2** contains the description of neuropathological changes of a presymptomatic carrier of a frontotemporal dementia (FTD) tau mutation, a related hereditary tauopathy. Finally, in the general discussion **(chapter 5)** the main findings in light of the current knowledge about the disease are discussed and recommendations for future research are made.

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# 1.2

## Recent advances in progressive supranuclear palsy: a review

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## ABSTRACT

Progressive Supranuclear Palsy (PSP) has been used to denote a unifying disorder with progressive parkinsonism with early falls, vertical supranuclear gaze palsy, pseudobulbar dysfunction and cognitive decline. Over the last decade, heterogeneity of the disease into different clinical subtypes has been recognized in clinicopathological studies. Although neuroimaging features and laboratory findings may support the diagnosis, true biomarkers are still lacking in the clinical setting. Neuronal and glial tau-positive aggregates are predominantly found in basal ganglia and brainstem, and the significant association of PSP with the common H1 tau haplotype likely points to a pathophysiological role of the tau protein in the disease process. Future genetic studies of familial cases and an ongoing genome-wide association study of large series of pathological-proven cases may reveal additional genetic factors in the near future.

## INTRODUCTION

In 1964 Steele *et al.* described a uniform clinical and neuropathological picture in 9 patients, and designated it with the term progressive supranuclear palsy (PSP).[1] Since its discovery, much work has been conducted on clinical, pathological and genetic aspects. The disorder has now been recognised as the second most common parkinsonian neurodegenerative disorder after Parkinson's disease (PD). Although clinical criteria for PSP have proven to be very useful and have been widely accepted,[2] recent studies have emphasized that clinical presentations do not always fulfil the criteria for possible or probable PSP. An important contribution to the clinical setting and future therapeutic interventions is the PSP Rating Scale which can be used to semi-quantitatively evaluate disease progression.[3] PSP has classically been considered a sporadic disease, but recent studies have reported families with PSP and increased familial aggregation.[4] Characteristic neuroimaging features have been demonstrated and a recent study has reported a potential biomarker in cerebrospinal fluid.[5] PSP is classified as a tauopathy because of the neuropathological aggregates which consist of hyperphosphorylated Microtubule Associated Protein Tau (MAPT). The genetic association of PSP with the H1 *MAPT* haplotype has been known for more than a decade, but it has been delineated in more detail in recent years. The type of tau pathology has been further characterized by the development of isoform-specific antibodies,[6] and a scoring system for tau severity has proven to be useful for the morphologic assessment of PSP tau pathology.[7] Several trials with different agents have been carried out, of which Coenzyme Q10 appears to improve cerebral energy metabolism.[8] In light of these recent developments, a review on recent advances in clinical, pathological and genetic research is warranted.

## EPIDEMIOLOGY

PSP accounts for approximately 5% of all parkinsonian disorders.[9] The age-adjusted prevalence of PSP has been estimated at 5–6.4 per 100.000,[10,11] and the incidence of PSP increases with age, from 1.7 (per 100.000 person-years) for those aged 50 to 59, to 14.7 for those aged 80 to 99.[12] Lower incidence rates have recently been reported in Russia (0.14/100.000) and Sweden (1.2/100.000).[13,14] The disease might affect men more frequent than women.[15]

In Guadeloupe, a French Caribbean island, an unexpectedly high frequency (75%) of atypical parkinsonism unresponsive to levodopa has been reported; only 25% fulfilled the Brain Bank criteria for PD.[16] Half of the patients with atypical parkinsonism had a PSP like syndrome with oculomotor disturbances and postural instability with falls. However, only a small subset fulfilled the criteria for PSP, whereas the majority differed from classic

PSP because of the presence of hallucinations (unrelated to medication), dysautonomia and tremor.[17] Among these patients with atypical parkinsonism, a strong association was found with the consumption of herbal tea and tropical fruits containing acetogenins, which are potentially toxic inhibitors of the mitochondrial respiratory chain.[16] This has given impetus to investigate the role of annonacin (the major acetogenin in these tropical fruits) as a strong mitochondrial complex I inhibitor, which induces neurodegeneration with GABAergic cell loss in the striatum and cholinergic and dopaminergic cell loss of the substantia nigra in animal models.[18] In case-control series of Caucasian origin, no significant association has been found with environmental factors, although some evidence has been revealed for lower education levels in PSP patients compared to controls.[19,20]

Familial aggregation in PSP is an issue of controversy. Although classically considered a sporadic disease, a non-significant trend towards a positive family history was found and several families with clustering of PSP-like disorders have been reported in literature.[19,21–24] More recently, a large case-control study has shown that the occurrence of parkinsonism in first-degree relatives of PSP patients (12%) is higher than in controls (3%), whereas equal frequencies of dementia were found in both groups (25% versus 23%).[4]

## CLINICAL FEATURES AND DIAGNOSIS

Progressive parkinsonism starting in the seventh decade with prominent disequilibrium problems and falls, is the typical presentation of the disease. Other less frequently reported symptoms at onset are memory impairment, personality change, pseudobulbar problems, blurred vision or diplopia.[25] Vertical gaze palsy, the most characteristic feature of PSP is usually absent in the initial phase, whereas slowing of vertical saccades can often be observed at neurological examination at this stage.[2]

High predictive values have been found for possible and probable PSP based on the international consensus criteria (NINDS-SPSP, Table 1).[2,26,27] A few shortcomings of these criteria have to be mentioned. First of all, a considerable number of patients with a full-blown clinical picture of PSP did not have frequent falls in the first year, which classifies them as possible PSP and excludes the diagnosis probable PSP; Secondly, the existence of a parkinsonism subtype (PSP-P) in a large clinicopathological study[28] has broadened the clinical spectrum, but has therefore lowered the sensitivity of the criteria. In contrast to the classical picture of PSP with falls, gaze palsy and cognitive dysfunction (the so-called Richardson's syndrome), PSP-P is characterized by an asymmetric onset, tremor, a good response to levodopa, and longer disease duration.[28] This latter presentation accounts for 8–32% of all PSP patients and is often mistaken for PD.[28,29] Another clinical presentation of PSP is the syndrome of Pure Akinesia with Gait Freezing (PAGF), which includes a gradual onset with early freezing of gait or speech, without rigidity, tremor,

**Table 1.** Clinical consensus criteria for PSP. (Litvan *et al.* 1996)

| **NINDS-SPSP criteria for PSP** |
|---|
| **Possible PSP**<br>Gradually progressive disorder; onset age 40 or later; *either* vertical supranuclear palsy or both slowing of vertical saccades and postural instability with falls within a year of disease onset; no evidence of other diseases that could explain the foregoing features |
| **Probable PSP**<br>Gradually progressive disorder; onset age 40 or later; vertical supranuclear palsy *and* prominent postural instability with falls within a year of disease onset; no evidence of other diseases that could explain the foregoing features |
| **Definite PSP**<br>Clinically probable or possible PSP *and* histopathological evidence of typical PSP |

dementia, or eye movement abnormalities during the first 5 years of the disease and without benefit from levodopa therapy.[30]

A frontal presentation with prominent cognitive dysfunction and behavioural changes has been identified in 20 percent of a large population based cohort, which may suggest an alternative neurological or psychiatric diagnosis, for example FTD or depression in the initial phase.[29] Apart from mental slowness and apathy, executive dysfunction is one of the characteristic cognitive features in PSP and includes reduced verbal fluency, impaired abstract thinking, and difficulty planning and set shifting. The Frontal Assessment Battery (FAB) is a simple test of executive function and helps to differentiate PSP from MSA and PD (cut off score of 15).[31,32] The applause sign is frequently present in PSP patients and demonstrates the reduced motor control which is thought to be mediated by frontal and/or basal ganglia dysfunction.[33] Its specificity for PSP, however, is a current subject of debate, as it is present in several other neurodegenerative disorders.[34] In a recent paper, recognition of negative emotions appears to be impaired in PSP patients, but this has to be replicated.[35]

The fixed or surprised facial expression characteristic for PSP is presumed to result from focal dystonia of facial muscles.[36] Blepharospasm, limb dystonia and retrocollis are other dystonic features which may evolve during the disease.[37] The rapidly progressive nature of the disease is reflected by an average interval of five to six years between onset and a wheelchair-requirement stage.[38] Dysarthria and dysphagia develop much earlier in PSP than in PD, and an unintelligible speech occurs after a mean disease duration of six years.[38,39] Patients with the Richardson type of PSP have a survival of seven to eight years, whereas PSP-P patients tend to have a much longer survival.[40,41] The early occurrence of falls, dementia, and oculomotor dysfunction as well as male gender and older age at onset is associated with increased mortality risk.[25,41] Also, a high score on the PSP rating scale has proven to be a good independent predictor of survival and may be helpful to determine the prognosis in individual patients.[3,41]

## DIFFERENTIAL DIAGNOSIS

PSP is often misdiagnosed in the early phase of the disease. This is reflected by a mean interval of 4 years between onset and time of correct diagnosis, often because ophthalmoplegia is lacking in this stage.[29,42] PD or unspecified parkinsonism, balance disorder, cerebrovascular disease and dementia are the most common misdiagnoses.[25] On the other hand, in approximately 80 percent, pathological examination confirms the clinical diagnosis PSP established over the course of the disease.[27,43] Vertical gaze palsy occasionally occurring in PD, multiple system atrophy (MSA), corticobasal degeneration (CBD) and dementia with Lewy Bodies (LBD), may have misled the clinician in false-positive cases.[27,44–46] Usually, there is an isolated upward gaze limitation in these disorders (or even in normal aging) and therefore downward gaze palsy may be more discriminative for PSP. Tremor, psychosis, dementia and asymmetry are supportive findings against the diagnosis PSP.[43] Also, drug induced dyskinesia, late autonomic dysfunction and visual hallucinations are more supportive of PD, LBD or MSA than for PSP-P.[47] Patients with MSA are usually younger, commonly show signs of severe autonomic dysfunction and develop falls, unintelligible speech and cognitive impairment later in the disease course than in PSP.[40] Vascular Parkinsonism is characterized by more asymmetric signs, lower body involvement and a later occurrence of falls.[48,49] Differentiating PSP from CBD can be very challenging, as both disorders show considerable overlap in clinical features suggesting that both disorders represent different points of a single disease spectrum. In a few published case series, PSP may present with CBS, including asymmetrical features, apraxia and alien limb phenomena (PSP-CBS subtype).[50] Finally, PSP (and CBD) may present with a progressive apraxia of speech, nonfluent aphasia (PNFA), or a combination of these.[51,52] It has been suggested as a new variant within the clinical spectrum of PSP, designated as PSP-PNFA (Table 2).

**Table 2.** Clinical subtypes of PSP

| PSP- Subtypes | Clinical presentation |
|---|---|
| **Richardson's syndrome** | Early falls and postural instability; early vertical gaze palsy; early cognitive decline |
| **PSP-parkinsonism** | Asymmetric onset; levodopa response; tremor |
| **PSP-PAGF** | Gradual onset of freezing of gait or speech; no tremor; no sustained response to levodopa; and no dementia, rigidity and ophthalmoplegia in the first 5 years of disease. |
| **PSP-PNFA** | Difficulty with speech production (progressive apraxia of speech, nonfluent aphasia (PNFA), or a combination of these) |
| **PSP-CBS** | Asymmetrical features; apraxia; alien limb phenomena |
| **PSP-FTD** | Early cognitive and behavioural symptoms |

## INVESTIGATIONS

Characteristic neuroimaging features may improve the diagnostic accuracy in individual PSP patients, although visual interpretations are highly influenced by radiological expertise. Prominent midbrain atrophy is often present in PSP and is visualized as a "penguin" or "hummingbird" sign on midsagittal MRI[53–55], and "morning glory sign" on axial MRI,[56] although this feature may also be seen in MSA.[57] A significantly smaller anterior-posterior midbrain diameter measured in axial view has been found in some, but not all studies. [55,58,59] Other quantitative studies of the midbrain include two- or three dimensional measurements,[60,61] which can be useful to rapidly differentiate PSP from other parkinsonian syndromes and to follow up disease progression.[61–63] MRI may also show atrophy of the superior cerebellar peduncle (SCP) in PSP, although its measurement has shown overlap with MSA.[60,63,64] Recently, a so-called MR parkinsonism index has been proposed that combines measurements of structures mainly involved in PSP (midbrain and SCP) and MSA (pons and MCP), and could accurately differentiate PSP from PD and MSA.[60] The diagnostic value of diffusion-weighted MRI is relatively limited, as apparent diffusion coefficient (ADC) in basal ganglia has found to be higher in PSP compared to Parkinson's disease in some,[65,66] but not all studies.[67] In contrast, the superior cerebellar peduncle has shown higher ADC values in PSP than in PD and MSA, which indicates that demyelination and gliosis occur early in the course of the disease.[59] Finally, lower volumes of frontal cortex and subcortical nuclei in PSP patients have been correlated with executive deficits.[68–70]

Hypometabolism of the brainstem and anterior cingulate cortex (ACC) on PET scan is a disease-specific pattern of PSP with a high sensitivity and specificity,[71,72] and is in accordance with regional neuropathological changes. An interesting question is whether specific loss of neurotransmitter receptors accompanies this pattern of hypometabolism in ACC, as has been demonstrated in the neocortex of PD patients. PET scanning with an *in vivo* marker of peripheral benzodiazepine site expression (the radioactive ligand [$^{11}$C] PK11195), has visualized activated microglia in brainstem, cerebellum, basal ganglia, and frontal cortex, probably reflecting the glial response to the degenerative process.[73] However, this ligand causes a great amount of non-specific binding with considerable variation across individual patients.[74]

Reduced binding of striatal pre-synaptic dopamine transporters (DAT) is found in several parkinsonian disorders, including PSP, but is not helpful in differentiating them.[75] There is some evidence that statistical parametric mapping applied to [(123)I]beta-CIT SPECT in midbrain can enhance differentiation between PD and atypical parkinsonism.[76] Reduction of postsynaptic D2 receptors is suggestive for MSA or PSP, but cannot discriminate between them, whereas a normal postsynaptic D2 receptor status cannot

exclude atypical parkinsonism.[77] Moreover, some late stage PD patients can show low striatal postsynaptic radiotracer binding as well.[78]

Iodine-123-meta-iodobenzylguanidine ([$^{123}$I]-MIBG) is a radio-iodinated analogue of norepinephrine and used to visualize the myocardial sympathic nerve terminals. The uptake is significantly lower in PD patients compared to PSP patients.[79] However, in a more recent study, nearly 70% of the patients without PD (including 7 PSP patients) had decreased uptake, with considerable overlap between PD patients, indicating that MIBG cannot necessarily distinguish PD from PSP patients.[80] Mitochondrial dysfunction in the pathophysiology has been suggested by the observation that high-energy metabolites on phosphorus MR spectroscopy are significantly reduced in basal ganglia and frontal cortex of patients with early-stage PSP.[81] This mitochondrial role is consistent with experimental studies with annonacin, which is linked to a PSP-like syndrome on Guadeloupe.[82]

A potential biomarker to improve the diagnostic accuracy of PSP may be the quantitative analysis of cerebrospinal fluid on tau products or isoforms. Although total and phospho-tau levels in PSP have proven to be similar to controls,[83] Borroni *et al.* have recently developed an immunoprecipitation assay recognizing proteolytic tau products and has found a significantly lower ratio (33kDa/55 kDa) in the CSF of PSP patients compared to that of other neurodegenerative disorders, like AD, FTD and MSA.[5] However, a recent study was not able to confirm the presence of these tau forms in CSF.[84]

Sandwich ELISAs for quantification of three-repeat and four-repeat tau isoforms have recently been developed, and have successfully shown increased four-repeat tau in brain homogenates from frontal cortex and caudate nucleus of PSP brains.[85] The next step will be to use these assays to study tau isoform changes in CSF.

## NEUROPATHOLOGY

In contrast to PD, LBD and MSA where the accumulation of alpha-synuclein is the prominent neuropathological feature, PSP belongs to the "tauopathies": a group of neurodegenerative disorders characterized by aggregates of hyperphosphorylated tau protein. Globoid neurofibrillary tangles (NFTs), neuropil threads (NT), tufted astrocytes (TA) and oligodendroglial coiled bodies (CB) can be visualized with antibodies against tau and are found in basal ganglia, diencephalon and brainstem.[86] The insoluble aggregates of tau protein in PSP are made up of ultramicroscopic straight filaments in contrast to the paired helical filaments seen in AD. The severity of tau pathology varies considerably between different brain regions and between individual cases, and cortical tau pathology has been associated with cognitive impairment.[87] The subthalamic nucleus, globus pallidus and substantia nigra are the most severely affected brain regions. The motor

cortex and anterior cingulate cortex are often involved with variable neuron loss and NFTs, whereas parietal and temporal cortex shows no neuron loss and only sparse NFTs. The presence of TA, commonly found in motor cortex and striatum, is highly specific for PSP and may represent a central degenerative process rather than a reactive change to gliosis.[88] Spinal cord may also be involved in the disease process, although this structure has not been routinely investigated.[89,90] The overall tau lesion severity in all brain regions has been significantly correlated to the CB + NT score in substantia nigra, caudate and dentate nuclei using a five-point grading system.[7] Interestingly, this PSP-tau score has shown a negative correlation with disease duration.[7,41]

Through alternative splicing of the *MAPT* gene, six tau isoforms are generated. The in- or exclusion of exon 10 results in tau isoforms with four-repeat (4R) or three-repeat (3R) mictrotubule binding sites respectively. In normal situation, the level of 3R and 4R tau is equal, whereas in PSP there is an increased 4R/3R ratio. The concept of PSP as a 4R tauopathy has been confirmed by immunoblotting, where abnormal insoluble tau migrates as two bands (68 and 64kDa, which comprise 4R tau),[91] and positive immunohistochemical staining of tau aggregates with specific antibodies against 4R tau isoforms, and negative staining with antibodies against 3R tau isoforms.[92] Under normal conditions, tau is bound to microtubules and regulates the assembly and stabilization of microtubules which is essential for intraneuronal vesicle and_organelle transport.[93]Unbound phosphorylated tau (particularly 4R tau) has a tendency to aggregate, causing a toxic gain of function. Furthermore, the lack of tau to stabilize the microtubules leads to loss of the physiological function of microtubules.

Recent studies have shown differences in pathological severity in clinical subtypes in PSP. PSP-P showed relatively more 3R tau isoforms in the insoluble tau fraction and significantly less tau burden compared to Richardson's syndrome. In atypical PSP syndromes as PSP-PNFA, PSP-CBS and PSP-FTD, greater tau pathology in the cortical areas are found (cortical predominant atypical PSP), while PSP-P and PSP-PAGF show more tau burden in the globus pallidus, diencephalon and brainstem (brainstem predominant atypical PSP). [94]

Cholinergic deficits are thought be responsible for motor and cognitive symptoms in PSP, which is confirmed by the observation of reduced cholinergic receptors (M2 and M4 receptors) in the posterior striatum and thalamus,[95,96] but normal cholinergic receptor density in the frontal cortex.[97]

Concurrent pathologies in PSP have been reported and include AD,[98]Lewy bodies[99], argyrophilic grain disease[100] and CBD[101–103] and is thought to occur independently of PSP pathology. Their clinical relevance has been difficult to determine due to the limited number of cases. Increased age, female sex and APOE ε4 carrier status are risk factors for AD pathology in PSP.

## GENETICS

The involvement of *tau* in the pathogenesis of PSP is further supported by results from genetic studies. The initial association of PSP with the dinucleotide repeat (A0) in intron 9 of *MAPT* has been subsequently extended to other polymorphisms in linkage disequilibrium with the A0 polymorfism.[104–106] The high degree of linkage disequilibrium in the *MAPT* region is thought to result from an inversion of 900 kb occurring 3 million years ago, producing the two extended *MAPT* haplotypes H1 and H2.[107] Both the H1 haplotype (including the A0 polymorphism) and the H1/H1 genotype are found significantly more often in PSP patients.[106–108] Fine-mapping of this region has revealed a subhaplotype (H1c) with a variation in intron 0 of *tau*, which seems to influence the expression of tau.[109] Very recently, another subhaplotype including a variant 5′ upstream of *MAPT* and *CRHR1* genes has been associated with an earlier age at onset and its location suggests a *cis* element regulating gene expression.[110]A single genome wide association study has found a second major locus on chromosome 11 containing several interesting candidate genes,[111] but this has to be replicated by other groups. An interesting finding from a recent genome wide association study in PD patients, revealed besides an association in the gene encoding alpha-synuclein, a second locus with strong association at the *MAPT* locus.[112] These data suggest a link between molecular pathways between both disorders.

Mutations in *MAPT* are commonly associated with frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17). In some families, the clinical phenotype is consistent with PSP including supranuclear gaze palsy, saccadic eye movements and axial rigidity, although age at onset is usually much younger than classical PSP.[113–117] Screening of large cohorts of sporadic and familial PSP cases, however, do not reveal these mutations.[118] In two series, ~7% of all PSP patients fulfilled the criteria for an autosomal dominant mode of transmission.[4,119] The phenotype varied among PSP, dementia, tremor, and parkinsonism within these pedigrees. For one large family with an autosomal dominant form of PSP, linkage to the chromosome 1q31.1 region has been found and awaits identification of the causal gene defect.[120] Pathological examination in one affected family member within this pedigree confirmed the clinical diagnosis PSP, but the occurrence of action or postural tremor with facial tics or synkinesias in others, suggests independent segregation from the PSP phenotype. Familial clustering has also been described in several other studies, sometimes with pathological confirmation of PSP in affected relatives, but most of these families were too small for linkage analysis.

## MANAGEMENT

To date, no effective therapy to delay or stop the progression of PSP is available. Levodopa may have a moderate but transient response and it is worthwhile to attempt in the early stages. Amitriptyline has shown a beneficial effect on motor and bulbar problems in a few case reports.[121] Neurotransmitter replacement approaches are unsuccessful in PSP,[122] possibly due to the widespread neuronal loss. Riluzole has also proven to be unsuccessful as a disease-modifying agent in a recent multicenter double-blind randomized placebo-controlled trial.[123]

Mitochondria are the major source for the generation of reactive oxygen species and several studies provide evidence for mitochondrial dysfunction in PSP.[93] Imaging studies with proton and phosphorus MR spectroscopy showed decreased concentrations of high energy phosphates in basal ganglia and frontal lobes, which was unlikely due to neuronal death only. Furthermore, oxidative stress and reactive oxygen species activate tau kinases which causes tau to hyperphosphorylate and aggregate more easily. Finally, annonacin (the toxic substance associated with PSP on Guadeloupe) inhibits complex 1 which reduces ATP levels and induces tau redistribution from the axons to the cell body and leads to cell death. A recent phase II clinical trial with Coenzyme Q10, a physiological cofactor of complex I, showed significant improvement of cerebral energy metabolism and mild clinical improvement in the short term.[8] However, more research is required to confirm these findings and to investigate long term effects. Results from other clinical studies with tau-kinase inhibitors, tau-aggregation inhibitors and microtubule stabilizers like davunetide will be awaited in the near future.

Relief of symptoms with palliative therapies remains the keystone of disease management and includes different aids (shoes with heels, weighted walker, angled glasses) and forms of rehabilitation programs for balance, gait, speech, swallowing and vision problems. In later stages however, insertion of PEG may be necessary. Botulinum toxin injections may lead to functional improvement in all forms of dystonia, especially blepharospasm.[124] The burden for caregivers is related to the disease severity and disability; this increases during the first 18 months after diagnosis and then stabilizes.[125] Psycho-educational programmes and supportive care can help lighten the burden for caregivers.

## FUTURE RESEARCH

Over the last decade, clinical advancements have been achieved by refining the clinical spectrum of PSP into different subtypes. In this context, it is an intriguing question whether these subtypes represent pathophysiological heterogeneity or only reflect the

effect of a modulating factor. One of the challenges in the field of PSP research will be to develop biomarkers to establish the diagnosis of clinically typical and atypical PSP during life. A considerable number of PSP cases present with non-classical symptoms and for future trials, it is important to identify these patients. A second challenge will be to identify genetic networks involved in PSP, starting from the coming results of a genome-wide association study. For AD and PD, recent studies have demonstrated the early synaptic changes in mouse models of presenilin 1- , Pink1 gene mutations and overexpression of alpha-synuclein.[126–128] As PSP lacks a transgenic mouse model, an alternative approach might be to carry out proteomics of the synaptosome on fresh-frozen brain samples of patients died from PSP. Finally, the identification of annonacin as toxic agent in a PSP-like disorder may give further impetus to research on the possible role of mitochondrial dysfunction in PSP pathophysiology. Trials with Coenzyme Q10 and davunetide are hopefully the first steps in the strategy to delay the progression of this devastating and disabling disease.

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# 2

# CLINICAL ASPECTS OF PROGRESSIVE SUPRANUCLEAR PALSY AND FRONTOTEMPORAL DEMENTIA

# 2.1

## Survival in progressive supranuclear palsy and frontotemporal dementia

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## ABSTRACT

**Objective:** To compare survival and to identify prognostic predictors for progressive supranuclear palsy and frontotemporal dementia.

**Background:** Progressive supranuclear palsy (PSP) and frontotemporal dementia (FTD) are related disorders. Homozygosity for H1 haplotype is associated with PSP, whereas several *MAPT* mutations have been identified in FTLD-tau. Survival duration probably reflects underlying pathophysiology or disease.

**Methods:** Patients with PSP and FTD were recruited by nationwide referral. Survival of 354 FTD patients was compared with that of 197 PSP patients. Cox regression analysis was performed to identify prognostic predictors. FTLD-tau was defined as Pick disease and FTDP-17 with *MAPT* mutations. Semiquantitative evaluation of tau-positive pathology was performed on all pathologically proven cases.

**Results:** The median survival of PSP patients (8.0 years; 95% CI 7.3 to 8.7) was significantly shorter than that of FTD patients (9.9 years; 95% CI 9.2 to 10.6). Corrected for demographic differences, PSP patients were still significantly more at risk of dying than FTD patients. In PSP, male gender, older onset-age, and higher PSP Rating Scale score were identified as independent predictors for shorter survival, whereas in FTD a positive family history and an older onset-age were associated with a poor prognosis. The difference in hazard rate was even more pronounced when comparing pathologically proven cases of PSP with FTLD-tau.

**Conclusion:** Survival of PSP patients is shorter than that of FTD patients, and probably reflects a more aggressive disease process in PSP. Independent predictors of shorter survival in PSP were male gender, older onset-age and higher PSP rating scale score, whereas in FTD a positive family history and higher onset-age were predictors for worse prognosis.

## INTRODUCTION

Progressive supranuclear palsy (PSP) is clinically characterized by parkinsonism, supranuclear gaze palsy and cognitive decline,[1,2] and shows clinical, pathological and genetic overlap with frontotemporal dementia (FTD).[3–5] A frontal presentation has been identified in 20 percent of PSP cases,[6] whereas FTDP-17 associated with microtubule associated protein tau (*MAPT)* mutations may present with the clinical picture of PSP.[7,8] Neuronal and glial tau-positive inclusions are found in PSP and consist mainly of hyperphosphorylated four-repeat tau isoforms. In contrast to this, a subset of FTLD with Pick bodies (so-called Pick disease) is characterised by the accumulation of three-repeat tau isoforms,[9] whereas inclusions in FTDP-17 with *MAPT* mutations variably consist of three- and four-repeat tau isoforms, depending on the location of the mutation.[10] The relevance of clinical and pathological overlap is further emphasised by the strong association between *MAPT* H1/H1 genotype and PSP.[11] Determining survival within this FTLD-PSP spectrum is of important clinical relevance and may give insight into the underlying disease process. However, only a few small studies compared survival between PSP and FTD and did not find any differences.[12,13] Small pathological series of PSP and tau-positive and tau-negative FTLD patients have shown conflicting results regarding the effect of tau pathology on survival.[14–16] In a recent study, specific neuropsychological profiles in FTLD have been correlated to disease duration, whereas onset-age or positive family history were not.[12] Early falls and gaze palsy have been found as prognostic features in retrospective studies on PSP,[17–20] whereas the PSP Rating Scale (PSPRS) has also proven to be of predictive value in survival in a prospective longitudinal study.[21] However, this still has to be replicated. Severity of tau pathology has shown an inverse correlation with prognosis in PSP,[22,23] whereas conflicting results have been reported in FTLD.[14–16,24]

The aim of this study is to prospectively investigate the survival in two large cohorts of PSP and FTD patients in relationship to demographic and clinical features, and to the presence and severity of tau pathology in a subset of patients who underwent brain autopsy.

## METHODS

Patients with PSP and FTD were recruited by nationwide referral from neurologists and by visiting patients in nursing homes.[6,25] Detailed clinical history, including the first presentation of symptoms, was obtained from patients and their family members, and by reviewing medical records. The onset-age was defined as the age at which the first symptom attributable to PSP and FTD appeared according to the patient's caregiver

and from medical records. In case of discrepancies, data from medical records were used. Data on family history were obtained using a structured questionnaire provided by spouse or first-degree relative. Family history was defined positive if at least one first-degree relative suffered from dementia, parkinsonism or motor neuron disease. All available hard copies of neuroimaging of both PSP and FTD patients were reviewed by the investigators in order to exclude other structural causes of both conditions and to semiquantitatively measure the severity of lobar atrophy.

PSP patients were neurologically examined and videotaped and the severity of their cognitive and motor functioning was scored by means of Mini-Mental State Examination (MMSE), Frontal Assessment Battery (FAB), Unified Parkinson's Rating Scale-III (UPDRS-III) and PSPRS.

FTD patients underwent neurological examination, neuropsychological evaluation and neuroimaging (CT, MRI or SPECT with 99mTc-hexamethyl propyleneamine oxime (HMPAO)). The clinical diagnosis of all patients was established in a consensus meeting according to the National Institute of Neurological Diseases and Stroke-Society for PSP (NINDS-SPSP) criteria[2] and the Lund and Manchester criteria for FTD.[26] PSP patients were subdivided according to phenotype as described by Williams *et al.*[27] One hundred and twenty-one patients were classified as Richardson's syndrome (RS), and seven cases of PSP-parkinsonism (PSP-P) were identified in our cohort. Of 18 patients, there was insufficient data available on the first two years after onset. The remainder of the patients (n = 51) could not be subdivided into a phenotype. Both studies on PSP and FTD patients were approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam. Informed consent for participation (including blood collection) was obtained from the spouse or a first-degree relative of each patient. *MAPT, CHMP2B* and *GRN* genes were sequenced in all familial FTD patients, as has been previously described.[28–30] In PSP patients with a positive family history, screening of *MAPT*, *GRN,* and *LRRK2* was performed according to previously described methods.[29,31,32]

The possibility of post-mortem examination was discussed with patients and their relatives during follow-up. Brain autopsy of patients who gave consent and who died during follow-up was conducted by The Netherlands Brain Bank according to their Legal and Ethical Code of Conduct. All brains that became available for autopsy were processed for routine staining and immunohistochemistry with AT8 (1:40, Innogenetics, Ghent, Belgium), ubiquitin (1:500, Dako, Glostrup, Denmark), three-repeat tau isoform (RD3, Upstate, Charlottesville, VA; 1:3000) and four-repeat tau isoform (RD4,Upstate, Charlottesville, VA; 1:100), p62 (BD Biosciences Pharmingen, San Diego, California, USA; 1:200, following 80° C antigen retrieval), TDP-43 (Proteintech, Chicago, Illinois, USA; 1:100, following pressure-cooking), β-amyloid (anti-β-amyloid, DAKO, Glostrup, Denmark, 1:100, following formic acid pre-treatment) and α-synuclein (anti-α-synuclein, Zymed Laboratories, San Francisco, California, USA; undiluted, following formic acid pre-

treatment). These were incubated overnight at 4 °C. Endogenous peroxidase activity was inhibited by 30 min incubation in PBS–hydrogen peroxide–sodium azide solution (100 ml 0.1M PBS, 2 ml 30% $H_2O_2$, 1 ml natriumazide). The Histostain-Plus broad-spectrum kit DAB (Zymed, San Francisco, California, USA) was used as a detection system. Slides were counterstained with Mayer's haematoxylin and mounted in Entellan.

The neuropathological diagnosis FTLD was classified into FTLD-tau and FTLD-U (with or without TDP-43-positive inclusions).[33] FTLD-tau was defined as Pick disease and FTDP-17 with *MAPT* mutations. Cases with FTD-MND were excluded from this study. In FTLD-tau cases, neuronal loss and tau-staining reactive neurons and glial cells were visually quantified (none, mild, moderate and severe) in the following regions: frontal lobe, temporal lobe, hippocampus, parietal lobe, caudate nucleus and substantia nigra.

The neuropathological diagnosis PSP was established according to international criteria,[34] and a semiquantitative assessment of neurofibrillary tangles (NFT), tufted astrocytes (TA), oligodendroglial coiled bodies (CB) and thread pathology (Th) in all regions was carried out by two raters (WK, JvS) using a five-point grading scale according to Williams *et al.*[23] The PSP-tau score was calculated from the combined grade of coiled bodies and thread lesions in the substantia nigra, and caudate and dentate nucleus.

Follow-up of PSP and FTD patients was performed by visits to the outpatient department of the Erasmus Medical Center or by telephone interview with relatives up to 1 August 2008.

### Statistical analysis

SPSS 15.0 for Windows (SPSS, Chicago, Illinois, USA) was used for analysis. Onset-age, gender and family history were analyzed by independent sample t-test or Chi-square test. Actuarially corrected median survival was calculated, as well as the mean survival in deceased cases. Survival analysis was performed using the Cox proportional hazard model, using a backward selection procedure model. Only results of multivariate analyses are shown, with variables that were significant in the univariate analysis. As the early occurrences of clinical symptoms are incorporated into the PSPRS, these symptoms were not analyzed together with the PSPRS in one model. However, different sections of the PSPRS (history, mentation, bulbar, ocular, limb and gait sections) were analyzed separately. Entry date was set as time of first symptoms. Censoring date was either date of death or end of follow-up (1 August 2008). The assumption of proportionality of hazards was examined by log-log plots. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated. Onset-age and PSPRS score were categorised into quartiles. Correlation between tau pathology and disease duration and onset-age was examined using Spearman calculation. All statistical testing took place at a 0.05 level of significance (two-tailed).

## RESULTS

The demographic data of patients with PSP and FTD are summarized in Table 1. FTD-MND patients (n = 30) were excluded, due to their known shorter disease duration. Two PSP patients died of non-natural cause and have not been included in the survival analyses. The mean onset-age and age at death of PSP patients were significantly higher than that of FTD patients. During follow-up, 133 of 197 patients with PSP died at a mean disease duration of 7.2 ± 2.6 years, whereas 242 out of 354 FTD patients had died after a mean disease duration of 9.2 ± 4.1 years.

**Table 1.** Demographic characteristics of patients with PSP and FTD

| | PSP | FTD | p-value |
|---|---|---|---|
| **N** | 197 | 354 | |
| **Age at symptom onset , years*** | 66.2 ± 8.1 | 57.5 ± 8.9 | < 0.001 |
| **Male gender, n (%)** | 102 (51.8) | 164 (46.3) | 0.220 |
| **Presence of family history, n (%)** | 62 (31.5) | 169 (47.7) | < 0.001 |

*Mean ± SD. PSP, progressive supranuclear palsy; FTD, frontotemporal dementia.

### Survival and hazard analysis of PSP and FTD

The median disease duration in PSP patients (8.0 years; 95% CI 7.3 to 8.7) was significantly shorter than in FTD patients (9.9 years; 95% CI 9.2 to 10.6) (Chi-square 17.1, $p < 0.001$) (Fig. 1). This worse prognosis for PSP patients than FTD patients in a univariate analysis (HR 0.634; 95% CI 0.509 to 0.788) remained significant after adjustment for gender, onset-age and family history (HR 0.766; 95% CI 0.603 to 0.975). Comparing PSP phenotypes, RS (6.8 years; 95% CI 6.3 to 7.4) was found to have a shorter median survival than PSP-P (10.9 years; 95% CI 7.5 to 14.2) and the non-conclusive group (8.8 years; 95% CI 8.2 to 9.3).

A Cox proportional hazards regression model of PSP patients (Table 2) revealed male gender, older onset-age (> 72 years) and higher score on the PSPRS to be independent predictors for shorter disease duration, after adjustment for the interval between onset and ascertainment (mean of 5.3 ± 2.6 years).

When entering separate sections of the PSPRS in the model, only supranuclear ocular motor exam (HR 1.195; 95% CI 1.090 to 1.310) remained significant, whereas bulbar exam (HR 1.144; 95% CI 0.997 to 1.312) and gait exam (HR 1.063; 95% CI 0.999 to 1.131) were near significant.

In FTD patients, positive family history (HR 1.438; 95% CI 1.114 to 1.858) and onset-age > 64 years (HR 1.656; 95% CI 1.160 to 2.363) were significantly associated with poor survival. Looking into family history in FTD in more detail, the mean disease duration of deceased FTD patients with a negative family history (9.9 years; 95% CI 9.1 to 10.6) was

significantly longer than that of FTD patients with a positive family history (8.4 years; 95% CI 7.7 to 9.1; $p = 0.006$). In this latter group a trend towards longer mean disease duration of patients with a *MAPT* mutation (n = 36 from 10 families; 9.3 years; 95% CI 7.8 to 10.8) was found compared with patients without a *MAPT* mutation (n = 83, including 17

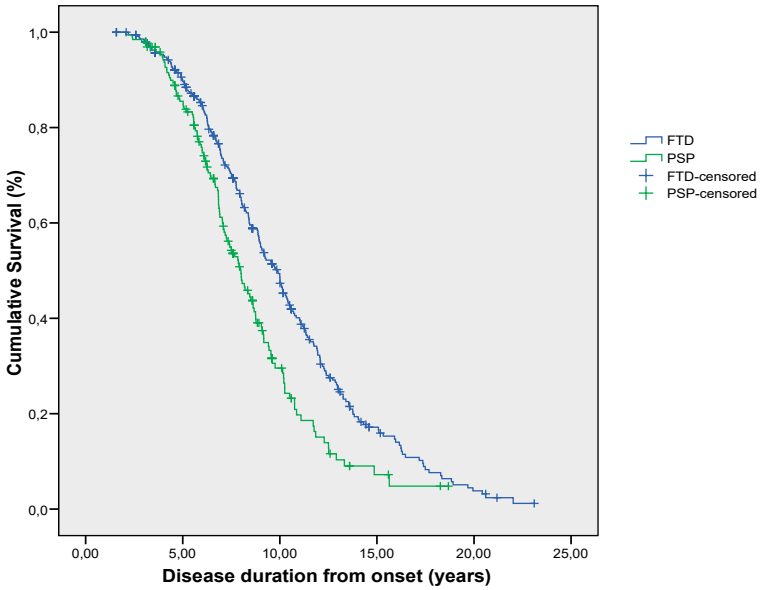


**Figure 1.** Kaplan Meier survival curve for PSP and FTD patients

**Table 2.** Multivariate Cox models in PSP patients (HR with 95% CI)

| | | p-value |
|---|---|---|
| **Gender** | 0.627 (0.428 to 0.918) | 0.016 |
| **Positive family history** | | ns |
| **Estimated onset-age** | | |
| < 62 | 1 (reference) | |
| 62 to 66 | 0.878 (0.515 to 1.498) | 0.634 |
| 66 to 72 | 1.453 (0.846 to 2.498) | 0.176 |
| > 72 | 2.028 (1.211 to 3.398) | 0.007 |
| **PSPRS*** | | |
| 0 to 35 | 1 (reference) | |
| 35 to 48 | 1.956 (1.068 to 3.584) | 0.030 |
| 48 to 62 | 2.991 (1.646 to 5.435) | < 0.001 |
| > 62 | 8.553 (4.478 to 16.337) | < 0.001 |

*10 patients had missing values on the PSPRS;
HR, Hazard ratio; CI, confidence interval; ns, not significant

patients with a *GRN* mutation from three families; 8.1 years; 95% CI 7.3 to 8.8; $p = 0.105$). Of the *MAPT* mutations, L315R had the shortest mean disease duration (n = 5; 5.7 ± 1.9 years), followed by P301L (n = 20; 8.2 ± 3.0 years), whereas R406W had the longest mean disease duration (n = 4; 17.5 ± 3.2 years). The remaining *MAPT* mutations, S320F (n = 1), G272V (n = 5), and ΔK280 (n = 1) all had a mean disease duration of just above 10 years. Patients with a *GRN* mutation had a survival of 7.7 ± 2.8 years.

### Pathology

Pathological examination was available for 24 PSP patients (all RS) and 61 FTLD patients (FTLD-tau n = 32 and FTLD-U n = 29). Men were over-represented (70.8%) in the PSP series, and the FTLD series showed a higher percentage of a positive family history (57.6%) and younger onset-age (55.3 years) than the total group, due to significant lower onset-age for cases with *MAPT* mutations (50.9 years).

After adjustment for gender, onset-age, and family history, FTLD-tau patients remained less at risk than PSP patients (HR 0.524; 95% CI 0.282 to 0.974), and a trend towards longer survival was found compared to FTLD-U patients (HR 0.608; 95% CI 0.361 to 1.024).

The FTLD-tau group consisted of 15 sporadic cases, all of which showed pure three-repeat tau pathology, and 17 cases with *MAPT* mutation, with pure three-repeat (G272V and ΔK280), pure four-repeat (P301L), or a mix of three-repeat and four-repeat (S320F, R406W and L315R) tau pathology depending on the location of the mutation. All PSP cases showed four-repeat tau pathology. The mean disease duration of sporadic Pick disease cases was 12.1 years and was similar to that in *MAPT* cases with three-repeat tau pathology (n = 6) of 10.2 years, whereas a trend ($p = 0.098$) could be observed towards shorter survival in *MAPT* cases with four-repeat tau pathology (n = 7) of 8.6 years. Disease duration in *MAPT* mutations with a mix of three-repeat and four-repeat tau pathology varied considerably.

The FTLD-U cohort consisted of nine type 1, 16 type 2 and four type 3 (all four with *GRN* mutation) cases. Survival of pathological *GRN* cases did not significantly differ from the total group of deceased *GRN* cases or FTLD-U type 2 cases (8.4 ± 3.1 years) but was significantly shorter than survival of FTLD-U type 1 cases (11.6 ± 5.0 years).

### Tau pathology quantification

Neuronal loss in PSP cases was most prominent in subthalamic nucleus, globus pallidus, dentate nucleus and substantia nigra. Tau pathology consisting of globoid neurofibrillary tangles, tufted astrocytes and glial coiled bodies varied considerably between cases, with the subthalamic nucleus, thalamus, substantia nigra, basal pontine nuclei, locus coeruleus and dentate nucleus regions most severely involved. The severity of tau

pathology, expressed in the PSP-tau-score, showed a significant negative correlation with disease duration (Fig. 2), but was not correlated with onset-age.

For the FTLD-tau group, neuronal loss in frontal, temporal and hippocampus regions was severe in most cases, whereas parietal, caudate nucleus and substantia nigra regions showed a variable neuron loss. Tau-positive inclusions showed a similar pattern of topographic distribution with severe tau pathology in frontal and temporal cortex and hippocampal regions, whereas the severity of tau pathology was more variable in parietal cortex, caudate nucleus and substantia nigra. Astrocytic tau pathology was severe in the L315R mutation, but only mild in other *MAPT* mutations and sporadic Pick disease. No significant correlation could be found between disease duration and either neuronal loss or tau-reactivity in any region.

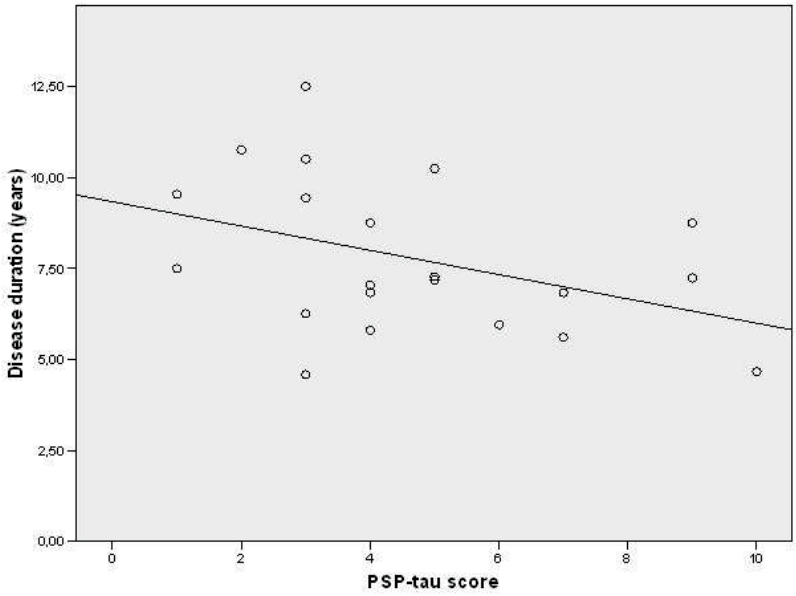


**Figure 2.** Disease duration of pathologically proven PSP cases according to PSP-tau score (Spearman's rho –0.44, $p = 0.045$)

## DISCUSSION

This study is the largest prospective population-based study comparing the survival between patients with PSP and FTD, and showed a significantly shorter disease duration in PSP. This difference was even more pronounced when comparing pathologically proven cases of PSP with FTLD-tau. This study replicates, for the first time, the prognostic value of the PSPRS with a sharp increase of probability of death above a score of 60. In

PSP patients, male gender and older onset-age were also independent predictors for shorter disease duration, whereas a positive family history and an older onset-age were associated with a poor prognosis for FTD.

Our observation of a shorter disease duration in PSP than in FTD contrasts with two other studies,[12,13] in which the small number of PSP patients may explain the lack of correlation. Our findings are probably close to true survival rates, as the patients were population-based ascertained. Looking into the natural history of PSP separately, the mean disease duration of deceased cases of 7.2 years in the present study comes very close to 6.8 years found in the only other large prospective study by Golbe *et al.*,[21] whereas a large retrospective study[20] showed shorter survival of 5.7 years. This was also true for RS cases in the clinicopathological study by O'Sullivan *et al.* (6.2 years),[35] whereas a much longer survival was found for PSP-P patients (11.6 years). Although our PSP-P group consists of only seven cases, due to the strict use of NINDS-SPSP criteria, the difference in survival compared with our RS cases was striking as well. The effect of higher onset-age on survival in the present study was also found in retrospective studies,[20,21,35] whereas our observed predictive value of gender contrasted to a weak or absent effect on survival in several other studies,[18,20,21] but not all.[35] The prognostic significance of older onset-age in PSP resembles observations made in Alzheimer's disease[36] and Parkinson's disease (PD), whereas there is conflicting evidence regarding effect on prognosis of male gender in PD.[37] A good explanation for lower survival in men with PSP in our study is lacking. The finding might perhaps be explained by differences for gender in co-morbidity at higher age. However, such data are not available in our study.

The predictive value of the PSPRS score for survival in PSP patients confirms the first observations made in a tertiary referred cohort of Golbe *et al.*[21] and also proves its predictive value in a population-based cohort. In line with Golbe's observations, a sharp rise in mortality risk was seen in patients with a PSPRS score above 60. Only the subsections supranuclear ocular motor exam, bulbar exam and gait exam were of prognostic value in our study. The replication of Golbe's findings on the PSPRS has implications for its potential use in clinical trials.

Shorter survival in FTD patients with a positive family history in this study contrasts with other studies on the natural history of FTD,[12,13,24,38,39] and may suggest a more malignant disease process for hereditary forms. This is especially true for patients with *GRN* mutations and hereditary FTLD with an unknown genetic defect, both groups exhibiting ubiquitin pathology,[40] whereas *MAPT* mutations showed a trend towards longer disease duration. However, as several mutation carriers were related, we cannot exclude other familial genetic factors influencing the disease duration within the families. The absence of an association between positive family history and survival in other studies may be explained by a low number of patients or an unknown family history.

The longer survival of the FTLD-tau group compared to pathologically proven PSP cases supports the hypothesis of a different disease process. The mean disease duration in the present series of 11.1 years is similar to that in the study by Hodges *et al.* (9.0 years).[24] The shorter survival of tau-positive cases (6 years) in the study by Xie *et al.*[16] can be explained by the inclusion of PSP and CBD cases. Our findings are very similar to the observations made by Hu *et al.,*[14] which showed that three-repeat FTLD-tau have a longer survival than four-repeat FTLD-tau and four-repeat controls, comprising PSP and CBD patients, and supports the idea that FTLD-tau patients tend to have a more indolent disease course than PSP.

The observed negative correlation between the severity of glial tau pathology and disease duration in PSP patients is in line with the study by Josephs *et al.*[22] The severity of oligodendroglial tau pathology in the substantia nigra and caudate and dentate nucleus represented the overall tau pathology reliably in the study by Williams *et al*, which again correlated negatively with disease duration,[23] and was shown to be higher in RS than in PSP-P. Due to the absence of PSP-P in our pathological cohort, we could not replicate the latter finding. The correlation between the type and severity of tau pathology indicates that pathophysiological mechanisms determine the disease progression. Small sample size, a semiquantitative method of scoring and different *MAPT* mutations with different functional effects may have hampered our analysis in FTLD. The association in FTLD between shorter survival and abundant tau pathology in basal ganglia in the study by Xie *et al.*[16] could not be confirmed by our study and should probably be explained by the inclusion of PSP and CBD cases in their analysis. The best strategy would be to extend the survival analysis to a much larger series of pathologically proven FTD cases, which have been prospectively ascertained during life in order to collect reliable clinical information.

One of the drawbacks of the present study is a selection bias towards typical cases, and therefore missing cases with PSP-P, a subgroup that usually has a longer disease duration and an atypical presentation. Furthermore, as the population of the study consists of cases alive at the time of entry, there may be some degree of survival bias. A final drawback is that there was pathological confirmation in only 24 of 133 of the deceased patients. However the NINDS-SPSP criteria show a good positive predictive value for probable PSP (100%) and possible PSP (83%) in patients presenting with parkinsonism,[34] but also in patients presenting with dementia (96% for combined possible and probable PSP).[41] Also, no large differences were found between our clinical and our pathological cohort.

In conclusion, this large prospective study showed that survival in PSP is shorter than in FTD. This difference in prognosis was even more pronounced when comparing pathological PSP cases with FTLD-tau. Within the PSP group, male gender, older onset-age and higher PSPRS score were independent predictors for shorter disease duration, whereas

a positive family history and an older onset-age were associated with a poor prognosis in FTD. The significant effect of diagnosis on survival may suggest that the underlying pathophysiology in PSP is more aggressive than in FTD. This perspective should help clinicians anticipate disease progression of patients with PSP and FTD.

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# 2.2

# Midcingulate involvement in progressive supranuclear palsy and tau-positive frontotemporal dementia

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## ABSTRACT

**Background:** Progressive supranuclear palsy (PSP) patients often exhibit cognitive decline and behavioural changes during the disease course. In a subset, these symptoms may be the presenting manifestation and can be similar to those in frontotemporal dementia (FTD). However, correlation studies between quantitative imaging measures and detailed neuropsychological assessment are scarce. The aim of this study was to investigate the functional role of affected brain regions in cognition in PSP compared with controls and subsequently examine these regions in FTD patients with known tau pathology (FTD-tau).

**Methods:** 21 PSP patients, 27 healthy controls, and 11 FTD-tau patients were enrolled**.** All participants underwent neuropsychological testing and technetium-99m-hexamethylpropylenamine-oxime single photon emission computed tomography. Regression slope analyses were performed in statistical parametric mapping to find significant associations between neuropsychological test results and brain perfusion.

**Results:** PSP patients showed hypoperfusion in the midcingulate cortex of which the posterior part correlated with Stroop III and Weigl. In FTD-tau patients, midcingulate cortex involvement was located more anterior and correlated with Stroop III and Wisconsin Card Sorting Test concepts. The degree of hypoperfusion in the anterior and midcingulate cortex in the disorders differed in the subgenual anterior cingulate cortex only.

**Conclusions:** The posterior part of the midcingulate cortex is prominently involved in the neurodegenerative process of PSP, and the severity of its hypoperfusion correlated with the extent of executive dysfunction. In FTD-tau, this cognitive domain was associated with anterior midcingulate cortex involvement. The degree of hypoperfusion in these regions did not differ between PSP and FTD-tau. These observations provide insight into the role of the cingulate cortex in cognitive dysfunction in these neurodegenerative disorders and warrant further investigations.

## INTRODUCTION

Progressive supranuclear palsy (PSP) is a neurodegenerative disorder characterised by early postural instability, supranuclear gaze palsy, parkinsonism, pseudobulbar palsy and cognitive decline.[1,2] PSP patients often exhibit mental slowness and a dysexecutive syndrome, as well as behavioural changes, commonly considered as frontal-subcortical dementia.[3] This cognitive dysfunction in PSP has a significant effect on the quality of life of patients for which effective therapeutic interventions are lacking.[4] In a subset of PSP patients, these cognitive and behavioural symptoms may be the predominant presenting manifestation, and can be similar to those observed in the behavioural variant of frontotemporal dementia (bvFTD).[5] Overlap between PSP and FTD is further emphasised by tau-positive inclusions in the brain, which characterise PSP, but are also found in a large subset of bvFTD patients, denoted as frontotemporal degeneration with tau pathology (FTD-tau).

Various brain imaging modalities, including technetium-99m-hexamethyl-propylen-amine-oxime ($^{99m}$Tc HMPAO) single photon emission CT (SPECT), $^{18}$F-fluorodeoxyglucose (FDG)-positron emission tomography (PET) and magnetic resonance imaging (MRI), have found involvement of the caudate nuclei, thalamus and midbrain in PSP.[6–11] In addition, frontal brain regions and specifically the cingulate cortex have shown involvement in PSP as well as in FTD.[12–16] However, the few published cognition-imaging correlation studies in PSP lacked quantitative imaging measures,[6] extensive neuropsychological assessment,[8–10,17] or detailed division of cortical areas.[7] We sought to overcome these limitations by determining perfusion measures on $^{99m}$Tc-HMPAO SPECT using statistical parametric mapping (SPM), and correlating these with neuropsychological test scores.

Our aim was to elucidate the functional role of affected brain regions in cognition in PSP compared with healthy controls in order to gain insight into the underlying mechanisms of cognition in this disorder. To assess the specificity of regions that are affected in PSP, we also specifically examined these areas in FTD-tau.

## METHODS

### Participants

PSP patients with subjective cognitive complaints were recruited between 2002 and 2010 by nationwide referral to the outpatient department of the Erasmus University Medical Center as part of a large longitudinal study.[5,18] FTD patients were recruited in a similar fashion.[19] All patients were examined by either the research physician (WZC) or a neurologist (AJWB and JCvS). A detailed clinical history was obtained from patients and their family members, and by reviewing the medical records. The neurologic ex-

amination was videotaped according to a standard protocol. Structural neuroimaging of patients was reviewed by the investigators to exclude other structural causes.

The clinical diagnosis of all patients was established in a consensus meeting according to the National Institute of Neurological Diseases and Stroke-Society for PSP (NINDS-SPSP) criteria,[2] and the Lund and Manchester criteria for FTD.[20] All PSP patients in the current study were typical Richardson's syndrome cases (10 probable and 11 possible cases). The PSP Rating Scale (PSPRS) quantified the measure of disability in PSP patients.[21] According to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV), one PSP patient was found to suffer from prolonged periods of a depressed mood and suicidal thoughts but without intent, and without vegetative symptoms other than apathy.

Healthy controls were recruited from the Rotterdam Elderly Study and had no neurological disorders or abnormalities at neurological and neuropsychological examination.[22]

In the current study, FTD patients consisted of a subset of patients described in a previous study of our group.[18] FTD-tau patients were selected for this study and comprise four pathologically proven tau-positive FTD patients without and seven FTD patients with *MAPT* mutations (two G272V and five P301L) that underwent $^{99m}$Tc-HMPAO SPECT scan and neuropsychological examination. As was the case in our previous study, FTD-tau patients were significantly younger than PSP patients.[18]

The PSP, FTD and Rotterdam Elderly studies were approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam and all participants or first-degree relatives signed informed consent. Neuropsychological testing and $^{99m}$Tc HMPAO SPECT of patients were a part of the diagnostic workup.

### Neuropsychological assessment

The neuropsychological test battery for all patients and controls, carried out by a single experienced clinical neuropsychologist (IdK), included the Mini-Mental State Examination (MMSE), the Dutch version of the Rey Auditory Verbal Learning Test (15-Word Test)[23] or Word List Memory Test (CERAD), Trailmaking Test A and B, the Stroop Color-Word Test, phonological fluency (DAT), and semantic fluency (animals and occupations). Higher scores on the Trail Making and Stroop Tests correspond to worse performance. Additional tests for patients consisted of the Boston Naming Test, Wisconsin Card Sorting Test (WCST) or Weigl Color-Form Sorting Test. Differences in test batteries between patients and controls are due to the clinical setting in which PSP and FTD patients were assessed as opposed to the Rotterdam Elderly Study. Education was categorised according to the system of Verhage,[24] which consists of seven increasing levels of education.

### $^{99m}$Tc-HMPAO SPECT scanning and image processing

$^{99m}$Tc HMPAO SPECT brain perfusion scans of all participants were exclusively carried out at the Department of Nuclear Medicine of the Erasmus Medical Center. After injection of 740 MBq of $^{99m}$Tc HMPAO, SPECT scans of PSP and bvFTD patients were acquired on a Prism 3000XP Philips (Picker) three-headed system, with a fan-beam collimator. Only controls that underwent SPECT imaging using the same three-head camera were selected from the Rotterdam Elderly Study.[22] On average, scans were started 20 minutes after injection of $^{99m}$Tc HMPAO while resting in a quiet room. Duration of scanning was 30 minutes. A total of 120 projections (3 x 40 steps of 3 degrees, and 20 seconds per step) were acquired. Image reconstruction was performed by a ramp filtered back projection and three dimensionally smoothed with a Metz Filter. No attenuation or scatter correction was performed.

After gross manual image reorientation and approximate definition of the image center point (anterior commissure), the SPECT scans were spatially processed using Statistical Parametric Mapping (SPM5; Wellcome Trust Center for Neuroimaging, London, UK) implemented in Matlab 7.9.0 (MathWorks, Natick, Massachusetts, USA). The effect of differences in spatial resolution was minimised by masking the images. SPECT images in native space were re-sliced into the same orientation, and spatially normalised onto the SPM5 MNI SPECT brain template with a 12 parameter affine transformation followed by non-linear transformations and a trilinear interpolation. Dimensions of the resulting voxel were 3x3x3 mm. In this normalisation step, estimation of individual SPECT normalisation parameters was constrained by a source weighting image. This method is used to correct for registering lesioned brains. Hereafter images were smoothed using a Gaussian filter of 16 mm full width at half maximum, limited to measured brain tissue. For SPECT data, smoothing with at least twice the full width at half maximum of the imaging system was shown to provide good detection sensitivity,[25] and is used in pathological brain studies with SPECT and PET.[26] Voxel based image analyses were conducted within the framework of the General Linear Model as provided by SPM.[27] Proportional scaling to the mean global image intensity was used to remove confounding effects due to variations in individual $^{99m}$TC-HMPAO uptake.[28] We performed two group analyses. In the first analysis we compared PSP, FTD-tau and controls in a full factorial design with gender as the nuisance variable. We chose not to adjust for age in this analysis as we expected patients to exhibit decreased perfusion, which likely reflects underlying disease and not effects of age, seeing that patients were younger than controls. The results of the full factorial design were thresholded at $p < 0.05$, Family Wise Error (FWE) corrected. The contrasts PSP versus controls and FTD-tau versus controls were used to define an overlapping region of interest (ROI), that is, regions involved in both conditions. Group analysis in PSP showed significant hypoperfusion of the anterior cingulate cortex (ACC) and midcingulate cortex (MCC) relative to controls. To assess the association between these

regions and cognitive dysfunction in PSP as well as FTD-tau, we performed a second group analysis. The design matrix of this ANCOVA model contained SPECT scans of both patient groups as the dependent variable and regressors for group and neuropsychological data per patient group. We correlated neuropsychological data with perfusion in PSP and FTD-tau separately within an ROI comprising the entire ACC and MCC. We then performed F test interaction analyses of the neuropsychological data and group within an ROI of overlapping hypoperfusion in PSP and FTD-tau relative to controls consisting of part of the MCC (Fig. 1B), to make inferences on differences in regression slope between the patient groups in a region that was affected in both conditions. The results of the slope and interaction analyses were thresholded at $p < 0.005$, uncorrected. Peak values of the significant regression slopes and interaction effects were used to extract individual proportional perfusion within a 5 mm radius of the peak voxel, using Matlab toolbox MarsBaR.[29] These perfusion measures were subsequently exported to SPSS for correlation analyses with neuropsychological data. In a post hoc analysis, we examined hypoperfusion of the cingulate cortex in PSP and FTD-tau more carefully by performing the contrast PSP versus FTD-tau and vice versa within the ROI of the entire ACC and MCC. MNI anatomical labelling of significant clusters was performed using WFU Pickatlas software extension to SPM5 (Functional MRI laboratory – Wake Forest University School of Medicine, Winston Salem, North Carolina, USA). The various subregions of the cingulate cortex are designated according to the four region model as proposed by Vogt *et al.*[30] MNI coordinates were assigned to specific parts of the MCC following subregion division of the cingulate cortex by Yu *et al.*[31]

### Statistical analysis

SPSS 15.0 for Windows (SPSS, Chicago, Illinois, USA) was used for statistical analysis. Age at examination, gender and education levels were analysed by independent sample t-test or Chi-square test, whereas ANOVA was used for analysis of neuropsychological data. For reasons explained above, we did not adjust for age in between group comparisons. Statistical testing took place at a 0.05 level of significance. Perfusion measures were entered as regressors in a stepwise regression analysis, with neuropsychological data as dependent variables ($p < 0.05$), adjusted for demographic data.

## RESULTS

The characteristics of the patients and healthy controls are summarised in Table 1. The mean PSPRS score of < 30 in 19 PSP patients reflects an early and mild disease stage.[21] Two patients were recruited before the introduction of the PSPRS.

**Table 1.** Clinical features of patients and controls.

| | PSP | FTD-tau | Controls | p |
|---|---|---|---|---|
| **n** | 21 | 11 | 27 | |
| **Age at examination** | 69.1 ± 5.5 | 54.8 ± 7.6 | 76.3 ± 5.8 | < 0.001[a] |
| **Male gender, n (%)** | 12 (57.1) | 4 (36.4) | 13 (48.1) | 0.531 |
| **Education** | 4.5 ± 1.7 | 4.0 ± 1.5 | 3.8 ± 1.5 | 0.297 |
| **Disease duration at examination** | 3.4 ± 1.3 | 3.0 ± 1.4 | n/a | 0.341 |

Mean ± standard deviation. n/a, not applicable; a, between all groups

## Cognitive function and cerebral perfusion in PSP

PSP patients performed significantly worse than controls on all examined cognitive domains of memory, attention and concentration, executive function, and language (Table 2).

**Table 2.** Cognitive test scores of patients and controls.

| | n | PSP | n | FTD-tau | n | Controls | p |
|---|---|---|---|---|---|---|---|
| **MMSE** | 20 | 25.7 ± 3.0 | 7 | 23.6 ± 4.0 | 27 | 27.7 ± 2.1 | 0.001[a] |
| **Memory** | | | | | | | |
| Learning (%) | 21 | 37.1 ± 12.0 | 10 | 32.6 ± 20.0 | 26 | 63.0 ± 13.9 | < 0.001[a] |
| Delayed Recall (%) | 21 | 35.2 ± 13.7 | 10 | 24.3 ± 27.7 | 26 | 63.1 ± 18.5 | < 0.001[a] |
| **Attention and concentration** | | | | | | | |
| Trail Making A | 21 | 93.9 ± 42.2 | 11 | 94.6 ± 66.3 | 25 | 58.1 ± 25.1 | 0.009[a] |
| Stroop I | 16 | 100.4 ± 29.0 | 9 | 80.6 ± 29.0 | 23 | 47.7 ± 6.7 | < 0.001[a] |
| Stroop II | 16 | 118.4 ± 32.4 | 9 | 105.9 ± 34.6 | 23 | 66.1 ± 14.8 | < 0.001[a] |
| **Executive function** | | | | | | | |
| Trail making B | 21 | 274.3 ± 111.1 | 11 | 221.2 ± 69.2 | 24 | 122.8 ± 54.6 | < 0.001[a] |
| Stroop III | 16 | 211.2 ± 111.1 | 9 | 202.2 ± 93.8 | 23 | 128.6 ± 47.0 | 0.001[a] |
| WCST, concepts | 17 | 3.1 ± 1.8 | 9 | 1.8 ± 2.5 | n/a | n/a | 0.146 |
| Weigl | 12 | 8.1 ± 2.8 | 6 | 4.3 ± 5.5 | n/a | n/a | 0.167 |
| **Language** | | | | | | | |
| Semantic fluency | 21 | 17.4 ± 5.3 | 11 | 11.7 ± 10.9 | 24 | 34.5 ± 9.1 | < 0.001[a] |
| Phonological fluency | 21 | 14.3 ± 11.9 | 9 | 10.6 ± 17.6 | 26 | 37.7 ± 13.8 | < 0.001[a] |
| Boston Naming Test | 18 | 47.8 ± 5.1 | 5 | 32.2 ± 11.2 | n/a | n/a | 0.034[b] |

Mean (range). MMSE Mini-Mental State Examination; n/a, not applicable; [a] between patients and controls; [b] between PSP and FTD-tau

SPM analysis with a threshold of $p < 0.05$ with FWE correction yielded relative hypoperfusion in PSP patients compared with controls in predominantly the ACC and MCC (Fig. 1A). A more lenient analysis ($p < 0.001$ uncorrected) also resulted in relative

hypoperfusion in frontal regions, the cerebellum, basal ganglia and midbrain, as shown in Fig. 2A.

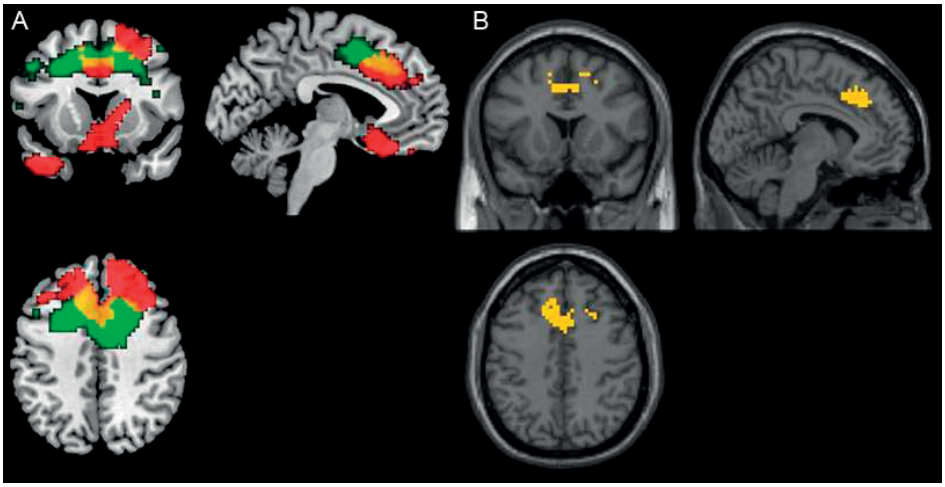


**Figure 1.** Technetium-99m-hexamethyl-propylenamine-oxime single-photon emission computed tomography ($^{99m}$Tc HMPAO SPECT) findings showing top, right lateral and midsagittal views. Images are displayed in neurological convention. (A) Statistical parametric mapping (SPM) of the comparison between PSP patients and controls revealed hypoperfusion in predominantly the posterior part of the midcingulate cortex in PSP ($p < 0.05$ with family wise error (FWE) correction). SPM of the comparison between FTD-tau patients and controls revealed hypoperfusion in the anterior part of the midcingulate cortex, temporal cortex and subgenual part of the anterior cingulate cortex in FTD-tau ($p < 0.05$ with FWE correction). (B) Region of interest of overlapping regions of hypoperfusion in PSP and FTD-tau patients.

Based on the results from the FWE corrected analysis of PSP patients versus controls, we restricted analyses of the ANCOVA model to the ACC and MCC. Regression slope analyses yielded significant results for the executive tests Stroop III (MNI X, Y, Z=-15, 6, 39) and Weigl (MNI X, Y, Z = 6, 3, 45) only ($p < 0.005$), indicating significant associations between these test results and perfusion in the posterior part of the MCC. The magnitudes of these associations were assessed in SPSS by correlating extracted perfusion measures with Stroop III (adjusted $r^2$ 0.245, β -0.543, p 0.03) and Weigl (adjusted $r^2$ 0.418, β 0.686, p 0.014).

### Cognitive function and cerebral perfusion in FTD-tau

FTD-tau patients performed worse than controls but equal to PSP patients on MMSE and all subtests of the examined cognitive domains. Only scores on the Boston Naming Test were significantly worse in FTD-tau patients (Table 2).

The FWE corrected analysis revealed relative hypoperfusion in the frontal, cingulate and temporal regions in FTD-tau patients compared with controls (Fig. 1A). The results of the uncorrected analysis are shown in Fig. 2B.

Regression slope analyses in the ACC and MCC ROI yielded significant results in the anterior part of the MCC for the executive tests Stroop III (MNI X, Y, Z = 6, 30, 27) and WCST (MNI X, Y, Z = 3, 33, 27) only ($p < 0.005$). The extracted perfusion correlated with Stroop III (adjusted $r^2$ 0.638, β -0.826, p 0.006) and WCST (adjusted $r^2$ 0.662, β 0.839, p 0.005).

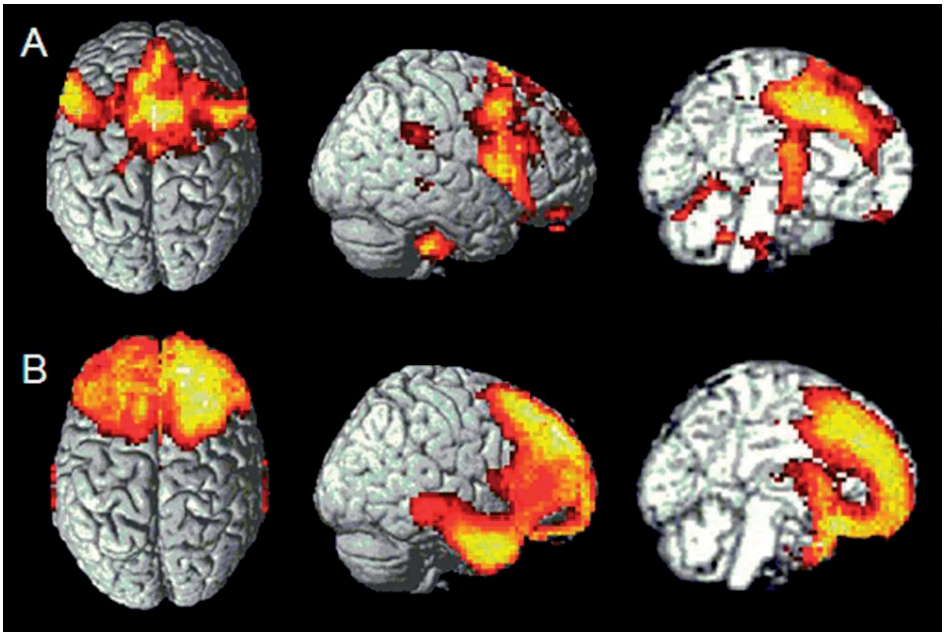


**Figure 2.** Technetium-99m-hexamethyl-propylenamine-oxime single-photon emission computed tomography ($^{99m}$Tc HMPAO SPECT) findings showing top, right lateral and midsagittal views. Images are displayed in neurological convention. (A) Statistical parametric mapping (SPM) of the comparison between PSP patients and controls showing significant hypoperfusion in the midcingulate, frontal gyri (orbitofrontal, superior, inferior, middle and medial frontal gyri), cerebellum, caudate nucleus, thalamus, putamen, globus pallidus and midbrain ($p < 0.001$ uncorrected). (B) SPM of the comparison between FTD-tau patients and controls showing significant hypoperfusion in the superior, medial, middle, inferior frontal and mid- and anterior cingulate gyri and medial, middle, superior temporal and inferior temporal lobe and caudate nucleus ($p < 0.001$ uncorrected).

### PSP versus FTD-tau

To assess whether there is a difference in slope in regions that are involved in both PSP and FTD-tau between the disorders, we performed a group by neuropsychological test interaction analysis in an overlap ROI (Fig. 1). This showed that the disorders differed significantly on only one of the executive tests, namely Weigl, in the posterior part of the

MCC (MNI X, Y, Z = 9, 12, 42); PSP patients had a significantly steeper slope than FTD-tau patients ($p < 0.005$). Comparison of anterior and midcingulate hypoperfusion between PSP and FTD-tau patients revealed differences in the subgenual part of the ACC (sACC) only, at an FWE corrected threshold, with higher perfusion values in PSP patients.

## DISCUSSION

The present study revealed prominent hypoperfusion in the posterior part of the MCC in PSP patients compared with controls using quantitative SPM analysis. This hypoperfusion correlated with the extent of executive dysfunction, as was the case for hypoperfusion in the anterior part of the MCC in FTD-tau patients. The degree of anterior- and midcingulate hypoperfusion in the disorders differed in the sACC only.

The robust finding of cingulate involvement in PSP patients of the present study has also been described by other investigators.[9,14–16] Salmon *et al.* detected impairment of glucose metabolism in the MCC in 10 PSP patients compared with healthy controls, and patients with Alzheimer's disease.[16] Involvement of the MCC was also found in PSP patients compared with Parkinson's disease patients,[14] whereas similar findings were reported in FTD compared with Alzheimer's disease patients and controls.[32] However, the functional role of the cingulate cortex in PSP and FTD remained speculative as correlation with neuropsychological evaluation was not performed in these studies.

We found that hypoperfusion in the posterior part of the MCC correlates with impairment of specific frontal functions in PSP. Several studies have linked frontal involvement to executive dysfunction and behavioural changes,[7–10] but these studies did not distinguish between different regions of the frontal lobe,[7] or correlate frontal regions to a more global measure such as the Frontal Behavioural Inventory instead of specific frontal tests.[8–10] Furthermore, compared with these studies, our PSP patients were evaluated early in the disease course. Only Blin *et al.* had a comparable mean disease duration at examination of 3.1 years.[8]

In addition to the correlation of executive function with the posterior part of the MCC in PSP patients, we found this cognitive domain to be associated with the anterior part of the MCC in FTD-tau patients. This observation is in line with a recent structure based meta-analysis by Torta *et al.,* who found multiple portions of the cingulate cortex activated for executive tasks, adding to the notion of multifunctionality of parts of the cingulate cortex.[33]

The difference in anterior and midcingulate hypoperfusion patterns between PSP and FTD patients when compared with controls was also found by other investigators; the posterior part of the MCC was predominantly affected in PSP compared with controls, whereas the most affected part of the cingulate in FTD compared with controls was

located more anteriorly. However, direct comparison of cingulate involvement between PSP and FTD was not assessed in their study.[15] Our observation that the extent of hypoperfusion in the cingulate cortex in PSP did not differ from that in FTD-tau, except for the sACC, emphasises a remarkable degree of involvement of the anterior and midcingulate regions in PSP. This is in accordance with other studies that have found reduced cingulate cortex metabolism to differentiate PSP from Parkinson's disease.[34] The regression slope in the overlapping region between PSP and FTD-tau was similar for both disorders, except for the Weigl. Based on this observation, a differential effect on the Weigl may exist, but further studies with larger sample sizes are needed to confirm this.

An interesting issue is whether the correlation in PSP might reflect the impairment of cortical-subcortical connections. The frontal hypometabolism in PSP is thought to be either secondarily caused by damage to subcortical projections, which disrupt cortical-subcortical circuits, or the primary accumulation of cortical tau pathology. The MCC in a post-mortem cohort of 24 PSP patients showed neurodegenerative involvement reflected by variable tau pathology, despite the visually assessed absence of neuronal loss.[18] These observations are similar to findings by Schofield *et al*.,[35] adding to the growing evidence for cortical tau pathology being at least in part directly responsible for the impaired functionality. The cingulate cortex also plays a role in social cognition,[36] which may be explained by the exclusive presence of von Economo neurons (VENs) in the cingulate cortex and frontal insula. Selective loss of VENs has been found in bvFTD,[37] and the question is whether the impaired recognition of negative emotions in PSP[38] is related to changes in the number of VENs. Future studies are needed to investigate the involvement of VENs in the cingulate cortex in PSP.

A limitation of the current study is the lack of pathological confirmation in our PSP patients. The NINDS-SPSP criteria however have shown a good positive predictive value of diagnosing typical cases of PSP.[39] Also, our sample of FTD-tau patients with full neuropsychological data and a perfusion scan is relatively small. Another limitation is that controls were slightly older than PSP patients. However, this means that the observed findings in PSP patients are likely an underestimate rather than an overestimate of the difference in hypoperfusion between PSP and controls, as ageing is accompanied by regionally selective reduction of cortical perfusion.[40] Another issue is the larger difference in age between PSP and FTD patients. This is inherent to the underlying disorders, as FTD is characterised by a presenile onset.

In conclusion, we have gained insight into the involvement and role of the MCC in cognition in PSP. The extent of hypoperfusion in this region in PSP did not differ from that in FTD-tau, and was correlated to executive function in both disorders. Our observations support the idea that further investigations into the role of this intriguing region in PSP, in particular, but also in FTD, are warranted, as the cingulate cortex has not been

a focus in PSP research. Understanding this region may prove key to finding a treatment for cognitive symptoms in these neurodegenerative disorders.

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# 3

# NEUROCHEMICAL ASPECTS OF PROGRESSIVE SUPRANUCLEAR PALSY

# 3.1

## Multireceptor fingerprints in progressive supranuclear palsy

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## ABSTRACT

**Background:** Progressive supranuclear palsy (PSP) with a frontal presentation, characterized by cognitive deficits and behavioural changes, has been recognized as an early clinical picture, distinct from the classical so-called Richardson and parkinsonism presentations. The midcingulate cortex is associated with executive and attention tasks and has been consistently found to be impaired in imaging studies of PSP patients. The aim of the present study was to determine alterations in neurotransmission underlying the pathophysiology of PSP, and their significance for clinically identifiable PSP subgroups.

**Methods:** *In vitro* receptor autoradiography was used to quantify densities of 20 different receptors in the caudate nucleus and midcingulate area 24′ of PSP patients (n = 16) and age- and gender-matched control subjects (n = 14).

**Results:** Densities of γ-aminobutyric acid type B, peripheral benzodiazepine, serotonin receptor type 2 and *N*-methyl-D-aspartate receptors were significantly higher in area 24′ of PSP cases, where tau impairment was stronger than in the caudate nucleus. Kainate and nicotinic cholinergic receptor densities were significantly lower, and adenosine receptor type 1 ($A_1$) receptors significantly higher, in the caudate nucleus of PSP cases. Receptor fingerprints also segregated PSP subgroups when clinical parameters such as occurrence of frontal presentation and tau pathology severity were taken into consideration.

**Conclusions:** We demonstrate for the first time to our knowledge that kainate and $A_1$ receptors are altered in PSP, and that clinically identifiable PSP subgroups differ at the neurochemical level. Numerous receptors were altered in the midcingulate cortex, further suggesting that it may prove to be a key region in PSP. Finally, we add to the evidence that non-dopaminergic systems play a role in the pathophysiology of PSP, thus highlighting potential novel treatment strategies.

## BACKGROUND

Progressive supranuclear palsy (PSP) is a neurodegenerative disorder clinically characterized by early postural instability, supranuclear gaze palsy, parkinsonism and cognitive decline.[1] Frontal presentation characterized by cognitive deficits and behavioural changes has recently been recognized as an early clinical picture,[2,3] distinct from the classical so-called Richardson and parkinsonism presentations.[4] Accordingly, the focus of research in PSP has expanded over the years from neuropathological studies of subcortical structures to investigations of the disease as a more diffuse condition with varying cortical involvement.[5]

The midcingulate cortex, comprising areas 24′ and 32′,[6] is associated with executive and attention tasks[7] and has consistently been found to be impaired in PSP patients.[3] A recent perfusion single-photon emission computed tomography (SPECT) study confirmed and extended these findings; the degree of midcingulate cortical hypoperfusion correlated with the extent of executive dysfunction in patients,[8] the cardinal feature of cognitive dysfunction in PSP.[9] Therefore, understanding the neurochemical changes in this region may prove crucial to finding a treatment for cognitive symptoms.

Previous neurotransmitter studies in PSP have been focused mainly on nigrostriatal dopaminergic and cholinergic systems. Authors of a comprehensive review of *in vivo* imaging studies addressing PSP-associated alterations of synaptic transmission revealed that most existing studies showed decreased dopamine transporter and dopamine receptor type 2 (D2) binding densities in the striatum, whereas dopamine receptor type 1 (D1) densities were demonstrated to be unaltered.[10] In studies focused on the cholinergic system, researchers reported significant reductions of muscarinic and nicotinic receptors in the striatum.[11,12] Despite these findings, dopamine and cholinergic replacement therapies in PSP have not proven to be effective.[13] Therefore, other neurotransmitter systems may be involved.

An intriguing question is whether the midcingulate hypometabolism found in PSP is accompanied by alterations in the densities of specific neurotransmitter receptors. Therefore, we applied quantitative *in vitro* receptor autoradiography on unfixed brain tissue from PSP patients and control subjects to quantify the densities of 20 different receptor binding sites and determine PSP-related alterations in the "receptor fingerprints"[14] of midcingulate area 24′. Furthermore, because the midcingulate cortex and caudate nucleus differ considerably in their neurochemical composition in the healthy brain,[6,14] and because different brain regions are not necessarily affected in the same way by disease,[15] we also examined caudate nucleus tissue obtained from the same PSP patients.

## METHODS

### Subjects

Brains were obtained from PSP patients (aged 72 ± 7 years old, 9 male, 7 female) recruited in a nationwide study on PSP between 2000 and 2009. Brain autopsy was conducted by the Netherlands Brain Bank according to its Legal and Ethical Code of Conduct. Control subjects consisted of age- and gender-matched subjects (aged 76 ± 10 years old, 9 male, 5 female) without a history of neurological or psychiatric diseases.

Patients were examined after referral to the outpatient department of the Erasmus University Medical Center and by visiting patients in nursing homes as part of a large longitudinal study[2,16] approved by the Medical Ethics Committee of the Erasmus University Medical Center. All participants or their first-degree relatives signed informed consent forms. All patients were examined by a research physician (WZC or LDK) or a neurologist (AJWB or JCvS). A detailed clinical history was obtained from patients and their family members and by reviewing medical records. The neurological examination was videotaped according to a standardized protocol. Structural neuroimaging of patients was reviewed to exclude other disease causes. Family history was considered positive when at least one first-degree relative had dementia or parkinsonism. The possibility of post-mortem examination was discussed with patients and their relatives. Relevant medication used in the last three months of life was recorded. Clinical diagnosis of patients was established in a consensus meeting according to the National Institute of Neurological Diseases and Stroke-Society for PSP criteria.[1] Neuropathological diagnosis of PSP was established according to international criteria.[17]

### Standard neuropathology

At The Netherlands Brain Bank, the right hemispheres of all brains are processed for routine staining and immunohistochemistry against several antibodies: AT8 (Innogenetics, Ghent, Belgium; 1:40), ubiquitin (Dako, Glostrup, Denmark; 1:500), three-repeat tau isoform (Upstate, Charlottesville, Virginia, USA; 1:3000), four-repeat tau isoform (Upstate, Charlottesville, Virginia, USA; 1:100), p62 (BD Biosciences Pharmingen, San Diego, California, USA; 1:200, following 80°C antigen retrieval), TDP-43 (Proteintech, Chicago, Illinois, USA; 1:100, following pressure-cooking), β-amyloid (anti-β-amyloid, DAKO, Glostrup, Denmark, 1:100, following formic acid pre-treatment), and α-synuclein (anti-α-synuclein, Zymed Laboratories, San Francisco, California, USA; undiluted, following formic acid pre-treatment). Slides were incubated overnight at 4°C. Endogenous peroxidase activity was inhibited by 30-minute incubation in a phosphate buffered saline–hydrogen peroxide–sodium azide solution (100 ml of 0.1M phosphate buffered

saline, 2 ml of 30% $H_2O_2$, 1ml of $NaN_3$). The Histostain-Plus broad-spectrum immunohistochemistry kit DAB (Zymed, San Francisco, Califonia, USA) was used as a detection system. Slides were counterstained with Mayer's haematoxylin and mounted in Entellan medium (EMD Millipore, Billerica, Massachusetts, USA).

A separate semiquantitative assessment of tau pathology for area 24′ and caudate nucleus was carried out by two raters (WZC and JCvS) using a two-point grading scale, which is an adaptation of the visual guide proposed by Williams *et al.*[18] We defined Williams's grades 1 and 2 as "mild" and grades 3 and 4 as "moderate to severe".



### *In vitro* receptor autoradiography

Probes from midcingulate area 24′ and the caudate putamen were taken from the left hemisphere, frozen in isopentane at -40°C with a postmortem delay of 6 ± 1 (PSP cases) and 8 ± 1 hours (control subjects), and serially sectioned at -20°C in 10 µm-thick sections with a cryostat. Alternating sections were processed for the visualization of 20 transmitter receptors according to standard protocols (Table 1) comprising a preincubation to remove endogenous ligands and external substances such as medication, a main incubation to label binding sites with a tritiated ligand in the presence (nonspecific binding) or absence (total binding) of a nonlabeled displacer, and a rinsing step to eliminate unbound radioactivity.[14] Nonspecific binding was less than 5% of total binding for all examined binding sites and thus was ignored in the present study. All sections intended for the visualization of a given receptor type were incubated in the same radioactive solution.

Radioactively labeled sections were coexposed against tritium-sensitive films (Amersham Hyperfilm®; GE Healthcare Life Sciences, Braunschweig, Germany) with plastic [$^3$H]-standards of known concentrations of radioactivity (Amersham Microscales®; GE Healthcare Life Sciences). Upon purchase, Microscales® were calibrated with the aid of brain homogenate standards for which total protein content had been determined by means of the Bradford assay. Resulting autoradiographs were processed by densitometry with a video-based image-analyzing technique.[19] The Microscales® were used to compute a calibration curve, which, together with the parameters specific for each binding experiment (i.e., specific activity, dissociation constant, and concentration of the ligand), enabled transformation of grayscale values in the autoradiographs of samples into a binding site density per unit of protein (femtomoles per milligram of protein). Mean densities were thus obtained for a series of three or four sections per receptor type in the area 24′ and caudate nucleus probe of each case.

**Table 1.** Ligands and binding protocols used for receptor autoradiography.

| Transmitter | Receptor | [$^3$H]-Ligand | Displacer | Incubation buffer | Preincubation | Main incubation | Final rinse** |
|---|---|---|---|---|---|---|---|
| **Glutamate** | AMPA | AMPA [10 nM] | quisqualate [10µM] | 50mM Tris-acetate (pH 7.2) + 100mM KSCN* | 3 x 10 min, 4°C | 45 min, 4°C | 1. 4 x 4 sec 2. acetone/ glutaraldehyde (100ml/2.5ml), 2 x 2 sec, 4°C |
| | kainate | kainate [9.4nM] | SYM 2081 [100µM] | 50mM Tris-acetate (pH 7.1) + 10mM $Ca^{2+}$-acetate | 3 x 10 min, 4°C | 45 min, 4°C | 1. 4 x 4 sec, 4°C 2. acetone/ glutaraldehyde (100ml/2.5ml), 2 x 2 sec, 4°C |
| | NMDA | MK-801 [3.3nM] | (+)MK-801 [100µM] | 50mM Tris-acetate (pH 7.2) + 50µM glutamate + 30µM glycine* + 50µM spermidine* | 15 min, 4°C | 60 min, 22°C | 2 x 5 min |
| | mGluR2/3 | LY 341,495 [1nM] | L-glutamate [1mM] | 10mM phosphate buffer (pH 7.6) + 100mM KBr* | 2 x 5 min, 22°C | 60 min, 4°C | 2 x 5 min |
| **GABA** | $GABA_A$ | muscimol [7.7nM] | GABA [10µM] | 50mM Tris-citrate (pH 7.0) | 3 x 5 min, 4°C | 40 min, 4°C | 3 x 3 sec |
| | $GABA_B$ | CGP 54626 [2nM] | CGP 55845 [100µM] | 50mM Tris-HCl (pH 7.2) + 2,5mM $CaCl_2$ | 3 x 5 min, 4°C | 60 min, 4°C | 3 x 2 sec |
| | BZ | flumazenil [1nM] | clonazepam [2µM] | 170mM Tris-HCl (pH 7.4) | 15 min, 4°C | 60 min, 22°C | 2 x 1 sec |
| | pBZ | PK 11195 [0.1nM] | PK 11195 [10µM] | 50mM Tris HCl (pH 7.4) | 15 min, 22°C | 60 min, 22°C | 3 x 5 min |

**Table 1.** Ligands and binding protocols used for receptor autoradiography. (*continued*)

| Transmitter | Receptor | [$^3$H]-Ligand | Displacer | Incubation buffer | Preincubation | Main incubation | Final rinse** |
|---|---|---|---|---|---|---|---|
| **Acetylcholine** | $M_1$ | pirenzepine [1nM] | pirenzepine [2µM] | Krebs buffer (pH 7.4) + 4mM KCl + 120mM NaCl | 15 min, 22°C | 60 min, 22°C | 3 x 4 min |
| | $M_2$ | oxotremorine-M [1.7nM] | carbachol [10µM] | 20mM HEPES-Tris (pH 7.5) + 10mM $MgCl_2$ + 300nM Pirenzepine | 20 min, 22° C | 60 min, 22° C | 2 x 2 min |
| | $M_3$ | 4-DAMP [1nM] | atropine sulfate [10µM] | 50mM Tris-HCl (pH 7.4) + 0.1mM PSMF + 1mM EDTA | 15 min, 22° C | 45 min, 22° C | 2 x 5 min |
| | nACh | epibatidine [0.5nM] | nicotine [100µM] | 1mM HEPES (pH 7.5) + 120mM NaCl + 5.4mM KCl + 0.8mM $MgCl_2$ + 1.8mM $CaCl_2$ | 20 min, 22° C | 90 min, 22° C | 5 min |
| **Noradrenaline** | $\alpha_1$ | prazosin [0.2nM] | phentolamine mesylate [10µM] | 50mM Na/K-phosphate buffer (pH 7.4) | 15 min, 22° C | 60 min, 22° C | 2 x 5 min |
| | $\alpha_2$ | UK 14,304 [0.64nM] | phentolamine mesylate [10µM] | 50mM Tris-HCl (pH 7.7) + 100µM $MnCl_2$ | 15 min, 22° C | 90 min, 22° C | 5 min |
| **Serotonin** | 5-$HT_{1A}$ | 8-OH-DPAT [1nM] | 5-hydroxy-tryptamine [1µM] | 170mM Tris-HCl (pH 7.4) + 4mM $CaCl_2$* + 0.01% ascorbate* | 30 min, 22°C | 60 min, 22°C | 5 min |
| | 5-$HT_2$ | ketanserin [1.14nM] | mianserin [10µM] | 170mM Tris-HCl (pH 7.7) | 30 min, 22° C | 120 min, 22° C | 2 x 10 min |

**Table 1.** Ligands and binding protocols used for receptor autoradiography. (*continued*)

| Transmitter | Receptor | [$^3$H]-Ligand | Displacer | Incubation buffer | Preincubation | Main incubation | Final rinse** |
|---|---|---|---|---|---|---|---|
| **Dopamine** | D1 | SCH 23390 [1.67nM] | SKF 83566 [1µM] | 50mM Tris-HCl (pH 7.4) + 120mM NaCl + 5mM KCl + 2mM $CaCl_2$ + 1mM $MgCl_2$ | 20 min, 22° C | 90 min, 22° C | 2 x 10 min |
| | D2 | raclopride [0.55nM] | butaclamol [1µM] | 50mM Tris-HCl (pH 7.4) + 0,1% ascorbate + 150mM NaCl | 20 min, 22° C | 45 min, 22° C | 6 x 1 min |
| **Adenosine** | $A_1$ | DPCPX [1nM] | R-PIA [100µM] | 170mM Tris-HCl (pH 7.4) + 2 Units/l adenosine deaminase + 100µM Gpp(NH)p* | 15 min, 4° C | 120 min, 22° C | 2 x 5 min |
| | $A_{2A}$ | ZM 241385 [0.42nM] | 2-chloro-adenosine [20µM] | 170 mM Tris-HCl (pH: 7,4) + 2 Units/l adenosine deaminase + 10mM $MgCl_2$ | 2 x 10 min, 22° C | 120 min, 22° C | 2 x 5 min |

BZ $GABA_A$ associated benzodiazepine binding sites; pBZ peripheral benzodiazepine receptor; nACh nicotinic cholinergic receptor of the $\alpha_4/\beta_2$ subtype; pBZ peripheral benzodiazepine receptor; * Substance only included in the main incubation buffer solution; ** Final rinsing carried out in incubation buffer at 4°C and followed by 1–1 dips in distilled water at room temperature, unless otherwise specified.

### Statistical analysis

IBM SPSS Statistics version 21.0 for Windows software (IBM, Armonk, NY, USA) was used for analysis. Demographic features were analyzed by independent sample t-test or Chi-square test. Discriminant analyses were performed separately for data from area 24' and the caudate nucleus to visualize the multivariate distance between control subjects and PSP cases and between the two groups into which the patients could be subdivided on the basis of tau pathology severity or frontal versus non-frontal presentation. Only in the case of a significant result did we perform post hoc tests (univariate F-tests) to reveal which receptor types differed between control subjects and PSP cases or PSP subgroups. These p values were not corrected for multiple comparisons. Significance levels were set at $p < 0.05$ for the omnibus tests and $p < 0.01$ for the post hoc tests. The discriminant analysis was chosen as a global test because it offers several advantages over the procedures classically used to test group differences, [20] the most important of which are that it is nonparametric and that it supports the analysis of multivariate datasets with more dependent variables (receptor densities, comprising 20 in this study) than cases (individuals in this study, comprising 14 control subjects and 16 PSP cases). This is accomplished by reducing the receptor densities to a smaller number of discriminant scores (two in this study) for statistical testing and graphing.

## RESULTS

No cases of PSP-parkinsonism were identified in the present cohort, and relevant clinical data are summarized in Tables 2 and 3.

**Table 2.** Demographic features of progressive supranuclear palsy patients and healthy controls.

| | Patients | Controls | p |
|---|---|---|---|
| **n** | 16 | 14 | |
| **Age at death** | 72.5 (6.92)* | 75.7 (10.07) | 0.34 |
| **Male gender, n (%)** | 9 (56) | 9 (64) | 0.8 |
| **Disease duration (years)** | 8.2 (2.26)* | - | - |
| **Postmortem delay (hours)** | 6.29 (1.21) | 7.53 (1.31) | 0.01 |

Expressed as mean (standard deviation)

* Three cases that underwent euthanasia were not included

### Progressive supranuclear palsy cohort versus control subjects

On the basis of discriminant analyses of receptor densities, classification of PSP patients and control subjects was significant in the caudate nucleus (Wilks' lambda 0.103, Chi-square 34.127, p 0.025) and in area 24' (Wilks' lambda 0.071, Chi-square 47.592, $p < 0.001$)

**Table 3.** Detailed clinical features of progressive supranuclear palsy patients.

| Case | Gender | Age at onset (years) | Age at death (years) | NINDS-SPSP criteria during life | Frontal presentation | Family history | Relevant medication in the last three months of life | Brain weight (grams) | Tau pathology area 24' | Tau pathology caudate |
|---|---|---|---|---|---|---|---|---|---|---|
| **1*** | M | 71 | 73 | probable | non-frontal | negative | clonazepam, temazepam, piracetam | 1398 | Grade 3 | Grade 2 |
| **2** | M | 66 | 76 | probable | frontal | negative | oxybutinin, acetylcysteine, thiopental, pancuronium | 1060 | Grade 3 | Grade 2 |
| **3** | M | 63 | 68 | probable | non-frontal | positive | temazepam, oxybutynin | 1405 | Grade 3 | Grade 4 |
| **4** | F | 70 | 79 | possible | frontal | positive | tolterodine | 1069 | Grade 2 | Grade 2 |
| **5** | M | 51 | 60 | probable | frontal | negative | temazepam | 1256 | Grade 3 | Grade 4 |
| **6*** | F | 74 | 80 | possible | non-frontal | negative | thiopental, pancuronium | 1100 | Grade 2 | Grade 1 |
| **7** | M | 66 | 75 | possible | frontal | negative | levomeprazin | 1253 | Grade 3 | Grade 1 |
| **8** | M | 54 | 64 | possible | frontal | positive | amantadine | 1290 | Grade 3 | Grade 1 |
| **9** | M | 79 | 85 | possible | non-frontal | negative | oxazepam, nitrazepam, acetylcysteine | 1175 | Grade 2 | Grade 2 |
| **10** | F | 68 | 79 | probable | non-frontal | negative | amitriptyline | 922 | Grade 2 | Grade 1 |
| **11** | F | 60 | 72 | possible | non-frontal | positive | levodopa/carbidopa, amitriptyline | 1045 | Grade 2 | Grade 2 |
| **12** | M | 60 | 67 | probable | frontal | positive | levodopa/carbidopa, lormetazapam, nortriptyline | 1013 | Grade 4 | Grade 2 |
| **13** | F | 62 | 70 | probable | non-frontal | negative | amantadine, temazepam, diazepam, amitriptyline | 1160 | Grade 2 | Grade 3 |
| **14*** | M | 79 | 85 | possible | non-frontal | negative | alprazolam, tamsulosin, thiopental, pancuronium | 1305 | Grade 3 | Grade 3 |
| **15** | M | 67 | 72 | possible | non-frontal | positive | levodopa/carbidopa | 1525 | Grade 2 | Grade 4 |
| **16** | M | 61 | 69 | probable | frontal | positive | midazolam, clozapine | 1270 | Grade 3 | Grade 3 |

* Cases that underwent euthanasia by sodium thiopental and pancuronium bromide; NINDS-SPSP = National Institute of Neurological Disorders and Stroke and the Society for Progressive Supranuclear Palsy

Post hoc univariate F-tests revealed significantly higher densities of peripheral benzodiazepine (pBZ) and adenosine receptor type 1 ($A_{1)}$ receptors, but lower densities of kainate receptors and of nicotinic cholinergic receptors of the α4/β2type (nACh) in the caudate nucleus of PSP brains than in brains of control subjects (Table 4; Fig. 1A). In contrast to this, significantly higher N-methyl-D-aspartate (NMDA), γ-aminobutyric acid receptor type B ($GABA_{B)}$, pBZ, and serotonin receptor type 2 ($5\text{-}HT_2$) receptor densities were found in area 24′ of PSP patient brains than in control brains (Table 5; Fig. 1D).

Because PSP cases displayed a high interindividual variability in receptor density alterations (see large standard deviation (SD) in Fig. 1A and 1D), we subdivided the cohort on the basis of the clinical parameters presence of frontal presentation and severity of tau pathology in the examined regions, and we tested separately for data obtained from the caudate nucleus and area 24′ whether these factors were associated with receptor density alterations in PSP cases.

## Progressive supranuclear palsy subgroups

### *Frontal presentation versus non-frontal presentation*

Discriminant analyses of receptor densities when the PSP cohort was divided into cases with frontal (n = 7) and cases with non-frontal (n = 9) presentation resulted in a significant segregation of these two clinically relevant pictures in both the caudate nucleus (Wilks' lambda 0.016, Chi-square 29.107, p 0.01) and area 24′ (Wilks' lambda 0.029, Chi-square 24.737, p 0.037). However, this result revealed by the omnibus test could not be attributed to distinct receptors in the subsequent post hoc tests, because none of them reached significance in either brain structure. The significant omnibus test in the case of the caudate nucleus can be explained by the fact that 11 of 20 receptors presented lower densities in cases with frontal presentation than in those with non-frontal presentation, and the opposite situation was found for only 8 receptors (5-HT2 receptor densities were identical in both groups). The significant omnibus test in the case of area 24′ is due to the fact that 11 of 20 receptors presented higher densities in cases with frontal presentation than in those with non-frontal presentation, and the opposite situation was found for only 9 receptors. However, relatively large SDs resulted for both regions in a lack of significance at the post hoc test level.

Interestingly, discriminant analyses and subsequent post hoc tests revealed that PSP cases with and without frontal presentation also had different variations from control subjects in both the caudate nucleus (Fig. 1B) and area 24′ (Fig. 1E). In the caudate nucleus (Fig. 1B), frontal PSP patients (Wilks' lambda 0.009, Chi-square 37.711, p 0.002 by omnibus test) had significantly higher pBZ and D1 but lower nACh and D2 receptor densities than did control subjects, whereas non-frontal PSP patients (Wilks' lambda 0.008, Chi-square 43.257, p 0.001 by omnibus test) presented significantly lower nACh

**Table 4.** Mean receptor densities in the caudate nucleus of controls and progressive supranuclear palsy patients.

| | progressive supranuclear palsy vs. controls | | | progressive supranuclear palsy frontal vs. non-frontal presentation | | | progressive supranuclear palsy mild vs. severe tau pathology | | |
|---|---|---|---|---|---|---|---|---|---|
| **Receptor** | **Controls** | **Patients** | **p-value** | **frontal** | **non-frontal** | **p-value** | **mild** | **severe** | **p-value** |
| **AMPA** | 607 (108) | 583 (228) | 0.749 | 477 (248) | 665 (184) | 0.102 | 631 (220) | 503 (238) | 0.290 |
| **kainate** | 879 (61) | 800 (73) | **0.007** | 783 (70) | 813 (76) | 0.426 | 793 (68) | 812 (85) | 0.628 |
| **NMDA** | 1216 (42) | 1218 (115) | 0.951 | 1210 (122) | 1224 (118) | 0.823 | 1251 (116) | 1163 (99) | 0.413 |
| **mGlu2/3** | 9295 (625) | 9416 (1455) | 0.799 | 8735 (1746) | 9945 (980) | 0.100 | 9502 (1272) | 9272 (1843) | 0.771 |
| **$GABA_A$** | 1273 (164) | 1091 (203) | 0.021 | 986 (214) | 1173 (160) | 0.065 | 1133 (178) | 1021 (239) | 0.304 |
| **$GABA_B$** | 2477 (299) | 2684 (397) | 0.155 | 2529 (402) | 2805 (370) | 0.177 | 2788 (382) | 2511 (389) | 0.184 |
| **BZ** | 1684 (136) | 1458 (330) | 0.023 | 1463 (422) | 1454 (266) | 0.956 | 1635 (284) | 1163 (121) | **0.002** |
| **pBZ** | 1759 (89) | 2050 (347) | **0.005** | 2127 (392) | 1989 (317) | 0.450 | 2154 (365) | 1876 (251) | 0.123 |
| **$M_1$** | 1174 (35) | 922 (379) | 0.018 | 968 (351) | 886 (418) | 0.686 | 904 (412) | 952 (353) | 0.815 |
| **$M_2$** | 567 (56) | 497 (127) | 0.063 | 467 (140) | 520 (119) | 0.422 | 501 (158) | 490 (54) | 0.049 |
| **$M_3$** | 1755 (36) | 1740 (255) | 0.829 | 1753 (309) | 1731 (223) | 0.869 | 1751 (299) | 1723 (183) | 0.843 |
| **nic $\alpha_4/\beta_2$** | 200 (42) | 105 (43) | **< 0.001** | 97 (30) | 111 (52) | 0.522 | 119 (42) | 82 (38) | 0.094 |
| **$\alpha_1$** | 339 (28) | 338 (53) | 0.945 | 314 (51) | 356 (50) | 0.123 | 331 (47) | 349 (65) | 0.543 |
| **$\alpha_2$** | 467 (65) | 510 (92) | 0.199 | 502 (98) | 516 (94) | 0.767 | 498 (91) | 531 (99) | 0.500 |
| **5-$HT_{1A}$** | 129 (16) | 135 (27) | 0.543 | 139 (28) | 132 (28) | 0.646 | 139 (33) | 128 (12) | 0.428 |
| **5-$HT_2$** | 1069 (100) | 976 (142) | 0.074 | 976 (138) | 976 (153) | 0.996 | 1048 (105) | 856 (115) | **0.004** |
| **D1** | 291 (8) | 332 (49) | 0.010 | 341 (30) | 326 (61) | 0.456 | 349 (53) | 305 (25) | 0.085 |
| **D2** | 885 (50) | 818 (103) | 0.057 | 765 (108) | 860 (82) | 0.066 | 845 (99) | 774 (102) | 0.188 |
| **$A_1$** | 1522 (239) | 1952 (288) | **< 0.001** | 1966 (327) | 1942 (274) | 0.786 | 1938 (349) | 1977 (167) | 0.806 |
| **$A_{2A}$** | 1814 (195) | 2105 (327) | 0.014 | 2112 (409) | 2100 (273) | 0.944 | 2093 (391) | 2124 (212) | 0.863 |

Absolute densities (standard deviation) in fmol/mg protein as well as *p*-values for the *post hoc* tests (significant values are highlighted in bold font) are provided for each receptor type.

**Table 5.** Mean receptor densities in midcingulate area 24'of controls and progressive supranuclear palsy patients.

| | progressive supranuclear palsy vs. controls | | | progressive supranuclear palsy frontal vs. non-frontal presentation | | | progressive supranuclear palsy mild vs. severe tau pathology | | |
|---|---|---|---|---|---|---|---|---|---|
| **Receptor** | **Controls** | **Patients** | **p-value** | **frontal** | **non-frontal** | **p-value** | **mild** | **severe** | **p-value** |
| **AMPA** | 731 (39) | 739 (246) | 0.899 | 681 (216) | 785 (271) | 0.421 | 695 (314) | 773 (191) | 0.546 |
| **kainate** | 1129 (182) | 1138 (150) | 0.880 | 1102 (162) | 1166 (144) | 0.422 | 1161 (166) | 1120 (145) | 0.602 |
| **NMDA** | 1290 (102) | 1455 (188) | **0.007** | 1531 (155) | 1397 (198) | 0.164 | 1404 (272) | 1495 (79) | 0.350 |
| **mGlu2/3** | 7932 (1816) | 8576 (1960) | 0.361 | 8462 (1215) | 8665 (2464) | 0.845 | 8136 (2647) | 8918 (1283) | 0.448 |
| **$GABA_A$** | 1975 (188) | 2125 (241) | 0.070 | 2078 (204) | 2162 (272) | 0.506 | 2232 (323) | 2042 (112) | 0.181 |
| **$GABA_B$** | 4089 (484) | 5126 (801) | < **0.001** | 4826 (360) | 5360 (982) | 0.176 | 5322 (1155) | 4974 (376) | 0.406 |
| **BZ** | 2686 (296) | 3094 (1028) | 0.164 | 2676 (525) | 3419 (1226) | 0.158 | 3567 (1373) | 2726 (468) | 0.165 |
| **pBZ** | 1698 (227) | 2380 (416) | < **0.001** | 2444 (257) | 2330 (402) | 0.605 | 2452 (464) | 2325 (394) | 0.564 |
| **$M_1$** | 621 (62) | 505 (175) | 0.023 | 581 (186) | 446 (150) | 0.131 | 458 (204) | 542 (150) | 0.358 |
| **$M_2$** | 277 (43) | 306 (43) | 0.076 | 285 (29) | 322 (47) | 0.094 | 323 (54) | 293 (29) | 0.185 |
| **$M_3$** | 1071 (84) | 1135 (127) | 0.117 | 1137 (142) | 1133 (122) | 0.959 | 1123 (129) | 1144 (132) | 0.752 |
| **nic $\alpha_4/\beta_2$** | 116 (40) | 102 (40) | 0.325 | 104 (47) | 100 (35) | 0.833 | 111 (38) | 94 (41) | 0.425 |
| **$\alpha_1$** | 705 (57) | 805 (174) | 0.042 | 803 (204) | 807 (159) | 0.963 | 802 (195) | 808 (167) | 0.949 |
| **$\alpha_2$** | 1124 (190) | 1288 (220) | 0.039 | 1342 (201) | 1246 (237) | 0.407 | 1337 (221) | 1250 (225) | 0.453 |
| **$5\text{-}HT_{1A}$** | 542 (53) | 630 (119) | 0.017 | 660 (99) | 606 (133) | 0.380 | 621 (172) | 636 (65) | 0.817 |
| **$5\text{-}HT_2$** | 817 (74) | 963 (170) | **0.006** | 999 (192) | 934 (158) | 0.471 | 1001 (177) | 933 (169) | 0.446 |
| **D1** | 125 (22) | 143 (25) | 0.050 | 157 (17) | 132 (26) | 0.050 | 136 (29) | 149 (21) | 0.307 |
| **D2** | 71 (17) | 92 (41) | 0.092 | 75 (19) | 104 (49) | 0.165 | 107 (57) | 80 (18) | 0.191 |
| **$A_1$** | 1300 (151) | 1476 (318) | 0.069 | 1542 (407) | 1424 (241) | 0.479 | 1413 (281) | 1525 (352) | 0.504 |
| **$A_{2A}$** | 120 (9) | 134 (30) | 0.089 | 142 (24) | 128 (35) | 0.392 | 133 (38) | 134 (26) | 0.941 |

Absolute densities (standard deviation) in fmol/mg protein as well as *p*-values for the *post hoc* tests (significant values are highlighted in bold font) are provided for each receptor type. Note, that although comparison of mild versus severe tau pathology resulted in a non-significant Omnibus test, results of the *post hoc* tests are displayed.

and higher $A_1$ receptor densities than did control subjects. In area 24′ (Fig. 1E), frontal PSP patients (Wilks' lambda 0.035, Chi-square 31.799, p 0.033 by omnibus test) showed significantly higher NMDA, $GABA_B$, pBZ, 5-$HT_2$, D1 and adenosine receptor type 2A ($A_{2A}$) receptor densities than control subjects, whereas non-frontal PSP patients (Omnibus test: Wilks' Lambda 0.005, Chi-square 58.876 and $p < 0.001$) presented higher $GABA_B$ and pBZ densities, but lower muscarinic cholinergic receptor type 1 ($M_1$) densities, than control subjects.

***Mild versus moderate to severe tau burden***

The degree of tau pathology in both area 24′ and the caudate nucleus varied in our series of PSP brains. Tau pathology in area 24′ was mild in seven brains. In five of these cases, tau pathology in the caudate nucleus was also mild, but in two of them, it was moderate to severe. Tau pathology in area 24′ was moderate to severe in nine brains. In four of these cases, tau pathology in the caudate nucleus was also moderate to severe, but in five of them, it was only mild. Discriminant analyses of receptor densities resulted in a significant segregation of PSP cases into patients with mild tau burden and patients with moderate to severe tau burden in the caudate nucleus (Wilks' lambda 0.019, Chi-square 27.751, p 0.015), but not in area 24′ (Wilks' lambda 0.098, Chi-square 16.259, p 0.298). Post hoc univariate F-tests revealed significantly lower BZ binding site (p 0.002) and 5-$HT_2$ (p 0.004) receptor densities in the caudate nucleus of PSP brains with moderate to severe tau pathology than in PSP brains with mild tau pathology.

Discriminant analyses and subsequent post hoc tests revealed that PSP cases with no to mild and moderate to severe tau pathology also compared differently from control subjects in both the caudate nucleus (Fig. 1C) and area 24′ (Fig. 1F). In the caudate nucleus (Fig. 1C), PSP patients with no to mild tau pathology (Wilks' lambda 0.016, Chi-square 39.477, p 0.004 by omnibus test) presented higher pBZ, D1 and $A_1$ densities, but lower kainate and nACh receptor densities than did control subjects, whereas PSP patients with moderate to severe tau pathology (Wilks' lambda 0.012, Chi-square 33.177, p 0.004 by omnibus test) showed higher $A_1$, but lower BZ binding site as well as nACh, 5-$HT_2$ and D2 receptor densities, than control subjects.

In area 24′ (Fig. 1F), PSP patients with no to mild tau pathology (Wilks' lambda 0.003, Chi-square 55.124 and $p < 0.001$ by omnibus test) showed higher $GABA_B$, pBZ and 5-$HT_2$ receptor densities than did control subjects, whereas PSP patients with moderate to severe tau pathology (Wilks' lambda 0.017, Chi-square 44.973, p 0.001 by omnibus test) presented higher NMDA, $GABA_B$, pBZ and 5-$HT_{1A}$ receptor densities than control subjects.

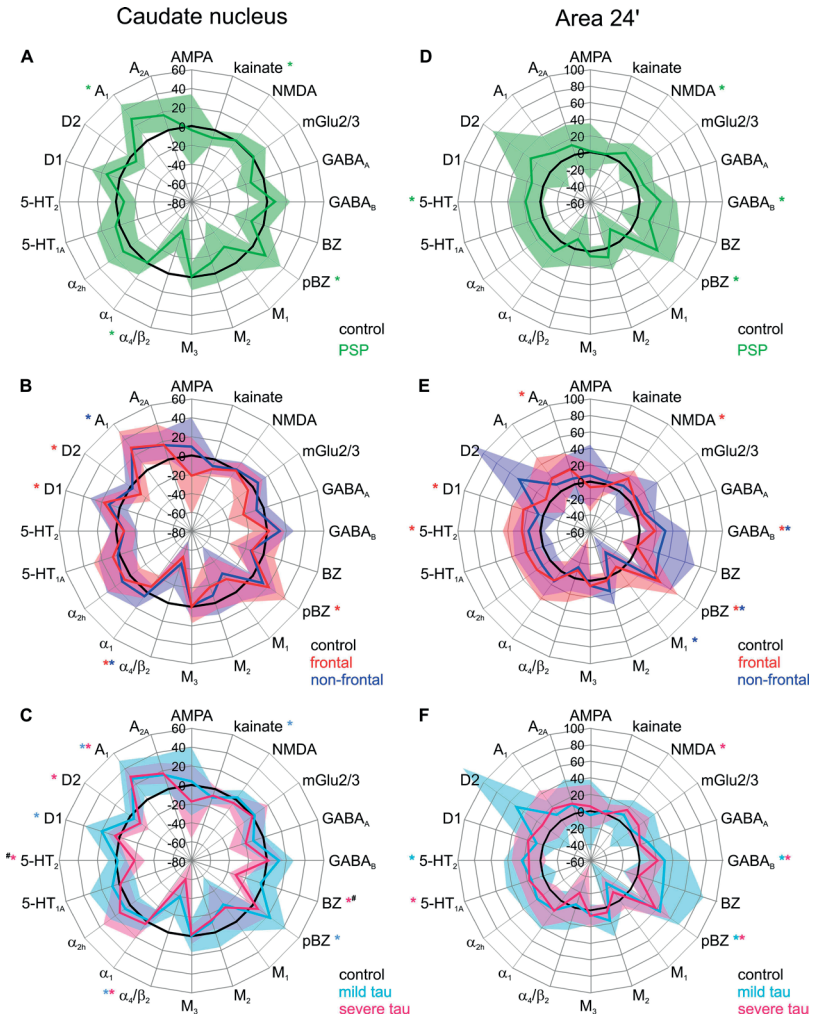


**Figure 1.** Receptor fingerprints of PSP-related receptor density alterations in the caudate nucleus (A-C) and area 24′ (D-F). Polar plots showing the mean relative changes (in %) in binding density of tissue obtained from the caudate nucleus (A) and area 24′ (D) of PSP patients (mean value coded by green line, s.d. given by transparent surface) with respect to controls (0%, in black). Polar plots showing the mean relative changes (in %) in binding density of tissue obtained from the caudate nucleus (B) and area 24′ (E) of PSP patients with frontal presentation (mean value coded by red line, s.d. given by red transparent surface) or with non-frontal presentation (mean value coded by blue line, s.d. given by blue transparent surface) with respect to controls (0%, in black). Polar plots showing the mean relative changes (in %) in binding density of tissue obtained from the caudate nucleus (C) and area 24′ (F) of PSP patients with no to mild tau pathology (mean value coded by turquoise line, s.d. given by turquoise transparent surface) or with moderate to severe tau pathology (mean value coded by pink line, s.d. given by pink transparent surface) with respect to controls (0%, in black). Colored asterisks highlight receptors which were significantly altered in a given PSP cohort compared to controls. Hashtags indicate receptors significantly different when comparing PSP cases with no to mild tau pathology and PSP cases with moderate to severe tau pathology. α4/β2nicotinic α4/β2 cholinergic receptors; BZ: GABA$_A$ associated benzodiazepine binding sites; pBZ peripheral benzodiazepine receptors.

## DISCUSSION

The present study shows a divergence in the severity of tau pathology between area 24′ and the caudate nucleus of PSP cases, as well as significant PSP-related alterations in the densities of multiple receptors from different neurotransmitter systems which differentially affected both structures. In the caudate nucleus of PSP brains, densities of pBZ and $A_1$ receptors were higher, and those of kainate and nACh receptors lower, than in control subjects. In area 24′, NMDA, $GABA_B$, pBZ, and 5-$HT_2$ receptor densities were higher in PSP than in control tissue. Furthermore, clinically relevant PSP subgroups could be differentiated on the basis of their receptor fingerprints.

To our knowledge, this is the first study to show that PSP patients with frontal and non-frontal presentation can be differentiated post-mortem with a high degree of accuracy on the basis of differences in receptor densities in both the caudate nucleus and area 24′. Receptor fingerprints also segregate mild from moderate to severe tau cases. We are aware that a drawback of our study is the fact that we were not able to assess the effect of medication on receptor densities, owing to the variability in drug therapy among patients.

Tau pathology is the histological hallmark of PSP, though the severity and distribution of tau pathology may differ between PSP subgroups.[4] The cerebral cortex and caudate nucleus are among the regions where the differences in severity are greatest.[4] This divergence in severity of pathology between cingulate cortex and caudate nucleus is supported by the present semi-quantitative evaluation and is reflected by our receptor data.

The widespread alterations in the GABAergic system highlight its importance in the pathophysiology of PSP. $GABA_B$ receptor densities were increased in area 24′ of PSP patients, but they were unaltered in the caudate nucleus. Because the increased density of $GABA_B$ receptors occurred in all PSP subgroups (frontal/non-frontal and mild/severe tau), they seems to be the most vulnerable receptor type in PSP. Furthermore, $GABA_B$ receptor increase in the midcingulate cortex is of particular interest because its activation is associated with the induction of long term potentiation,[21] and results in an amelioration of the cognitive impairment associated with chronic cerebral hypoperfusion.[22]

BZ binding sites were decreased in the caudate nucleus of PSP only in cases of moderate to severe tau pathology. This decrease may be caused by a loss of GABAergic projection neurons in this PSP subgroup, leading to a reduction of pre- and postsynaptic $GABA_A$ receptors and could explain the therapeutic effectivity of BZ agonists.[23] The $GABA_A$ receptor density demonstrated by the binding with the agonist [$^3$H]muscimol also showed a decrease, but this did not reach significance (Tables 4 and 5). Because the agonistic binding prefers high-affinity binding sites of the receptor, these data may

indicate a shift of the ratio between low- and high-affinity binding sites of the $GABA_A$ receptor in PSP.

Densities of pBZ receptors were higher in area 24′ and the caudate nucleus of PSP cases than in control subjects. This is in line with the increased PK11195 binding in these regions revealed by a positron emission tomography (PET) study[24] and reflects microglial activation. However, when the cohort was subdivided into frontal/non-frontal cases or mild/severe tau pathology, consistent alterations were found only in area 24′. Taken together, receptors of the GABAergic system are more affected in area 24′ than in the caudate nucleus, and impairment does not depend on the severity of tau pathology and frontal or non-frontal clinical type.



Our findings of widespread PSP-related changes in the glutamatergic system may be relevant for potential future treatment strategies in PSP, similar to recent studies in Parkinson's disease.[25,26] The divergence in the severity of receptor impairments between cortical and subcortical sites is further supported by our findings regarding NMDA receptors, which were altered only in area 24′. This increase in NMDA receptors is probably due to region-specific disease-induced alterations, and not caused by the long-term administration of amantadine, because patients treated with this NMDA receptor antagonist (cases #8 and #12, Table 3) presented normal NMDA receptor densities. The unchanged NMDA receptor density in the caudate nucleus is in accordance with the one other study investigating NMDA receptors in PSP patients.[27] Furthermore, we found a decrease of kainate receptors, but unaltered AMPA and mGlu2/3 densities, where up to now no information was available in PSP patients.

Drugs targeting the cholinergic system have failed to relieve the cognitive and motor impairments of PSP.[28] Interestingly, of the four cholinergic receptor types examined here, only the nACh receptors were found to be altered in the PSP cohort (as a whole and in all subgroups), though only in the caudate nucleus. Our results for nACh and $M_1$ receptors in the caudate nucleus are in line with previous findings.[11] The unaltered caudate nucleus $M_2$ receptor densities, however, contrast with the findings in another post-mortem study in which researchers reported reduced $M_2$ receptor densities in the posterior caudate nucleus.[12] The discrepancy may be explained by differences in post-mortem delay times (45 hours versus 6 hours in our study); ligands used (the antagonist [$^3$H]-AFDX 384 versus the agonist [$^3$H]-oxotremorine-M in our study); or the rostro-caudal anatomical, neurochemical and functional differences which characterize the caudate nucleus.[29]

Interestingly, the nACh receptor plays a major role in the control of dopamine release in the caudate nucleus.[30] Consequently, the remarkably strong decrease in nACh receptor densities leads to a reduction of dopamine release, which results in a global impairment of dopaminergic effects in the caudate nucleus of PSP patients.

The normal density of adrenoceptors in the caudate nucleus and area 24' in our cases, together with the normal adrenaline levels in various brain regions of PSP patients as found by Kish *et al.*,[31] as well as the ineffectiveness of noradrenergic replacement therapies,[32] suggests that this neurotransmitter system does not contribute significantly to the symptomatology of PSP. It must be noted, however, that researchers in the single other autoradiographic study on adrenoceptors in PSP to date found a generalized reduction of adrenoceptor type 2 receptors,[33] though their findings were based on a case report.

The 5-$HT_2$ receptor also emphasizes the divergent severity of alterations between area 24' and the caudate nucleus in PSP, since it was increased only in the former structure, preferentially in the frontal group. Our results are difficult to compare with those of an *in vivo* PET imaging study in which investigators reported normal densities of 5-$HT_2$ receptors in the neocortex, but higher 5-$HT_2$ receptor densities in the putamen,[34] because different regions were examined, and different ligands ([$^{18}$F]altanserin versus [$^{3}$H]ketanserin in our cases) were used. Furthermore, [$^{18}$F]altanserin PET does not directly reflect 5-$HT_2$ receptor density, as it is confounded by the uptake of the blood-brain barrier-penetrating metabolites and non-specific binding of [$^{18}$F]altanserin itself.[35]

The decrease of 5-$HT_2$ receptor densities in the caudate nucleus of PSP brains with moderate to severe tau pathology compared with those with mild tau pathology cannot be explained merely by a more severe neurodegeneration in the former group, because we did not observe an association between tau pathology and 5-$HT_2$ receptor alterations in area 24'. Interestingly, although a differential effect of serotonergic denervation on tau pathology in various brain regions has been previously described, the underlying explanation for this selective vulnerability remains unclear.[36]

Dopaminergic receptors are localized on medium spiny stellate cells. Cells expressing D1, or D1 co-localized with D2 receptors, preferentially project to the substantia nigra and the internal segment of the globus pallidus, whereas those expressing D2 receptors target the external segment of the globus pallidus.[37] Our finding of unaltered D1 receptor densities is in accordance with previous reports,[10,38] and the unchanged D2 receptor densities described here add to the controversial data concerning this receptor type.[10,11,38]

$A_1$ receptors are frequently localized presynaptically and control glutamate release. Thus, the significant PSP-related increase of receptor densities in the caudate nucleus may be a plastic reaction to (1) decreased inhibition resulting from BZ binding site downregulation and (2) increased excitation resulting from higher NMDA and lower kainate receptor densities, because the latter can also control glutamate release. The PSP-related increase in $A_1$ receptor densities in the caudate nucleus may be the result of an ongoing inflammatory process, because these receptors are expressed in microglia.[39] Furthermore, it could be a compensatory mechanism to counteract the decreased

concentrations of the adenosine precursors adenosine diphosphate and adenosine triphosphate measured in the basal ganglia of PSP patients.[40] Therefore, an intriguing question is whether modulation targeting the adenosine receptors may represent a therapeutic strategy in PSP.



## CONCLUSIONS

We have demonstrated the involvement of multiple non-dopaminergic neurotransmitter systems in the pathophysiology of PSP, which may be relevant for potential novel treatment strategies. We provide further evidence that the midcingulate cortex may prove to be a key region in this disease. GABAergic, glutamatergic and serotonergic receptors in PSP deviated most from those of controls in area 24′, where the highest frequency of tau pathology was found. This is in sharp contrast to dopaminergic, cholinergic and adenosine receptors, which were preferentially impaired in the caudate nucleus. Finally, "receptor fingerprints" not only differentiated PSP cases from control subjects neurochemically, but also segregated PSP subgroups when clinical parameters such as presence of frontal presentation and severity of tau pathology were taken into consideration.

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# 4

# GENETIC AND NEUROPATHOLOGICAL ASPECTS OF PROGRESSIVE SUPRANUCLEAR PALSY-LIKE DISORDERS

# 4.1

## *PRKAR1B* mutation associated with a new neurodegenerative disorder with unique pathology

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## ABSTRACT

Pathological accumulation of intermediate filaments can be observed in neurodegenerative disorders, such as Alzheimer's disease, frontotemporal dementia and Parkinson's disease, and is also characteristic of neuronal intermediate filament inclusion disease. Intermediate filaments type IV include three neurofilament proteins (light, medium and heavy molecular weight neurofilament subunits) and α-internexin. The phosphorylation of intermediate filament proteins contributes to axonal growth, and is regulated by protein kinase A. Here we describe a family with a novel late-onset neurodegenerative disorder presenting with dementia and/or parkinsonism in 12 affected individuals. The disorder is characterized by a unique neuropathological phenotype displaying abundant neuronal inclusions by haematoxylin and eosin staining throughout the brain with immunoreactivity for intermediate filaments. Combining linkage analysis, exome sequencing and proteomics analysis, we identified a heterozygous c. 149 T>G (p.Leu50Arg) missense mutation in the gene encoding the protein kinase A type I-beta regulatory subunit (*PRKAR1B*). The pathogenicity of the mutation is supported by segregation in the family, absence in variant databases, and the specific accumulation of PRKAR1B in the inclusions in our cases associated with a specific biochemical pattern of PRKAR1B. Screening of *PRKAR1B* in 138 patients with Parkinson's disease and 56 patients with frontotemporal dementia did not identify additional novel pathogenic mutations.

Our findings link a pathogenic *PRKAR1B* mutation to a novel hereditary neurodegenerative disorder and suggest an altered protein kinase A function through a reduced binding of the regulatory subunit to the A-kinase anchoring protein and the catalytic subunit of protein kinase A, which might result in subcellular dislocalization of the catalytic subunit and hyperphosphorylation of intermediate filaments.


**Abbreviations:** AKAP = A-kinase anchoring protein; D/D = dimerization/docking; FTD = frontotemporal dementia; FUS = fused in sarcoma; FTLD = frontotemporal lobar degeneration; NIFID = Neuronal intermediate filament inclusion disease; PKA = protein kinase A; SNP = single nucleotide polymorphism

## INTRODUCTION

Neurofilament proteins assembling into neuron-specific intermediate filaments type IV, are major constituents of the axonal cytoskeleton. Neurofilaments undergo significant changes in their subunit composition during development and in adult neurons, and play an essential role in axonal growth, axonal transport and signalling pathways.[1] In the CNS, the major neuronal intermediate filaments can be distinguished into three neurofilaments proteins: NF-L (light), NF-M (medium) and NF-H (heavy), and α-internexin, each composed of an N-terminal head domain, an α-helix-rich central rod domain, and a C-terminal tail domain.[2] Phosphorylation of the Lys-Ser-Pro repeat sites at the C-tail of NF-H and NF-M and at sites at the N-terminal domain, was proven to be essential for neurofilament-specific function.[3] The cyclic AMP-dependent protein kinase A (PKA) plays a major role in phosphorylation of neurofilaments,[4] and hyperphosphorylation of Lys-Ser-Pro repeat sites causes disrupted neurofilament axonal transport, prevents turnover of neurofilaments by ubiquitin proteasome system, and results in the accumulation of neurofilaments.[4–7]



PKA is an heterotetramer, consisting of two regulatory and two catalytic subunits, which is inactive in the absence of cyclic AMP.[8] Binding of cyclic AMP to regulatory subunits unleashes the catalytic subunit, thereby enabling PKA signalling. The regulatory subunits also provide binding sites for A-kinase anchoring protein (AKAP), a scaffold protein for targeting PKA signalling.[9,10] Mutation in these regulatory subunits have been shown to alter PKA function.[11,12]

Several neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, motor neuron disease, and frontotemporal dementia (FTD) show aggregation of neurofilaments in association with disease-specific accumulation of tau, α-synuclein or transactive response DNA-binding protein 43 (TARDBP, previously known as TDP-43), respectively.[5] Neuronal intermediate filament inclusion disease (NIFID), a rare neurodegenerative disorder, shows neurofilament and fused in sarcoma (FUS) protein-positive inclusions, which are negative for tau, TARDBP and α-synuclein.[13–18] NIFID is a non-familial disorder,[16,19,20] and neither pathogenic variants in any of the genes coding for intermediate filaments and FUS, nor biochemical modifications of intermediate filaments were found.[15,21,22]

Furthermore, neurofilament inclusions are found in patients with Charcot-Marie-Tooth disease type 2E and in transgenic mice with a mutation at the major phosphorylation site (Ser55Asp) of the *NEFL* gene.[23–25] Also, a transgenic mouse model overexpressing α-internexin, induces the formation of cerebellar torpedoes, and abnormal accumulation of neuronal intermediate filaments.[26]

In this paper, we report a novel familial neurodegenerative disorder with a highly specific neuropathological phenotype consisting of abundant α-internexin-positive, but

FUS-negative neuronal inclusions. By means of genome-wide linkage analysis, exome sequencing and proteomics of neuronal inclusions, we have identified a pathogenic mutation in the gene coding for the type I-beta regulatory subunit of protein kinase A, *PRKAR1B*. The mutant protein is found to be associated with aggregates of intermediate filaments in this disease.

## MATERIALS AND METHODS

### Subjects

We studied a three generation-large family with 12 affected patients presenting with dementia and/or extrapyramidal syndrome (Figure 1 and Table 1). Medical information was limited in the five deceased affected members (patients II:1, III:1, III:12, III:13 and III:14). One clinically unaffected patient, patient III:9, who died from myeloid leukemia at 57 years old, has been described with α-synuclein negative Lewy bodies previously.[27] The proband (patient III:4) from this family was evaluated for motor and mental complaints at the age of 56 years. The age at onset of the affected individuals varied between 45 and 64 years, the mean disease duration was 14.8 years, but varied from 5 to 25 years.

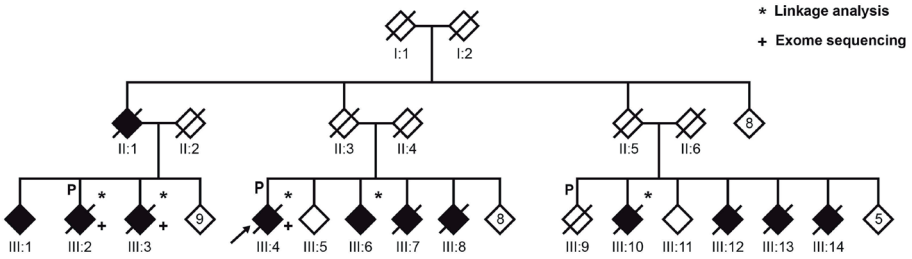


**Figure 1.** Pedigree of the family with *PRKAR1B* mutation. Filled symbols represent affected individuals by behavioural symptoms, dementia and or parkinsonism, and empty symbols represent unaffected individuals. Symbols with a diagonal line represent deceased individuals. Pathology is denoted by P, and the proband is indicated by arrow. Numbers in symbols indicates the number of individuals. Sex of the pedigree members are obscured to protect privacy. * = individuals included in linkage analysis; + = individuals included in exome sequencing.

DNA was extracted from peripheral blood samples of patients III:2, III:3, III:4, III:5, III:6, III:10 and III:11, and from the spleen of patient III:9. The study was approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam, and all family members participating in the study or their legal representatives gave informed consent. A series of 138 unrelated patients with Parkinson's disease and an autosomal dominant pattern of inheritance were also studied. The clinical diagnosis of Parkinson's disease was established according to widely used criteria.[28] The patients originated from Italy

(n = 114), Brazil (n = 14), Portugal (n = 9) and The Netherlands (n = 1). The average onset age of Parkinson's disease symptoms was 53.5 ± 11.7 years (range 20–75 years), and the average disease duration was 8.5 ± 7.1 years (range 1–36 years). In these patients the entire *PRKAR1B* coding region (10 exons) and exon-intron boundaries were sequenced by Sanger protocols to find possible pathogenic variants. Primers and PCR protocols are reported in the Supplementary Table 1. *PRKAR1B* variants were also analyzed in exome sequenced cohorts of familial FTD (n = 51) and FTD-FUS (n = 5). The average onset age of symptoms was 58.4 ± 8.6 years (range 36–73 years) in familial FTD and 33.7 ± 2.4 (range 30–36 years) in FTD-FUS. The average disease duration was 66.8 ± 8.8 (range 43–83 years) in familial FTD and 43.1 ± 3.7 (range 39–46 years) in FTD-FUS.



**Table 1.** Clinical characteristics of the individuals in the family

| Subject | Age at onset | Age at death | Duration | Current age | Dementia | Parkinsonism | Atrophy on MRI/CT |
|---|---|---|---|---|---|---|---|
| **II:1** | 45 | 70 | 25 | - | + | + | NA |
| **III:1** | NA | NA | NA | NA | - | + | NA |
| **III:2**[a] | 50 | 67 | 17 | - | + | _ | NA |
| **III:3** | 50 | 75 | 25 | - | + | NA | Frontal |
| **III:4**[a] | 56 | 61 | 5 | - | + | + | Generalized |
| **III:5** | - | - | - | 65 | - | - | NA |
| **III:6** | 60 | - | - | 67 | + | + | Generalized |
| **III:7** | < 57 | 63 | NA | - | + | NA | NA |
| **III:8** | < 60 | 62 | NA | - | + | NA | NA |
| **III:9**[a] | - | 57 | - | - | - | - | NA |
| **III:10** | 63 | 71 | 8 | - | + | + | Generalized |
| **III:11** | - | - | - | 80 | - | - | NA |
| **III:12** | < 65 | 67 | NA | - | + | NA | NA |
| **III:13** | 64 | 73 | 9 | - | + | NA | Generalized |
| **III:14** | NA | 74 | NA | - | + | NA | NA |
| **Range** | 45–54 | 61–15 | 5–55 | | | | |

+ or – indicate the presence or absence of the phenotype or information; NA, not available.
[a] Autopsy confirmed cases.

## Genomic analysis

CSV files containing single nucleotide polymorphism (SNP) call data from HumanCytoSNP-12v2.1(Illumina) arrays of five related patients (Figure 1) were adapted by GenomeStudio (Illumina) for linkage analyses using Allegro[29] implemented in easyLINKAGE Plus.[30] SNPs with a call rate < 95% were excluded from the calculations. Mendelian inheritance check was performed for all family members, with the program PedCheck.[31] SNPs showing Mendelian inconsistencies were excluded from the calculation. Individu-

als who were encoded by the pedigree information file were used for allele frequencies computation. Two separate multipoint linkage analyses were performed (affected only) on genotypes from five affected individuals using Allegro with a SNP spacing of 0.2 cM and one of 0.5 cM. Logarithm of the odds (LOD) scores in sets of 100 markers were calculated assuming the disease to be an autosomal dominant disorder with a gene frequency of 0.0001 in the population. Regions showing a LOD score > 1.5 in both models were used as candidate regions (Supplementary Figure 1). As borders, flanking SNP markers were used. Additionally, genome wide copy number analysis in genotyped individuals was performed using signal intensity files generated with GenomeStudio 2011, V2011.1 (Illumina) in Nexus Copy Number, Discovery Edition, ver. 5.1 (BioDiscovery).

Patients III:2, III:3 and III:4 were selected for exome sequencing (Figure 1). Whole exome capture and sequencing were performed by LGC Genomics. Exomes were captured by Agilent's SureSelect AllExon Kit, and were sequenced with 100-bp reads on the Illumina HiSeq2000 platform, according to the manufacturer's protocol. Reads were mapped to the human reference genome sequence (assembly GRCh37/hg19) using the Burrows-Wheeler Alignment Tool.[32] The identified variants per individual were called by using Genome analysis Tool Kit (GATK) and annotated by ANNOVAR.[33,34] GATK was also used for base quality recalibration, local sequence realignment and variant filtering to minimize base calling and mapping errors. Variants with quality score < 30, quality over depth < 5, strand bias >-0,10 and depth < 20 were filtered out. Additionally, Indels with strandbias >-1.0 instead of -0.10 were filtered out. We used the dbSNP129 (http://www.ncbi.nlm.nih.gov/projects/SNP/), the 1000 genome project (www.1000genomes.org/) and the National Heart Lung Blood Institute Exome Variant Server (https://evs.gs.washington.edu/EVS/) to filter out polymorphisms. The predicted functional effects of the novel sequence variants were assessed by PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant from Tolerant (SIFT) (http://sift.jcvi.org/www/SIFT_enst_submit.html), PROVEAN (http://provean.jcvi.org/seq_submit.php) and Mutation Taster (www.mutationtaster.org). The conservation of amino acid across different species was identified by MUCLES.[35]

### Histology and immunohistochemistry

The Netherlands Brain Bank performed brain autopsy (patients III:2 and III:4) within four hours of death according to their Legal and Ethical Code of Conduct of the Netherlands Brain Bank. Tissue blocks were taken from all cortical areas, hippocampus, amygdala, basal ganglia, substantia nigra, pons, medulla oblongata, cerebellum, and cervical spinal cord, and were embedded in paraffin blocks and subjected to routine staining with haematoxylin and eosin, periodic acid-Schiff reaction and silver staining. Brain autopsy and routine staining of the third case (patient III:9) was described by van Duinen (van Duinen *et al.*, 1999), and several regions were obtained for immunohistochemistry.

Immunohistochemistry was performed with antibodies directed against: hyperphosphorylated tau (AT-8, Innogenetics, 1:400); amyloid-β protein (DAKO, 1:100, following formic acid pre-treatment); α-synuclein (Zymed Laboratories, undiluted, after formic acid pre-treatment); poly-ubiquitin-binding protein p62 (BD Biosciences Pharmingen, 1:200, after pressure cooking); TARDBP (ProteinTech Group, 1:100, after pressure cooking); neuroserpin (Abcam; 1:100); SMI-31 (Sternberger; 1:5000); SMI-32 (Sternberger; 1:7000) α–internexin (Invitrogen; 1:100, after pressure-cooking); FUS (ProteinTech; 1:25–1:200 with initial overnight incubation at room temperature, after pressure cooking), and PRKAR1B (2x anti-PRKAR1B, Abcam ab38225; 1:50 and Santa Cruz Biotechnology SC-907, Inc; 1:125). Specificity of PRKAR1B antibody is described in other studies.[36,37] Primary antibodies were incubated overnight at 4°C followed with BrightVision horseradish peroxidase-linked secondary antibody (Immunologic). The immunoreactivity was visualized by freshly prepared Liquid DAB Substrate Chromogen solution (Dako). Slides were counterstained with Mayer's haematoxylin and mounted in Entellan®.

### Double immunofluorescence staining

For double staining, autofluorescence of brain tissue was quenched by treatment with 0.1% Sudan Black B (Sigma-Aldrich) in 70% ethanol. Secondary antibodies were Cy3-conjugated anti-mouse (Jackson ImmunoResearch; 1:100) and Alexa Fluor® 488-conjugated anti-rabbit secondary antibody (Invitrogen; 1:100). Slides were mounted in Mowiol and analysed by confocal microscope (Leica).

### Electron microscopy

Minute pieces of frontal cortex were fixed in 4% glutaraldehyde in 0.1 M phosphate (pH 7.2), postfixed in 1% osmium tetroxide and embedded in Epon®. Semi-thin sections (1 µm) were stained with Toluidine blue. Areas of interest were selected for ultrathin sectioning (50–60 nm). Contrast was enhanced by staining with lead citrate.

### Quantitative proteomics

Approximately 1000 positive inclusions were excised by laser capture microdissection (Carl Zeiss Microscopy) from each of the two brains (patients III:2 and III:4). In addition, tissues from the same brain areas of two healthy control brains without inclusions were collected in the same manner. Tissues were lysed in 25 µl SDS sample buffer, separated by 10% SDS PAGE, and stained with colloidal Coomassie Blue. Each gel lane was cut into two gel pieces of equal size, destained, and incubated with trypsin (1 µg, Promega) for 16h at 37°C.

Peptides were separated on a 200 mm reversed phase nano-column (100 µm ID packed with 3 µm Alltima™ C18 particle from Alltech) using an Eksigent NanoLC Ultra® system (AB-Sciex). The acetonitrile concentration in 0.1% acetic acid was increased

linearly from 4.5% to 38% in 40 min, and to 80% in 1 min. The flow rate was 400 nl/min. The eluted peptides were electro-sprayed into the LTQ-Orbitrap discovery (Thermo Electron). The mass spectrometer was operated in a data-dependent mode, in which one mass spectrometry (MS) full scan (m/z range from 330 to 2000) was followed by MS/MS scans on five most abundant ions. The exclusion window was 25s. The mass spectrometric data were searched against the IPI human database (ipi human v3.87) with MaxQuant software (version 1.3.0.5). The search parameters were MS tolerance, 20 ppm; MS/MS tolerance, 0.5 Da; enzyme, trypsin with maximum missed cleavages of 2.

### Sequential biochemical fractionation and immunoblot analysis

Post-mortem frozen brain tissue from two cases (patients III:2 and III:4), two frontotemporal lobar degeneration (FTLD) TDP cases and two Alzheimer's disease cases, were dissected, weighted, and sequentially extracted with buffers of increasing strength as previously described.[38] Briefly, grey matter was extracted at 5 ml/g (volume/weight) with low salt buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 1 mM DTT, 10% sucrose, and a cocktail of protease inhibitors), high salt-Triton buffer (low salt + 1% Triton$^{TM}$ X-100 + 0.5 M NaCl), myelin floatation buffer (30% sucrose in low salt + 0.5 M NaCl), and sarkosyl (SARK) buffer (1% N-Lauroyl-sarcosine in low salt + 0.5 M NaCl). The SARK insoluble material was extracted in 0.25 ml/g urea buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Tris, pH 8.5). Proteins were resolved by 7.5% SDS-PAGE and transferred to PVDF membranes (Millipore).

Following transfer, membranes were blocked with Tris buffered saline containing 3% powdered milk and probed with the mouse monoclonal antibody PKA [RI] raised against the C-terminal portion (amino acids 225–381) of the type I regulatory subunit (610166, Becton Dickinson Laboratories), the polyclonal antibody PKA Iβ reg (sc-907 (c-19), Santa Cruz) and a mouse monoclonal anti-α-internexin (32–3600, Invitrogen). Primary antibodies were detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch), and signals were visualized by a chemiluminescent reaction (Millipore) and the Chemiluminescence Imager Stella 3200 (Raytest).

For dephosphorylation experiments, urea fractions were dialyzed against RIPA buffer and treated with 400 units lambda phosphatase (New England Biolabs) for 30 min at 30°C.

## RESULTS

### Clinical and pathological features

Behavioural changes (self-neglect, delusions), with apathy and anxiety, and memory problems with disorientation, followed by stiffness, shuffling gait, and frequent falls

without tremor were the presenting symptoms in most of the patients (Table 1). Impaired attention and concentration, and deficits in memory, language, executive and visuo-constructional functions were found in two neuropsychologically evaluated patients (patients III:4 and III:6). Mini-Mental State Examination and Frontal Assessment Battery of the proband were 20 and 6, and 18 and 8, respectively for patient III:6. These two patients showed mild to moderate rigidity, bradykinesia, postural instability, small-stepped gait and normal ocular movements, and no cerebellar and motor neuron disease signs at neurological examination on the first evaluation. Unified Parkinson's Disease Rating Scale of the proband showed 6 points in part I, 13 points in part II, 16 points in part III, Hoehn and Yahr grade four and Schwab and England Activities of Daily Living scale of 40%. EMG in the proband revealed no evidence for motor neuron disease, myopathy or polyneuropathy. Generalized cerebral atrophy was seen on MRI or CT of the brain in patients III:4, III:6, III:10, III:13, and more prominent frontal atrophy in the proband (patient III:4) (Supplementary Fig. 2). Dopaminergic medication in two patients had only a modest effect. Moreover, $^{18}$F-fluorodeoxyglucose PET showed frontal hypometabolism in the proband (Supplementary Fig. 2), but a normal FP-CIT scan and normal CSF profile which is incompatible with Parkinson's disease and Alzheimer's disease. Brain autopsy was performed in patients III:2, III:4 and III:9.

Macroscopic examination showed mild cerebral atrophy (patients III:2, III:4 and III:9), slightly more pronounced in the frontoparietal cortex in two. An irregular tumor, defined as glioblastoma multiforme in the right temporal lobe was found in the brain of patient III:4 (absent on MRI one year earlier).

The neocortex showed normal cytoarchitecture in all three brains, with moderate neuron loss in the substantia nigra in two, and severe loss of Purkinje cells in one case. Abundant eosinophilic, periodic acid-Schiff reaction negative, cytoplasmatic neuronal inclusions with a glossy weakly stained core were observed in all neocortical regions in layers 3–6, hippocampus, substantia nigra, brainstem and spinal cord. Low to moderate number of inclusions were seen in the caudate nucleus, putamen, pallidum and the cerebellum (patient III:2), and in the dentate gyrus of patient III:9. These inclusions stained strongly with p62 and α-internexin antibodies (Fig. 2A-2C), and less intense with neurofilament antibodies, such as, SMI 31 and SMI 32. Larger inclusions showed a target-like picture with a halo and a weakly stained core, whereas smaller inclusions had a more homogeneous intense staining. Immunohistochemistry was negative for FUS, α-synuclein, AT-8, amyloid-β, neuroserpin, and TARDBP. No intranuclear inclusions were seen.

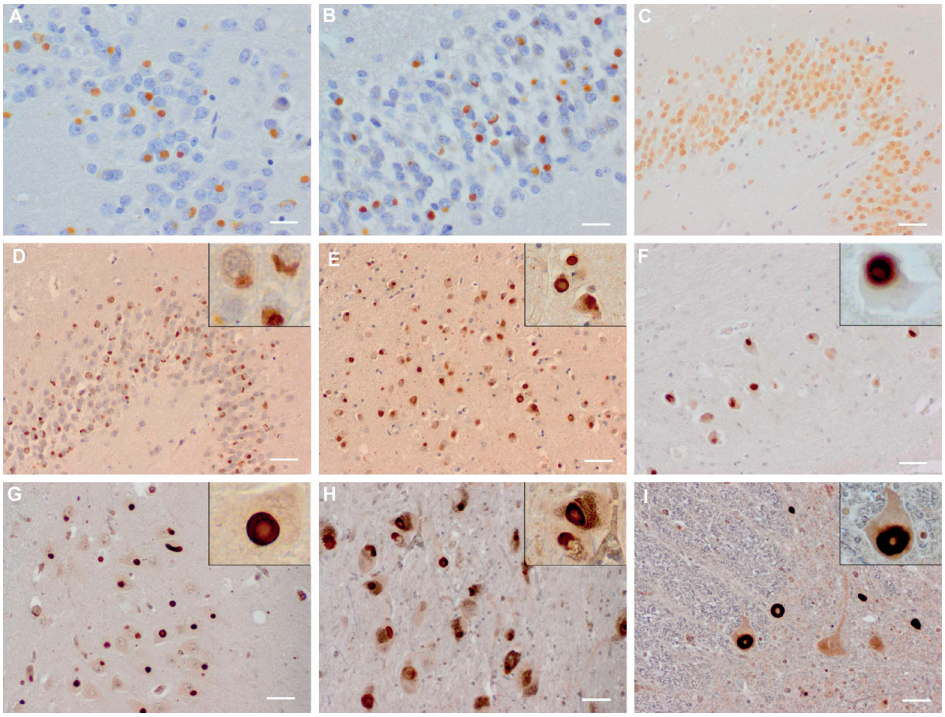


**Figure 2.** Distribution of neuronal cytoplasmatic inclusions found in familial neurofilamentopathy due to the mutation in the *PRKAR1B* gene. Strong immunoreactivity of neuronal cytoplasmatic inclusions with antibodies against α-internexin (A) and p62 (B) in the dentate gyrus of the hippocampus is seen in patient III:2. Granular cells of the dentate gyrus show diffuse weak nuclear staining without cytoplasmatic inclusions with FUS antibody (C). PRKAR1B-positive neuronal cytoplasmatic inclusions with various sizes are abundant in the hippocampus (D) and frontal region (E). A central unstained core surrounded by a strongly immunoreactive halo is found for larger inclusions in different cortices. Many inclusions are also found in the granular layer of the cerebellum (F). The same finding of PRKAR1B-positive neuronal cytoplasmatic inclusions is seen in the hippocampus (G) of the second patient (III:4). Substantia nigra show moderate neuron loss and positive immunoreactivity for PRKAR1B (H). These inclusions are also seen in lower motor neurons of the spinal cord (I). Scale Bars: A and B = 20 µ; C-I = 50 µm.

## Genomic and proteomic analysis

Two multipoint linkage analyses of 5 affected patients revealed six regions with LOD score > 1.5 (Supplementary Table 2). No overlapping copy number variant was detected in affected genotyped individuals. Exome sequencing produced ~6.4 Gb of reads per sample. The average coverage of targeted region was 57x, with 74%, 72%, and 74% covered at least 20x (Supplementary Table 3). This resulted into a calling of ~48.000 allelic variants per individual after quality filtering using GATK (Supplementary Fig. 3). We examined the exome data on known FTD and Parkinson's disease genes, but no pathogenic variants in known FTD genes (*GRN*, *MAPT*, *c9orf72*, *CHMP2B*, *VCP* and *FUS*)

and Parkinson's disease genes (*LRRK2*, *PARK2*, *PARK7*, *PINK1*, *SNCA*, *VPS35*, *ATP13A2*, *FBXO7*, *PANK2* and *PLA2G6*) were found.

We performed the genetic analysis in two steps. First, we filtered the exome sequencing data combining with linkage analysis to reduce the number of variants. An analysis of non-synonymous, splice-sites, stop and frameshift variants (SNPs and indels) in the six regions with LOD score > 1.5 showed seven variants shared by the three patients and not annotated by dbSNP129 and having an allele frequency < 1% in 1000 Genomes Project and Exome Variant Server.



Second, we combined proteomics data with the candidate variants found by genetic analysis to identify the causative gene. A total of 1000 inclusions were excised by means of microlaser dissection from fresh-frozen brain samples of patients III:2 and III:4, and corresponding inclusion-free tissue of the same size was obtained from two control brains. The dissection was repeated once from one of the patient samples to obtain a total of three replicates. Mass spectrometry of the inclusion bodies was sufficiently sensitive to identify proteins present, but not sufficient to detect protein modifications. Quantitative proteomics analysis revealed six proteins (Polyubiquitin-C, α-internexin, neurofilament light polypeptide, cAMP-dependent protein kinase regulatory subunit type I-beta, neurofilament medium polypeptide and heat shock cognate 71 kDa protein) that were present consistently and solely in the inclusion samples (Supplementary Table 4).

Combining proteomics analysis with the candidate variants from exome sequencing resulted in one single variant, the novel missense heterozygous variant in *PRKAR1B* on chromosome 7p22 (exon 2: c. 149T>G; p.Leu50Arg; RefSeq NM_002735.2), which is conserved and predicted as pathogenic by four different *in silico* methods that predict functional effects of sequence variations (Fig. 3 and Supplementary Table 5). No novel and pathogenic variants were found in the genes coding for the remaining five proteins identified in the proteomics analysis of the inclusion bodies. Sanger sequencing confirmed the presence of the p.Leu50Arg variant in the affected patients III:2, III:3, III:4, III:6, III:9 and III:10, and its absence in unaffected family members (III:5 and III:11). Sequencing of the *PRKAR1B* coding region (10 exons) and exon-intron boundaries in a cohort of autosomal dominant Parkinson's disease (n = 138) and basophilic inclusion body disease (n = 2) revealed several silent and intronic polymorphisms, which are all annotated in dbSNP, and two non-synonymous rare heterozygous variants, also present in dbSNP (Supplementary Table 6). The first, p.Ile40Phe, was detected in six Parkinson's disease probands (minor allele frequency 0.013, similar to dbSNP minor allele frequency 0.022), and is considered benign by most of the prediction programs (Supplementary Table 5). The other, p.Arg232Gln, was found in only one Parkinson's disease proband, and is considered pathogenic by all the prediction programs. However, this variant does not co-segregate with Parkinson's disease in the family (one affected sib was not a carrier). This variant is also annotated in dbSNP because of just one allele detected in the

1000 Genome Project, and is not present in the Exome Variant Server. Analysis of whole exome sequencing data in a cohort of familial FTD with unknown gene defect (n = 51) and FTD-FUS (n = 5) did not reveal any potential pathogenic rare variants in *PRKAR1B* (Supplementary Table 6).

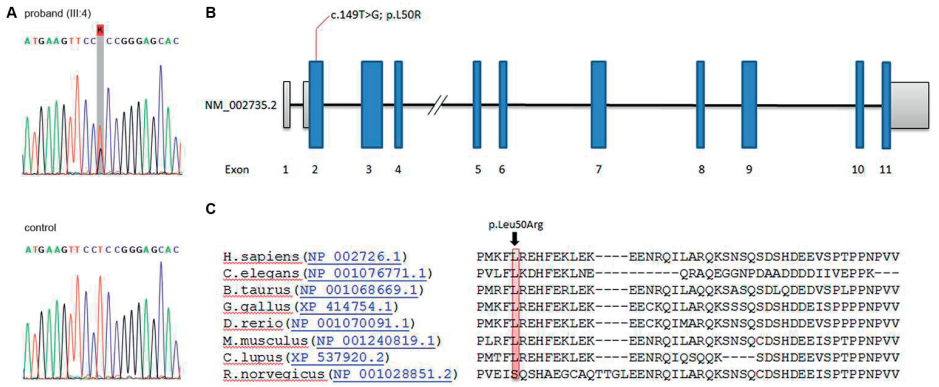


**Figure 3.** Schematic representation of the *PRKAR1B* genomic structure and conservation of the mutation. Electropherogram showing the *PRKAR1B* mutation at position chr7:750997 (A>C) in exon 2 (c.T149G; p.Leu50Arg) present in the affected individual (patient III-4) and absent in an unaffected control of the family (A). Schematic structure of *PRKAR1B* (isoform NM_002735.2) and the position of the mutation identified in the present study is shown. Exons are represented with blue boxes, untranslated regions in gray boxes (B). Alignment of the protein region containing the highly conserved leucine amino acid residue across different species is shown (C). The leucine at position 50 (NP_002726.1) is indicated in the red box.

## Immunohistochemistry and biochemical analysis

Immunohistochemistry with PRKAR1B antibodies revealed intense staining of the inclusions in brain of the three cases (Fig. 2). Cytoplasmatic inclusions were exclusively seen in neurons and had a variable size. Most inclusions were round and compact with strong PRKAR1B immunoreactivity, and some neurons showed a more granular cytoplasmatic staining (Fig. 2D-I). Round inclusions were seen in the dentate granule cells (Fig. 2D), cornu ammonis 1–4, subiculum and entorhinal cortex, and in all layers of the neocortex (Fig. 2E) of patients III:2 and III:4. Many inclusions were also observed in the hippocampus (Fig. 2G), substantia nigra (Fig. 2H), brainstem and spinal cord (Fig. 2I), whereas lower number of inclusions are found in the caudate nucleus, putamen and pallidum. The dentate nucleus and granular layer of the cerebellum showed many cytoplasmatic inclusions in patient III:2 (Fig. 2F), but only some in the same region of patient III:4. Available slices of three brain regions (pons and two neocortex) of patient III:9 also showed abundant PRKAR1B positive inclusions. Overall, more inclusions are found in patient III:2 than in similar regions of the other two cases. Large inclusions showed a faint core with strongly stained halo. The PRKAR1B antibody labelled the inclusions more intensely

than p62 and α-internexin. No glial cytoplasmatic inclusions were seen in the cerebral white matter.

Double-labelling immunofluorescence with α-internexin and PRKAR1B antibodies showed that nearly all α-internexin positive inclusions were also PRKAR1B-positive (Fig. 4A-C). Less than five percent of the inclusions, predominantly those with a smaller size, labelled either for α-internexin or PRKAR1B. Double labelling also showed the spatial relationship of these two proteins within the inclusions; PRKAR1B antibody often labelled the core, whereas α-internexin labelled a halo at the outer side of the inclusions (Fig. 4D-F).



Ultrastructurally, the core within the body consisted of electron-dense material, and fibrils were found in areas with remnants of vesicles and mitochondria (Fig. 4G and H). The body is composed of dense aggregates of filaments (densely packed fibrils with often a radiating aspect at the edges), whereas the paler halo consisted of bundles of both circularly and longitudinally arranged filaments.

Neurofibrillary tangles or plaques in Alzheimer's disease (n = 4), Lewy bodies in Parkinson's disease (n = 10) and Lewy body dementia (n = 5), glial inclusions in multiple system atrophy (n = 4), TARDBP-positive inclusions in FTLD (n = 4), Pick bodies, neurofibrillary tangles or pretangles in FTLD with MAPT gene mutations as well as healthy controls (n = 4), did not show any staining with the PRKAR1B antibody, demonstrating the specificity of PRKAR1B accumulation in our cases (data not shown).

To characterize potential biochemical alterations of PRKAR1B, proteins were sequentially extracted from temporal cortex from two cases (III:2 and III:4) as well as neurological controls with a series of buffers with an increasing ability to solubilize proteins and analysed by immunoblot using an antibody that recognizes both isoforms of the type I regulatory subunits of PKA (BD Laboratories).

Two bands at the expected molecular weights of PRKAR1A and B (~48 and 50kDa) were consistently present in the sarkosyl fractions in *PRKAR1B* mutation cases and FTLD and Alzheimer's disease. However, a strikingly different biochemical profile was seen in the urea fractions (fraction enriched for highly insoluble proteins) for the two *PRKAR1B* mutation cases compared with controls (Fig. 5A). Whereas controls showed only minimal reactivity in the urea fraction, a massive enrichment for PRKAR1B was observed in the two cases accompanied by the presence of additional bands of lower and higher molecular weight as well as a high relative molecular mass ($M_r$) smear. Similar results were observed with a second antibody raised against PRKAR1B (Santa Cruz, data not shown). As the appearance of higher migrating bands is suggestive for abnormal post-translational modifications such as hyperphosphorylation, we investigated the phosphorylation state of PRKAR1B by treating dialyzed urea fractions with lambda protein phosphatase. However, this did not reveal any obvious changes in the banding pattern (Supplementary Fig. 4). No biochemical alterations with respect to changes in

solubility or appearance of additional bands were observed for α-internexin between our *PRKAR1B* mutation cases and controls (Fig. 5B).

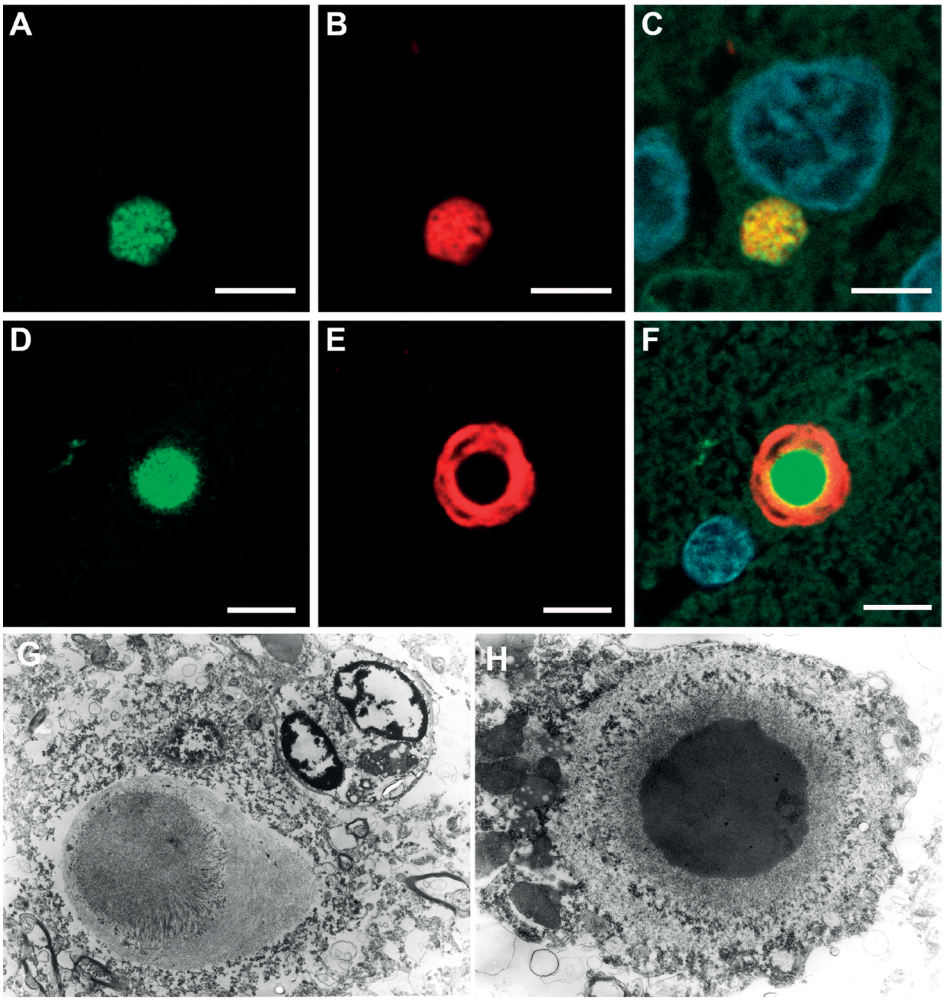


**Figure 4.** Double-label immunofluorescence for PRKAR1B (*green*) and α-internexin (*red*) in familial neurofilamentopathy due to *PRKAR1B* mutation. Immunofluorescence of the inclusions with PRKAR1B (A) and α-internexin (B). Some inclusions label homogeneous staining with co-localization of α-internexin and PRKAR1B (C). Merged images clearly show that both markers label distinct components of inclusions with a central core labelling for PRKAR1B antibody (D) surrounded by a halo labelling for α-internexin (E, F). Scale bars: A and B = 5 μm; D-F = 10 μm. (G and H) Ultrastructural examination of inclusions demonstrates dense aggregates of filaments of 11–16 nm surrounded by granule coated fibrils and cellular organelles, G = 9000x and H = 22500x enlarged.

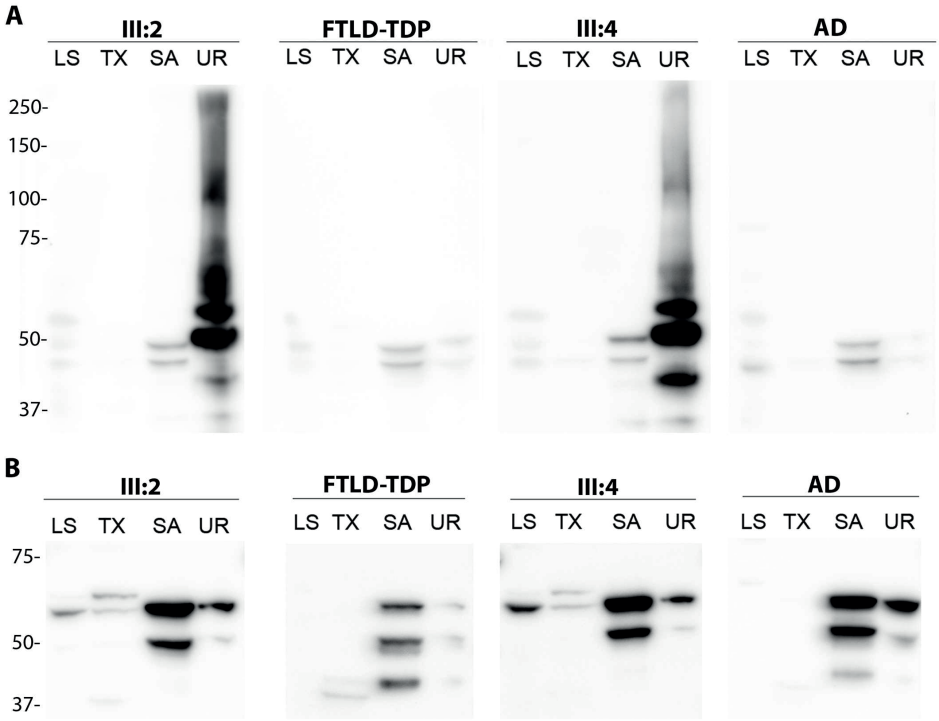


**Figure 5.** Biochemical analysis of PRKAR1B and α-internexin. (A) Proteins were sequentially extracted from temporal grey matter from two cases (patients III:2 and III:4) with the *PRKAR1B* mutation and neurological controls (Alzheimer's disease (AD) and frontotemporal lobar degeneration with TARDBP inclusions (FTLD-TDP)). Low salt (LS), Triton™ X-100 (TX), sarkosyl (SA) and urea (UR) fractions were separated by SDS-PAGE and immunoblotted with anti-PKA RI antibody (BD Laboratories). Cases and controls showed bands in the sarkosyl fraction corresponding to PRKAR1A (~48 kDa) and PRKAR1B (~50 kDa). Note the dramatic increase of highly-insoluble PRKAR1B in the urea fraction in cases compared with controls accompanied by appearance of additional bands of ~45 kDa and ~55 kDa and a high molecular mass smear. (B) The same protein fractions were analyzed by α–internexin immunoblot revealing no obvious changes with respect to changes in solubility or appearance of additional bands between cases and controls.

## In silico prediction of the *PRKAR1B* mutation on PRKAR1B protein structure

*PRKAR1B* codes for the R1β-subunit of cyclic AMP-dependent protein kinase A, which is a tetramer in its inactive form composed of two catalytic and two regulatory subunits (Fig. 6A and B).[39] Binding of cyclic AMP to the regulatory subunits unleashes the catalytic subunits, thereby allowing phosphorylation of PKA substrates. The regulatory subunits dimerize via their N-terminal dimerization/docking (D/D) domains. In the inactive PKA tetramer, the D/D domains of the R1β isoform form an integral part of the holoenzyme; while the cyclic AMP binding domains and the linker region tightly interact with one catalytic subunit additional *trans* interactions with the catalytic subunit in the other heterodimer (Fig. 6B). The leucine at position 50 of the PRKAR1B protein is located on

the dimer interface formed by the N-terminal D/D domains of the regulatory subunits (Fig. 6A). The leucine side chain forms a hydrophobic core together with several other conserved hydrophobic residues to create this dimer interface (Fig. 6B). A change of leucine to arginine at this position creates a steric hindrance, because of its larger

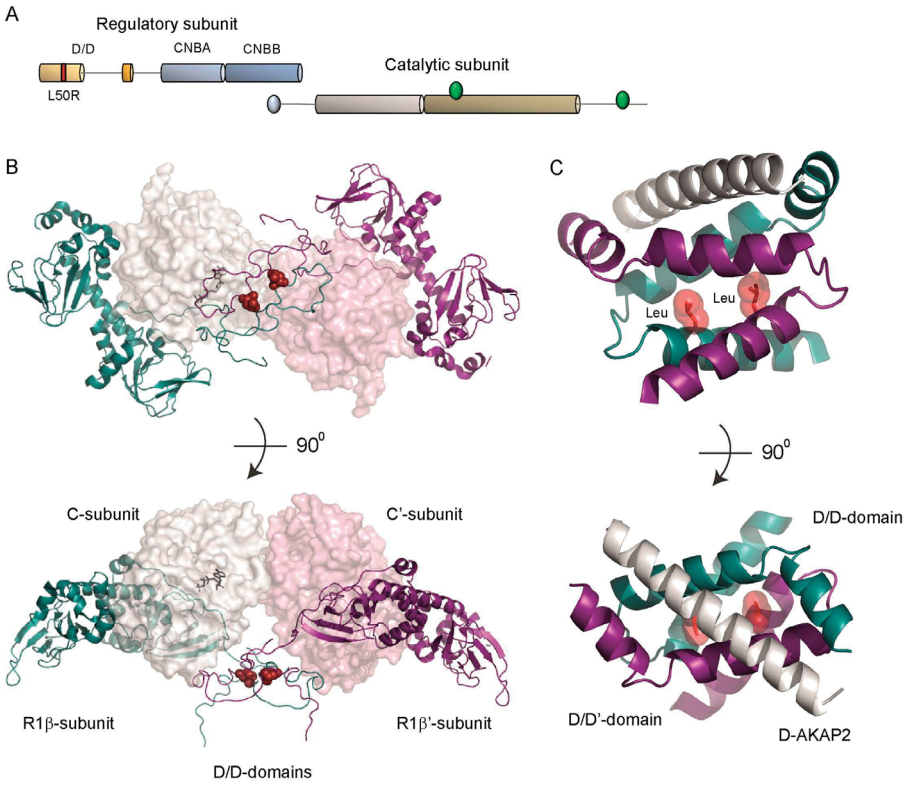


**Figure 6.** The L50R mutation is located on the interface between the docking/dimerization domains of the protein kinase A regulatory subunit β. (A) Organization of the PKA protein subunits. The regulatory subunits contain an N-terminal dimerization/docking (D/D) domain, followed by a linker region and two cAMP binding domains (CNBA and CNBB). The catalytic subunit contains an N-terminal myristylation site for membrane anchoring (blue circle), N-and C-terminal lobes and tails with phosphorylation sites (green circles). The p.Leu50Arg mutation maps to the D/D domain of the regulatory subunit. (B) Overall structure of the PKA tetrameric holoenzyme containing two catalytic and two regulatory subunits. The catalytic subunits C (white) and C′ (pink) are shown in space filling representation, with ATP bound to subunit C represented in black sticks. The secondary structure elements of the regulatory subunits are shown in cyan (subunit R1β=PRKAR1B) and in purple (symmetry-related subunit R1β′) with the leucine on the interface between the D/D domains in red space filling representation. The holoenzyme is shown in two different orientations. (C) The importance of the D/D-dimer interface formation for binding to A-kinase anchoring proteins is shown by the structure of the D/D domain of the homologous PRKAR1α subunit in complex with a peptide from dual-specific AKAP2. Secondary structures are shown in cyan and purple (regulatory subunit D/D domains) and white (dual-specific AKAP2). Side chains of Leu50 at the D/D interface are indicated in red stick representation surrounded by transparent spheres. Figure is created using Pymol (deLano Scientific).

size the arginine side chain will not fit within the hydrophobic core. Furthermore, the positive charge on the arginine side chain will potentially introduce unfavourable electrostatic interactions with its symmetry-related arginine across the dimer interface. In one scenario, the dimerization interface may still be formed but with local structural rearrangements resulting in an altered conformation. Due to the intimate connections between the D/D domains and the catalytic subunits, these local rearrangements may be propagated throughout the holoenzyme and affect the cyclic AMP-induced response and thus activation of the kinase.[39] Furthermore, the D/D dimerization interface creates the docking site for AKAPs (Fig. 6C),[40] and local rearrangements may affect AKAP binding and thereby PKA signalling and targeting PKA to a specific subcellular location.[10,40] In another scenario the arginine completely prevents formation of the dimerization interface between the D/D domains. This would affect correct holoenzyme formation and thereby PKA activation. In addition, the unassembled D/D domain interfaces would expose large hydrophobic areas which are prone to aggregation resulting in insoluble protein.



## DISCUSSION

This study describes a novel hereditary neurodegenerative disorder associated with a mutation (c.149T>G; p.Leu50Arg; RefSeq NM_002735.2, NP_002726.1) in the gene coding for the type I-beta regulatory subunit of the PKA with a unique neuropathological phenotype with PRKAR1B accumulation into abundant neuronal inclusions. The mutation is predicted to prevent or alter dimerization between the D/D domains within the PKA holoenzyme, thereby exposing hydrophobic protein regions that may result in aggregation, or reducing the binding of the regulatory subunits to both the catalytic subunits of PKA, and to AKAP. The frequency of this mutation appears to be rare, as the mutation is absent in dbSNP and Exome Variant Server. Moreover, no pathogenic mutation in *PRKAR1B* could be identified in a cohort of familial Parkinson's disease or frontotemporal dementia.

The present disorder has a rather unspecific phenotype consisting of dementia and parkinsonism with poor response to levodopa, normal FP-CIT scan, and normal CSF biomarkers, which have ruled out Parkinson's disease and Alzheimer's disease. The neuropsychological profile with impairment of multiple cognitive domains and clinical symptoms are consistent with the involvement of all cortical and subcortical regions of the brain. Cerebellar and motor neuron signs were lacking at neurological examination despite the widespread inclusions in cerebellum and spinal cord; however, we cannot exclude that the patients may develop such signs in the last stage of the disease during their stay in a nursing home. Its hereditary occurrence has initially not been recognized,[27] but distinguishes it from the mostly sporadic NIFID. Furthermore, the absence of

immunoreactivity with α-synuclein and FUS antibodies distinguishes this disorder from Parkinson's disease and NIFID. The co-occurrence with cancers (myeloid leukemia and glioblastoma) in two out five patients is quite remarkable. PKA stimulates the expression of the NR4 receptor, and NR4 is involved in several malignancies, such as glioblastoma and myeloid leukemia.[41] It might be worth screening for potential variants in *PRKAR1B* in cohorts of patients with these malignancies and to see whether the mutation alters the expression of NR4 receptor. However, this is beyond the scope of this research.

The present approach of combining genome-wide linkage analysis, exome sequencing and proteomic analysis of neuronal inclusions allowed us to identify a heterozygous p.Leu50Arg variant in *PRKAR1B* in five affected family members, consistent with an autosomal dominant mode of inheritance. The highly specific pattern of PRKAR1B accumulation in inclusions in the three autopsy-proven cases together with the dramatic enrichment of PRKAR1B in highly insoluble protein fractions with appearance of abnormal $M_r$ species, the negative PRKAR1B immunoreactivity in any other neurodegenerative disorders (Alzheimer's disease, Parkinson's disease, Lewy body dementia, multiple system atrophy, Pick's disease, and FTLD-TARDBP) and the absence of the mutation in variant databases, strongly argues for a causative role of this mutation in this family.

The pathophysiological mechanism how this mutation leads to neurodegenerative disease remains to be investigated by establishing cell culture and animal models. Our first hypothesis is that the mutation leads to impaired dimerization between the regulatory R1β subunits and catalytic subunits within the PKA holoenzyme. Structural changes within the holoenzyme may liberate the catalytic subunits which could be vulnerable to degradation, resulting in reduced PKA activity. This hypothesis is supported by loss of PKA catalytic subunit and PKA activity shown in PKA regulatory knockout mice.[11] However, catalytic subunits unleashed by mutation-induced structural changes may also lead to increased PKA activity, as has been found in the p.Ser9Asn mutation of PRKAR1A.[12] PRKAR1B knock-out mice have impaired long-term potentiation and long-term depression in the mossy fibers – cornu ammonis 3 region of the hippocampus and visual cortex.[42–44]

A second pathophysiological mechanism is that the Leu50Arg mutation on the subunit interface of the D/D-domain can induce structural changes to the docking site for AKAP which is located across this interface, and abolish the binding between AKAP and the regulatory subunit.[39,40] An important function of AKAP is to target the holoenzyme in close proximity to the dedicated substrates by binding to the D/D-domain.[39] which is important for creating the microenvironment for PKA signaling. It is likely that impaired binding to AKAP causes subcellular dislocalization of the complex, thereby disturbing PKA signaling on the dedicated substrates.

The Leu50Arg *PRKAR1B* mutation and subsequent change in PKA function probably leads to an imbalance of the phosphorylation status of N-terminal head and C-terminal

tail domain of neurofilaments. PKA is responsible for transient phosphorylation of specific sites on the N-terminal head domain of neurofilaments.[7] Phosphorylation levels of N- and C-terminal are related to each other, and are essential for axonal transport.[5,7] Therefore, the imbalance of phosphorylation would explain the additional accumulation of α-internexin and other neurofilaments in the cell soma in the present cases.

The aggregate formation of PRKAR1B, its biochemical enrichment and additional bands in urea fractions can be the result of post-translational modifications such as (hyper)phosphorylation. While our dephosphorylation experiments are not indicative of abnormal phosphorylation, more sophisticated biochemical analyses are needed to further address this. In addition, the high $M_r$ smear in the immunoblot might be explained by poly-ubiquitination of PRKAR1B protein in the proteosomal degradation.[45] The aggregation of PRKAR1B might entrap other proteins like intermediate filaments just as a secondary phenomenon, as we see in inclusions characteristic for other neurodegenerative diseases.[1]



Although we have not seen obvious changes and presence of abnormal $M_r$ α-internexin protein species in our cases by immunoblot, the analysis of phosphorylation alterations of intermediate filaments requires further investigation by more sensitive means such as mass spectrometry. Future studies to explore the phosphorylation of intermediate filaments in model systems carrying the Leu50Arg mutation are required to address this. Finally, the unique pathological phenotype of the present disorder is supported by the distinct pattern of PKA type I regulatory bands on western blots of brain tissue. To find out the reason of the different pattern between cases and controls, more sophisticated proteomic analysis would be required in the future.

In conclusion, we provide evidence that a mutation in *PRKAR1B* is associated with a new type of a familial neurodegenerative disease with dementia and parkinsonism characterized by specific and abundant accumulation of PRKAR1B into neuronal inclusions. Our findings link altered regulation of PKA by mutant PRKAR1B to human late-onset neurodegeneration.

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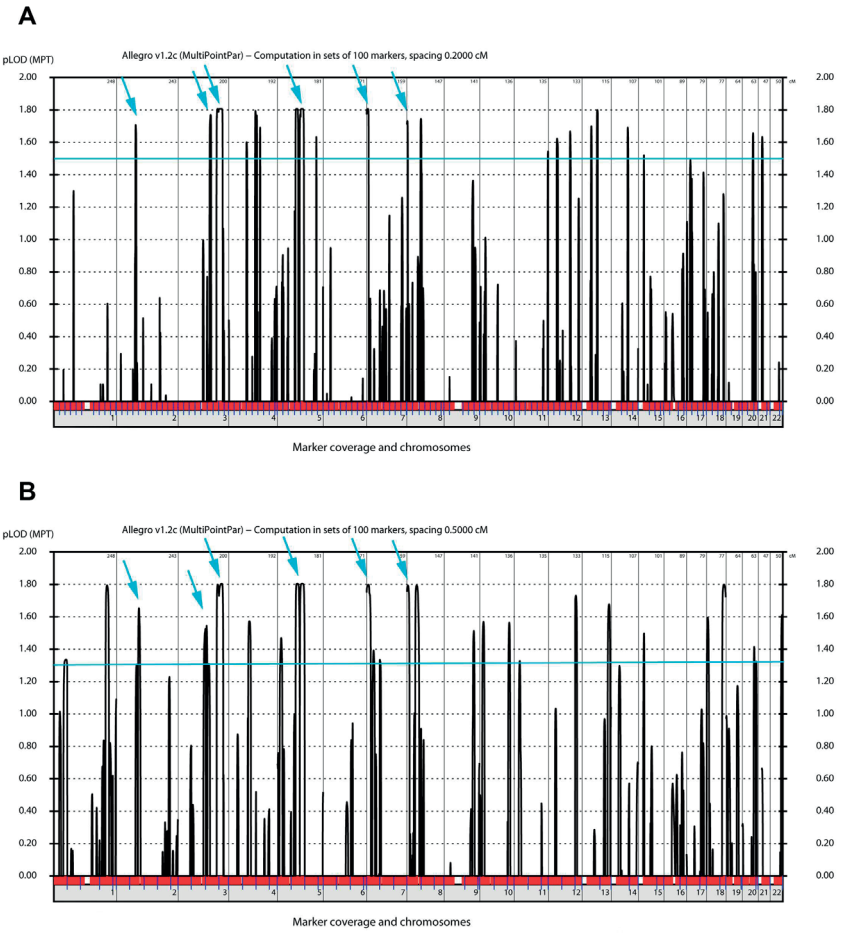
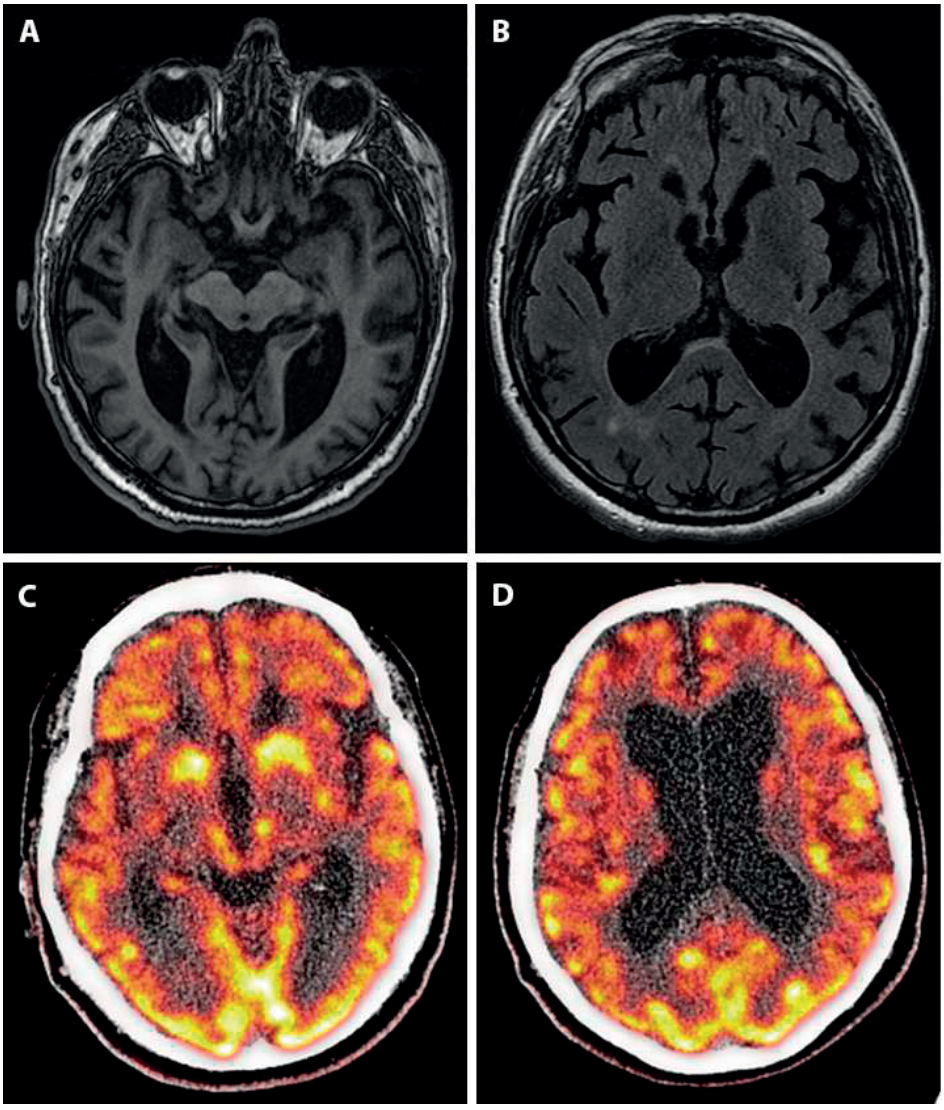
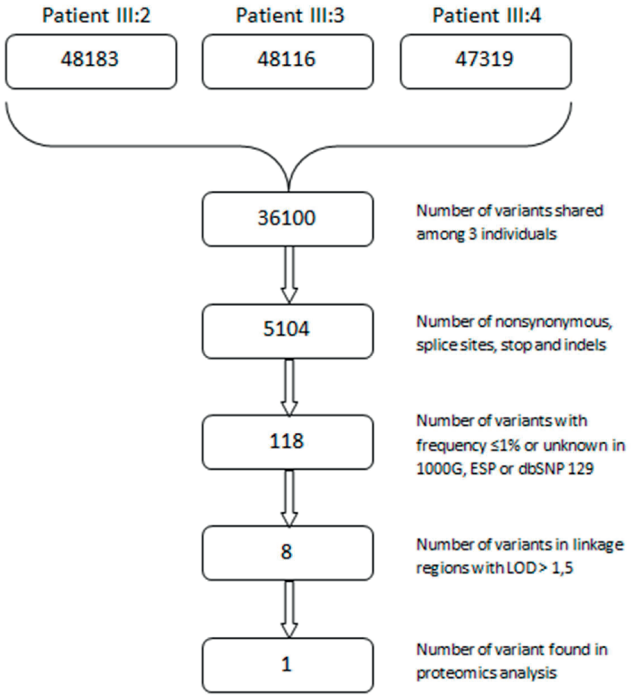
## SUPPLEMENTARY MATERIAL



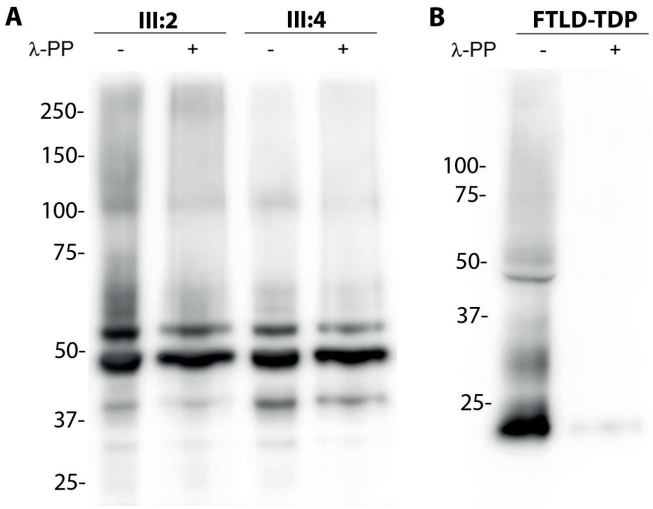

**Supplementary figure 1.** Plots of the LOD scores using Allegro. Two separate multipoint linkage analyses were performed (affected only) on genotypes from five affected individuals using Allegro with a SNP spacing of 0.2 cM (A) and one of 0.5 cM (B). Regions with LOD scores > 1.5 (blue line) in both models were used as candidate regions (blue arrows).




**Supplementary figure 2.** Neuroimaging findings from the proband (III:4). Axial images using FLAIR sequence showed symmetrical cortical atrophy and enlarged ventricles. Thinning of the mesencephalon (A) and atrophy of the caudate nucleus were observed (B). Imaging of the brain metabolic activity using [18F] fluorodeoxyglucose and PET showed symmetrical frontal hypometabolism (C and D). Metabolic deficient is also seen at the thalamus (C).




**Supplementary figure 3.** Bioinformatic analysis of exome sequencing




**Supplementary figure 4.** Biochemical analysis of phosphorylation state of PRKAR1B. (A) Dialyzed urea fractions from two cases with *PRKAR1B* mutation were either not treated (-) or treated (+) with lambda protein phosphatase (λ-PP), separated by SDS-PAGE and analyzed by immunoblot with anti-PKARI antibody (BD Laboratories). No obvious changes were observed in the banding pattern after dephosphorylation.
(B) To control for de-phosphorylation activity of the used enzyme and conditions, a urea fraction from a FTLD-TDP cases was used and analyzed by immunoblot with a phosphorylation-specific TDP-43 antibody (clone 1D3). Note the lack of immunoreactivity after λ-PP treatment.

**Supplementary table 1.** PCR primers and protocol for the amplification and sequencing of *PRKAR1B* fragments

| Oligoname[a] | Sequence | Size |
|---|---|---|
| **prkar1b-ex2F** | GGGCCGTCACGTTTAACACC | 465 bp |
| **prkar1b-ex2R** | ACCACGGGACAGAGGGAAGG | |
| **prkar1b-ex2SF** | GAGCCTGAAGGGCTGTGAGC | 183 bp |
| **prkar1b-ex2SR** | AGGACACGTGCGAAGGGAAG | |
| **prkar1b-ex3F** | AAGTGGGGATGATGGGGATG | 557 bp |
| **prkar1b-ex3R** | CTGAGACCCCCAGGAGGATG | |
| **prkar1b-ex4F** | TAACAGCAGGCTGAGGGTGGA | 350 bp |
| **prkar1b-ex4R** | CCGGAGAAGGCAGCTGTGAT | |
| **prkar1b-ex5F** | CTGTGAAATGAGGGGAGGAAGG | 436 bp |
| **prkar1b-ex5R** | CCCAGGTTCAAGCGATTCTCC | |
| **prkar1b-ex6F** | AATTGGTAGCACCCAGGATGTTG | 378bp |
| **prkar1b-ex6R** | CATCACCCTTGTTTCCCTCTGC | |
| **prkar1b-ex7F** | CTCTGCCCACAAGCGAAAGG | 460 bp |
| **prkar1b-ex7R** | CCTCCACCCCTTTCCACTCC | |
| **prkar1b-ex8F** | AGCTCCCTGCCCTTCATGG | 529 bp |
| **prkar1b-ex8R** | TCCATAATACCAACAACACTCAACTGC | |
| **prkar1b-ex9F** | TGTCTTGGACTGTGGCTGTGG | 381 bp |
| **prkar1b-ex9R** | GGGCAGGAGGAATCTCAGTGG | |
| **prkar1b-ex10F** | ACGTGGTGTCGGCAGTGG | 396 bp |
| **prkar1b-ex10R** | TTGGGGGAACAGGACTGAGC | |
| **prkar1b-ex11F** | CAGGACAATGGCTAGCTGAACG | 504 bp |
| **prkar1b-ex11R** | GGCCCACACCTCACACAGC | |

[a] primers used for PCR amplification and Sanger sequencing reactions

PCR protocol

The amplification reactions were performed in a total volume of 20 µl, containing 1x FastStart Taq DNA Polymerase buffer, 200 µM of each dNTP, 10 µM forward primer, 10 µM reverse primer, 0.5 unit FastStart Taq DNA Polymerase (Roche Diagnostics) and 20 ng genomic DNA. PRKAR1B_2F/R was amplified with addition of 1XGC-melt (Roche Diagnostics). PRKAR1B-ex2SF and PRKAR1B-ex2SR were used to validate the variant in sample III:9 isolated from DNA extracted from the spleen.

The PCR conditions were as follows: initial denaturation, 7 min 30 sec at 96ºC, followed by 9 cycles of: 30 sec denaturation at 96$^{o}$C; 30 sec annealing (1st cycle at 70$^{o}$C, with 1$^{o}$C/cycle decrease); 1 min extension at 72$^{o}$C. Then, 25 cycles of: 30 sec denaturation at 96$^{o}$C; 30 sec annealing at 60$^{o}$C; 1 min extension at 72$^{o}$C. Final extension: 5 min at 72$^{o}$C. The PCR reactions were purified using 5 units ExoI (Fermentas) and 1 unit Fast AP (Fermentas), 45′ 37oC, 15′ 80$^{o}$C. Direct sequencing was performed using Big Dye Terminator chemistry ver. 3.1 (Applied Biosystems) as recommended by the manufacturer. Dye terminators were removed using SephadexG50 (GE Healthcare) and loaded on an ABI 3130XL Genetic Analyzer (Applied Biosystems). For sequence analysis the software packages Sequence Analysis version 5.3 (Applid Biosystems) and Seqscape version 2.6 (Applied Biosystems) were used.

**Supplementary table 2.** Candidate regions from linkage analysis

| **UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly** | | |
|---|---|---|
| **chromosome: start-end** | **SNP: start-end** | **size (bp)** |
| Chr2:72013658–85537000 | rs898238-rs10165220 | 23523342 |
| chr3:97749272–229292001 | rs1498646-rs2244708 | 31542729 |
| chr3:149604549–978635542 | rs9815364-rs11715386 | 29030993 |
| chr5:63649860–007594079 | rs6449720-rs11240960 | 43944219 |
| chr7:45989–96995059 | rs6583338-rs13237658 | 16949070 |
| chr8:176568–80625104 | rs2003497-rs13270447 | 10448536 |

**Supplementary table 3.** Exome sequencing statistic per sample

| | **III: 2** | **III:3** | **III:4** | **average** |
|---|---|---|---|---|
| **Total reads** | 6776289400 | 6615714400 | 6449885600 | 6613963133 |
| **Total aligned reads** | 3975374358 | 3684889797 | 3408320419 | 3689528191 |
| **% aligned** | 58,66594715 | 55,6990459 | 52,84311429 | 55,736 |
| **% of 1X** | 95,7 | 96,2 | 96,1 | 96 |
| **% of 5X** | 89,7 | 90,2 | 90,8 | 90,23 |
| **% of 10X** | 83,3 | 84,1 | 85,4 | 84,27 |
| **% of 20X** | 71,7 | 74,2 | 73,9 | 73,24 |
| **Coverage** | 61,38 | 56,89 | 52,62 | 56,96 |

**Supplementary table 4.** Proteomics analysis of inclusion body and controls.

| Protein names | Gene names | control_1 | control_2a | control_2b | Inclusion_1 | Inclusion_2a | Inclusion_2b |
|---|---|---|---|---|---|---|---|
| Polyubiquitin-C | UBC;UBB; RPS27A; UBA52 | 0 | 0 | 0 | 39707000 | 123480000 | 23422000 |
| Alpha-internexin | INA | 0 | 0 | 0 | 15435000 | 54174000 | 34966000 |
| Neurofilament light polypeptide | NEFL | 0 | 0 | 0 | 11232000 | 47581000 | 32901000 |
| cAMP-dependent protein kinase type I-beta regulatory subunit | PRKAR1B | 0 | 0 | 0 | 3415200 | 11101000 | 407070 |
| Neurofilament medium polypeptide | NEFM | 0 | 0 | 0 | 1289300 | 12187000 | 9401100 |
| Heat shock cognate 71 kDa protein | HSPA8;HSPA2;HSPA1B;HSPA6;HSPA1A;HSPA 1L;HSPA7 | 0 | 0 | 0 | 299830 | 671950 | 214140 |

The values represent the MS1 peptide intensities generated by Maxquant. Samples were collected twice from control_2 and Inclusion_2 and were analyzed separately as control 2a and 2b versus Inclusion 2a and 2b, respectively. The protein isoforms are clustered.

**Supplementary table 5.** *In silico* prediction of functional effects of variants

| | | PRKAR1B p.Leu50Arg | PRKAR1B p.Arg232Gln | PRKAR1B p.Ile40Phe |
|---|---|---|---|---|
| **PolyPhen-2** | HumDiv[a] | **Probably Damaging** | **Probably Damaging** | Benign |
| | HumVar[b] | **Possibly Damaging** | **Possibly Damaging** | Benign |
| **SIFT[c]** | | **Damaging (score = 0,001)** | **Damaging (score = 0,001)** | Tolerated (0,878) |
| **PROVEAN** | | **Deleterious** | **Deleterious** | Neutral |
| **MUTATION TASTER** | | **Disease causing** | **Disease causing** | **Disease causing** |

[a] preferred model for evaluating rare alleles, dense mapping of regions identified by GWAS and analysis of natural selection.

[b] preferred model for the evaluation of Mendelian disease-causing variants, which requires distinguishing mutations with drastic effects from the remaining human variation, including abundant mildly deleterious alleles.

[c] the substitution is predicted to be damaging if the score is ≤ 0.05, and tolerated if the score is > 0.05.

**Supplementary table 6.** Variants detected in patients with frontotemporal dementia and Parkinson's disease
***PRKAR1B***[a]

| Function | dbSNP[b] | nucleotide change | protein change | freq FTD[c] n = 56 | freq PD[c] n = 138 | freq dbSNP[d] | freq EVS[e] |
|---|---|---|---|---|---|---|---|
| **Exon 11** | rs28488947 | c.1065C>T | p.Phe355= | 0,0000 | 0,0070 | 0,0470 | A = 154/G = 12778 |
| **Exon 11** | rs11545042 | c.1014T>C | p.Thr338= | 0,1786 | 0,0620 | 0,1730 | G = 1852/A = 11016 |
| **Exon 11** | rs370829885 | c.1008G>A | p.Ala336= | 0,0714 | 0,0000 | - | T = 11/C = 12851 |
| **Exon 11** | rs28626752 | c.984A>G | p.Ala328 | 0,0000 | 0,0070 | 0,0520 | C = 220/T = 12678 |
| **intron 10** | rs28585978 | c.974–4A>C | | 0,0000 | 0,0070 | 0,0510 | G = 130/T = 12716 |
| **Exon 10** | rs78260651 | c.903C>T | p.Ser301= | 0,0000 | 0,0040 | 0,0010 | A = 21/G = 12561 |
| **Intron 9** | rs71518309 | c.892–28G>C | | 0,4196 | 0,4750 | 0,3950 | G = 7290/C = 5128 |
| **Intron 9** | rs118004775 | c.892–29G>A | | 0,0804 | 0,0000 | 0,0720 | T = 863/C = 11311 |
| **Intron 9** | rs62431411 | c.891+38A>G | | 0,4554 | 0,3010 | 0,3910 | C = 5499/T = 7499 |
| **Intron 9** | rs62431412 | c.891+24C>T | | 0,5000 | 0,3010 | 0,3910 | A = 5498/G = 7500 |
| **Exon 9** | rs3211362 | c.846T>C | p.Ile282= | 0,5000 | 0,3040 | 0,4010 | G = 5629/A = 7369 |
| **Exon 9** | rs77809618 | c.810G>A | p.Ala270= | 0,0000 | 0,0040 | 0,0010 | T = 12/C = 12986 |
| **Intron 7** | rs9330368 | c.709–96A>C | | 0,5000 | 0,3480 | 0,2810 | G = 8773/T = 4225 |
| **Exon 7** | rs200069843 | c.695G>A | **p.Arg232Gln** | 0,0000 | 0,0040 | 0,0010 | - |
| **Exon 7** | rs76061469 | c.642C>T | p.Thr214= | 0,0000 | 0,0110 | 0,0010 | A = 22/G = 12976 |
| **Intron 4** | rs117395529 | c.440+34G>A | | 0,0179 | 0,0000 | 0,0270 | T = 71/C = 12935 |
| **Intron 4** | rs142693952 | c.440+25G>A | | 0,0000 | 0,0250 | 0,0080 | T = 172/C = 12834 |
| **Exon 2** | rs61732492 | c.118A>G | **p.Ile40Phe** | 0,0000 | 0,0220 | 0,0130 | C = 258/T = 12748 |

[a] References for annotation of variants: GenBank n. NM_002735.2 and NP_002726.1

[b] SNP reference number in dbSNP137

[c] Minor allele frequency in frontotemporal dementia (FTD) and Parkinson's disease (PD)

[d] Minor allele frequency in dbSNP

[e] Minor allele frequency in Exome variant Server

# 4.2

# Tau pathology in a presymptomatic *MAPT* L315R mutation carrier

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# 5

# GENERAL DISCUSSION

Classically PSP, corticobasal degeneration (CBD) and multi-system atrophy (MSA) have been designated forms of atypical parkinsonisms, due to their combination of extrapyramidal and non-extrapyramidal signs. The original paper by Richardson, Steele and Olzewski[1] already reported on cognitive and behavioural changes in several patients, but it was many years later that these symptoms were widely appreciated in PSP. In the study by Donker Kaat *et al.*, the frontal presentation of PSP with cognitive decline and behavioural disturbances, resembling the clinical presentation of the behavioural variant of frontotemporal degeneration (FTD), was identified in approximately 20 percent of cases.[2] The occurrence of this clinical presentation was recently confirmed in a multicenter study of 100 autopsy-proven PSP patients.[3] This subtype PSP-FTD was the third most common clinical presentation of PSP after typical PSP (Richardson's syndrome) and PSP-parkinsonism. Moreover, it is now recognized that patients with typical PSP will frequently develop behavioural and cognitive symptoms that affect quality of life and caregiver burden significantly during the course of the disease.[4,5]



The pathophysiological overlap between PSP and FTD is further emphasized by the aggregation of the hyperphosphorylated tau protein that characterizes both neurodegenerative disorders. There exist six distinct tau isoforms in the human brain, which are distinguished by their number of binding domains and generated through alternative splicing of the *MAPT* gene. The in- and exclusion of exon 10 results in 3 or 4 microtubule binding domains. Under normal circumstances, tau is bound to and stabilizes microtubules, which are involved in intraneuronal vesicle and organelle transport. Typically in PSP, brain immunohistochemistry is tau-positive, which is also the case for a large subset of FTD patients, whereas in Parkinson's disease and MSA alpha-synuclein is the culprit.

With this in mind, PSP can be considered part of the spectrum of frontotemporal lobar degenerations. The first part of this thesis focuses on clinical and imaging similarities and differences within this spectrum, and more specifically between PSP and FTD. In **chapter 2.1**, we investigated the survival and clinical predictors thereof in two large cohorts of PSP and FTD patients. Both are rapidly progressive disorders, but data on survival rates within this FTLD spectrum were scarce, mostly retrospective or small in sample size. We found a shorter mean disease duration in PSP (7.2 years) than in FTD as a whole (9.2 years), and this difference was even more pronounced between PSP and FTD with known tau pathology (pathologically proven cases with tau pathology and cases with *MAPT* mutations). These findings are in agreement with a very recent meta-analysis of survival in FTD.[6] Also, PSP subgroups differed in survival with a mean survival of 6.8 years in Richardson's syndrome and 10.9 years in PSP-Parkinsonism. Further evidence for the heterogeneity in survival rates of tauopathies is demonstrated by the large range in survival times of the different *MAPT* mutations and a trend towards shorter survival in FTD cases with 4-repeat versus 3-repeat tau pathology, similar to previous observations.[7] The question is whether the heterogeneous survival rates in tauopathies is due

to the extent of involvement of brain regions or the different types of tau aggregation or perhaps both.

Genetic factors may play a more significant role in survival in FTD than in PSP as a positive family history in only FTD had a negative effect on survival. On the other hand, the effect of gender on survival in PSP was not observed in FTD. An epidemiological study on the natural course of disease within the FTLD spectrum identified cardiovascular failure and cancer as comorbid factors influencing prognosis.[8] Differences in comorbidity between genders at later disease stages may have an effect on survival, but insufficient data was available to explore this more thoroughly in the current study. These findings may help clinicians anticipate disease progression of different phenotypes which is relevant for patient counseling.

Executive dysfunction is the most common cognitive impairment in PSP, whereas the behavioural changes are characterized by apathy which is paradoxically often accompanied by impulsivity. These symptoms are thought to be associated with frontal lobe dysfunction, but may also be caused by disruption of striatofrontal connections.[9] Correlation studies between imaging measures and cognition in PSP, however, are scarce. The few studies that have been published lacked quantitative imaging measures, extensive neuropsychological assessment or detailed compartmentalization of cortical areas. In **chapter 2.2** we sought to overcome these limitations by correlating brain perfusion measures assessed by statistical parametric mapping with neuropsychological test scores of PSP patients. Although executive dysfunction is assumed to be predominantly affected in PSP, our results showed a more global cognitive deterioration, including deficits in other domains such as memory, language and attention. Furthermore, we demonstrated these deficits to occur early in the disease course, as we ascertained PSP patients just over 3 years after onset, and at the time of ascertainment 19 out of 21 patients had a mean PSPRS score < 30 reflecting an early and mild disease stage. A recent review of cognition in PSP underlined this early occurrence of a wide range of cognitive and behavioural deficits.[5] In terms of imaging we found hypoperfusion in frontal regions, the cerebellum, basal ganglia, midbrain and cingulate cortex, but when applying a more stringent analysis only the cingulate cortex yielded significant hypoperfusion, mainly in the posterior part of the midcingulate cortex (MCC). Our observation that the extent of hypoperfusion in the cingulate cortex in PSP (classically thought of as a subcortical disorder) did not differ from that in FTD-tau (a cortical disorder), except for the subgenual part of the anterior cingulate cortex, emphasizes the remarkable degree of cingulate involvement in PSP. Regression analyses restricted to the cingulate cortex found hypoperfusion in the posterior part of the MCC to be correlated with the extent of executive dysfunction in PSP, whereas the anterior part of the MCC correlated with this cognitive domain in FTD-tau. This supports the differential vulnerability of MCC

subregions to neurodegeneration and resulting cognitive decline.[10] Loss of empathy and impaired social cognition are more and more recognized to be part of the cognitive syndrome in PSP, similar to behavioural variant FTD.[11] Such data are lacking so far, as social cognition tests, such as emotion recognition using Ekman faces and theory of mind tests were not included in our neuropsychological test batteries at the time. It would have been interesting to assess whether cingulate cortex perfusion measures also correlate with social cognition, as appears to be the case in schizophrenia.[12]

An intriguing question is whether the midcingulate hypoperfusion is accompanied by alterations in the densities of specific neurotransmitter receptors. The majority of neurotransmitter studies in PSP have focused on subcortical sites and found decreased D2 receptor binding densities and reductions of muscarinic and nicotinic receptors in the striatum. In spite of these findings, dopamine and cholinergic replacement therapies in PSP have not proven effective. Consequently, other neurotransmitter systems may be implicated. In **chapter 3** we investigated this by quantifying the densities of twenty different receptors from seven neurotransmitter systems by *in vitro* receptor autoradiography on unfixed brain tissue from the MCC and caudate nucleus of 16 PSP patients and 14 age- and gender-matched controls. GABAergic, glutamatergic and serotonergic receptors in PSP were altered predominantly in MCC, which is in sharp contrast to the preferential impairment of nicotinic, cholinergic and adenosine receptors in caudate nucleus, demonstrating the involvement of multiple non-dopaminergic neurotransmitter systems in the pathophysiology of PSP. Our findings suggest that the adrenoceptor system does not contribute significantly to the symptomatology in PSP. Perhaps surprisingly, alterations of the D2 receptors did not reach significance in either brain region, suggesting preservation of D2 receptor bearing neurons. This contrasts several previous *in vivo* and *in vitro* studies, but not all.[13] The discrepancy may be explained by the heterogeneity of PSP, differences in post-mortem delay times, ligands used, or the rostro-caudal anatomical, neurochemical and functional differences which characterize the caudate nucleus.[14] Furthermore, *in vivo* estimates of D2 binding may be confounded by the continued presence of endogenous dopamine and lack of pathological confirmation.[13] Moreover, although the distribution of tau severity between frontal and non-frontal PSP patients did not differ significantly, (unpublished data Chiu WZ) we showed that frontal and non-frontal presentation PSP patients can be differentiated post-mortem with a high degree of accuracy based on differences in both MCC and caudate nucleus receptor densities. This is interesting, as classical PSP symptoms develop during the course of the illness,[2,5] and it was unclear whether these PSP patients with frontal presentation at late stages could be post-mortem differentiated from typical PSP. The present results show that PSP with frontal presentation is not only a clinical but also a neurochemical distinctive entity. This differentiation was not due to a specific neurotransmitter, as no

post hoc test was significant, but rather to the sum of differences in receptors or "receptor fingerprints" between the subgroups. These results demonstrate that cognitive dysfunction is an early core feature of not only PSP with frontal presentation but also of typical PSP, which should be recognized in the upcoming revision of clinical criteria. Moreover, the correlation between executive dysfunction and MCC hypoperfusion, and the significant alterations of neurotransmitter receptors in this brain region provide further evidence that the MCC may prove to be a key region in PSP cognition, and warrants further investigations.

Chapter 4 consists of studies on PSP-like disorders. In **chapter 4.1** we identified a novel gene defect in a three generation-large family with 12 affected members presenting with dementia and/or extrapyramidal symptoms. The genetic analysis was performed in 2 steps. First, we combined regions from the linkage analysis with filtered exome sequencing data. Second, we combined proteomics data with the candidate variants found by genetic analysis to identify the causative gene, which resulted in one single variant. This variant in the gene coding for the type I-β regulatory subunit of protein kinase A (*PRKAR1B*) is located on chromosome 7p22, and is predicted to be pathogenic. Neuropathological examination displayed negative immunohistochemistry for FUS, α-synuclein, AT-8, amyloid-β, neuroserpin, and TDP, which distinguishes this disorder from more common neurodegenerative disorders. However, strong staining with p62 and α-internexin and to a lesser extent with neurofilament antibodies of abundant cytoplasmic neuronal inclusions was observed throughout the brain. The phenotype however is highly unspecific, even in late stage cases. Where most neurodegenerative diseases exhibit characteristic symptoms in the course of the illness, such as impaired eye movements in PSP, or hallucinations in LBD, the phenotype in our cases remained uncharacteristic during life. So, although this novel familial neurodegenerative disorder distinguishes itself from other neurodegenerative diseases by unique neuropathological findings, the clinical phenotype is characterized by being uncharacteristic. Mutations in the regulatory subunits of protein kinase A (PKA) have been shown to alter PKA function. In the present study, we predict the mutation to alter or even prevent dimerization and thus affecting activation of the kinase, as it is located on the interface between the docking/dimerization domains of the PKA regulatory subunit β. These findings demonstrate the effectiveness of combining not only linkage analysis with whole exome sequencing, but also genetic analyses with a proteomic approach to identify a genetic mutation and link altered regulation of PKA by mutant PRKAR1B to human late-onset neurodegeneration.

A most intriguing question is whether and if so which neuropathological changes develop before clinical symptoms manifest. Investigating genetic dementia makes

it possible to identify the very earliest features by studying presymptomatic subjects who are at risk of developing dementia. Neuroimaging abnormalities can be observed in mutation carriers prior to the onset of symptoms in different types of dementia, but no neuropathological reports of presymptomatic *MAPT* mutation carriers have been published to date to our knowledge. **Chapter 4.2** provides the first account of neuropathologic changes in a presymptomatic *MAPT* L315R carrier. The tau-immunoreactivity most likely represents the very earliest stage of *MAPT* mutation pathology, as its distribution is not consistent with early Braak stages of Alzheimer's disease. The confirmation of abnormal tau inclusions in our presymptomatic mutation carrier is of importance in light of the recent development of tau PET tracers, highlighting their potential utility as a presymptomatic biomarker. Severe and selective loss of these neurons has been demonstrated even in early-stage FTD.[15,16] This was not the case in our presymptomatic subject however. The remarkable discrepancy between the dominant 3R-tau pathology on immunoblot, and the immunostaining of inclusions with RD4 only, with RD3 showing mere diffuse background staining. One wonders whether this reflects a stage where the insoluble 3R-tau has not yet formed inclusions. This case gives valuable insight into the earliest neuropathological abnormalities of this tauopathy, and underlines the potential of tau PET imaging as a biomarker for presymptomatic mutation carriers.



## FUTURE DIRECTIONS

### Biomarkers

Pathological examination is still the gold standard for the diagnosis of definite PSP. Clinical diagnostic criteria for PSP are available, and have proven to be highly specific for predicting PSP pathology, but lack sensitivity especially in the first few years of the disease.[17] It is highly important to optimize diagnostic sensitivity in the initial stages of the disease, as patients included in clinical trials based on current clinical criteria are often already too advanced for any neuroprotective effect to be ascertained. A revision of clinical criteria by a consortium on PSP is underway and results are anticipated in the course of 2017.

Moreover, validated biomarkers contributing to establish the diagnosis PSP in the early stage are urgently needed. Magnetic resonance imaging in PSP may show certain patterns of atrophy, nigral hyperintensity, and diffusion tensor abnormalities but overlap with related disorders exists. In PD, high uric acid blood levels, an oxidative stress marker, is correlated with a decreased risk of PD, and a reduced rate of disease progression. The utility of serum uric acid level in PSP is so far uncertain as results are conflicting.[18,19] Recent advances in the field of biomarkers may prove more useful in upcoming years. In vivo positron emission tomography (PET) imaging with tau ligands may help

differentiate PSP and related disorders at an early stage by revealing specific patterns of aggregated tau. Various tau-ligands are currently being investigated, but results are still preliminary.[20,21]

Cerebrospinal fluid (CSF) may possibly be a reliable biomarker source, due to its direct contact with the central nervous system (CNS). Therefore, proteins that may represent disease pathology are likely to disperse into the CSF. A decrease in four-repeat microtubule binding domain (4R)-tau isoform was found in PSP and Alzheimer's disease (AD) and may represent a potential candidate marker.[22] A novel CSF tau ELISA designed to measure tau fragments detected lower N-terminal and C-terminal tau concentrations in PSP than in healthy controls and AD patients.[23] Neurofilament light chain (NFL) is another promising candidate, as CSF NFL concentrations in PSP, but also multisystem atrophy (MSA), were significantly higher than in Parkinson's disease (PD).[24] Interestingly, plasma NFL was also found to be elevated in PSP patients.[25] A proof of concept study showed a distinct lysosomal network protein profile in PSP CSF, with decreased early endosomal antigen 1 and increased lysozyme.[26] These markers show promise, but larger and longitudinal series are required to clarify their use.

## Pathophysiology

In 2011, a large multicenter genome-wide association study (GWAS) in PSP confirmed the role of H1 *MAPT* haplotype affecting risk for PSP, and in addition identified several risk loci: *STX6*, *EIF2AK3* and *MOBP* on chromosomes 1, 2 and 3 respectively.[27] A polymorphism in *STX6* and tau pathology may be associated as it is a strong expression quantitative trait locus, lowering expression of syntaxin 6 in white matter. Syntaxin 6 is believed to affect intracellular membrane trafficking, which may lead to abnormal tau aggregation, but this alluring hypothesis needs further investigation.[28] Equally intriguing is the recent link between a single nucleotide polymorphism (SNP) near the *MOPB* gene, which influences the expression of appoptosin, which in turn induces caspase-3-mediated tau cleavage, promotes tau aggregation, synaptic dysfunction and induces PSP-like dysfunction in transgenic mice.[29] Fascinatingly, caspase-3 inhibitors abolished the effects of appoptosin.

Until recently no risk factors for PSP other than age have been identified. However, a recent case-control study found evidence for a possible role of environmental factors in the etiology of PSP, particularly drinking well water and living in or near agricultural regions. The hypothesis being that these factors are related to exposure to mitochondrial function inhibiting chemicals, such as pesticides.[30] Yet exposure to pesticides in itself could not be linked with PSP, perhaps due to limited statistical power. These findings are supported however by a recent report of a cluster of PSP patients in Northern France in a geographical area with severe environmental contamination by industrial metals.[31] Environmental factors may also increase disease risk by epigenetic modification, as up-

regulation of specific microRNAs was found in post-mortem forebrains of PSP patients, and appeared to downregulate specific target genes that play significant roles in several cellular processes.[32]

Although there is consensus on the importance of tau in the pathophysiology of PSP, there is still much debate about what mechanisms underlie tau transmission. A theory has emerged that suggests that tau and other proteins can behave like a prion, that is, propagating the protein to other cells.[33] Studies have *in vitro* (cellular models) and *in vivo* (inoculating transgenic mice) supported this prion-like hypothesis,[34] though the term prion should be used with caution, as transmission between individuals has not been proven.

These exciting studies provide insight into the pathophysiology of tauopathies and may lead to new therapeutic advances in the near future.



**Treatment approaches**

Currently, no effective treatment is available for PSP. Neurotransmitter replacement strategies have yielded disappointing results in previous decades. More recent treatment strategies have focused on tau dysfunction, and particularly at preventing aggregation and/or phosphorylation of tau, decreasing tau levels, and microtubule stabilization.[35,36] So far with negative results, perhaps due to the aforementioned enrollment of relatively advanced patients, emphasizing the urgent need for early diagnosis. Very recently, rho-associated protein kinases (ROCK1 and ROCK2) were recognized as potential novel drug targets for PSP, as increased insoluble tau levels were associated with elevated ROCK1 and ROCK2 in PSP brains and pharmacologic inhibition of ROCKs lowered tau levels in cellular models.[37] Another promising therapeutic approach is immunization with anti-tau antibodies, as mouse models have shown hopeful results.[38] Indeed, several phase I trials with such antibodies have been initiated. It is clear that treatment approaches targeting tau are under further development, and time will tell whether these tau-focused strategies will prove effective in PSP.

Beyond this, results of a pilot phase-I study on autologous bone marrow mesenchymal stromal cell (MSC) infusion into cerebral arteries of PSP patients are encouraging, exploiting the regenerative properties of these cells. The authors demonstrated feasibility in 5 PSP patients, and reported clinical stabilization for at least 6 months, as assessed by commonly used rating scales, paving the way for a phase-II study.[39]

As new insights into the pathophysiology of PSP emerge, such as the prion-like and appoptosin mechanisms, new drug targets will be developed, increasing the likelihood of finding an effective treatment for this devastating disease.

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# 6

# SUMMARY / SAMENVATTING

## SUMMARY

Progressive supranuclear palsy (PSP) is a neurodegenerative disorder characterized by early postural instability, supranuclear gaze palsy and parkinsonism. Classically, PSP has been designated a form of atypical parkinsonism, emphasizing the motor disturbances that characterizes this disease. It is now clear however, that many PSP cases will develop prominent behavioural and cognitive symptoms. In a subset of PSP patients these non-motor symptoms may be the predominant presenting manifestation, resembling the clinical presentation of the behavioural variant of frontotemporal dementia (FTD). Overlap between PSP and FTD is further emphasized by tau-positive inclusions in the brain, which characterize PSP, but are also found in a large subset of FTD patients. For these reasons PSP can be considered part of the clinicopathological spectrum designated with the term frontotemporal lobar degeneration (FTLD).

The aim of this thesis was to assess clinical, neuropathological and neurochemical aspects within this spectrum, but focused mainly on PSP.

After a general introduction to the thesis in **chapter 1.1**, **chapter 1.2** provides a general overview of PSP, including clinical, genetic and pathological aspects. **Chapter 2** covers novel clinical aspects of PSP. In **chapter 2.1** we investigated the survival and clinical predictors thereof in two large cohorts of PSP and FTD patients. We found a shorter mean disease duration in PSP than in FTD as a whole, and this difference was even more pronounced between PSP and FTD with known tau pathology (FTD-tau). Also, PSP subgroups differed in survival with a mean survival of 6.8 years in Richardson's syndrome and 10.9 years in PSP-Parkinsonism. In PSP male gender, older onset-age and higher PSP Rating Scale score are independent predictors for shorter survival, whereas in FTD a positive family history and an older onset-age are associated with a poor prognosis. These findings may help clinicians anticipate disease progression of different phenotypes which is relevant for patient counseling.

In **chapter 2.2** we aimed to elucidate the functional role of affected brain regions in cognition in PSP by correlating brain perfusion measures with neuropsychological test scores. Although executive dysfunction is assumed to be predominantly affected in PSP, our results showed a more global cognitive deterioration. Moreover, we found a remarkable degree of hypoperfusion in the cingulate cortex in PSP, as the extent of hypoperfusion in the cingulate cortex in PSP (classically thought of as a subcortical disorder) did not differ from that in FTD-tau (a cortical disorder), except for the subgenual part of the anterior cingulate cortex. The extent of hypoperfusion in the midcingulate cortex (MCC) was correlated to executive dysfunction in both disorders.

An intriguing question is whether the midcingulate hypoperfusion is accompanied by alterations in the densities of specific neurotransmitter receptors. In **chapter 3** we investigated this by quantifying the densities of twenty different receptors from seven

neurotransmitter systems by *in vitro* receptor autoradiography on unfixed brain tissue from the MCC and caudate nucleus of 16 PSP patients and 14 controls. GABAergic, glutamatergic and serotonergic receptors in PSP were altered predominantly in MCC, which is in sharp contrast to the preferential impairment of nicotinic, cholinergic and adenosine receptors in caudate nucleus, demonstrating the involvement of multiple non-dopaminergic neurotransmitter systems in the pathophysiology of PSP. Moreover, we showed that frontal and non-frontal presentation PSP patients can be differentiated post-mortem with a high degree of accuracy based on differences in "receptor fingerprints" in both MCC and caudate nucleus, suggesting that PSP with frontal presentation is not only a clinical but also a neurochemical distinctive entity.

The correlation between executive dysfunction and MCC hypoperfusion and the significant alterations of neurotransmitter receptors in this brain region suggest that the MCC may prove to be a key region in PSP cognition. Further investigations are warranted.

**Chapter 4** comprises studies of PSP-like disorders. In **chapter 4.1** we studied a three generation-large family with 12 affected members presenting with dementia and/or extrapyramidal symptoms. Through the combination of linkage analysis regions with filtered exome sequencing data as well as proteomics data, we found one single variant. This variant in the gene coding for the type I-β regulatory subunit of protein kinase A (*PRKAR1B*) is located on the interface between the docking/dimerization domains of the PKA regulatory subunit β and is predicted to alter or even prevent dimerization and thus affecting activation of the kinase. Analysis of the *PRKAR1B* coding region in cohorts of autosomal dominant Parkinson's disease and familial FTD with unknown gene defect did not reveal any potential pathogenic rare variants that co-segregate with disease. The neuropathological phenotype is also unique, with abundant α-internexin-positive, but FUS-negative neuronal inclusions. The clinical phenotype however is characterized by being unspecific, even in the late stage. Where most neurodegenerative diseases exhibit characteristic symptoms in the course of the illness, such as impaired eye movements in PSP, or hallucinations in LBD, the phenotype in our cases remained uncharacteristic during life. These findings link altered regulation of PKA by mutant PRKAR1B to human late-onset neurodegeneration.

**Chapter 4.2** provides the first account of neuropathological changes in a presymptomatic *MAPT* L315R carrier, that died at age 68. Neurofibrillary tangle distribution was consistent with Braak stage III. However, the presence of tau-positive granule cells in dentate gyrus, tau-positive glial cells in white matter and diffuse neuronal immunoreactivity in temporal cortex were not consistent with early Braak stages of Alzheimer's disease. Rather, the tau-immunoreactivity most likely represents the very earliest stage of *MAPT* mutation pathology. The confirmation of abnormal tau inclusions in our presymptomatic mutation carrier is of importance in light of the recent development

of tau PET tracers, highlighting their potential utility as a presymptomatic biomarker. Furthermore, we assessed von Economo neuron (VEN) density, as these specific neurons are found exclusively in the cingulate cortex and frontal insula, suggesting a relation with social cognition. These neurons appear to be selectively targeted in early-stage FTD. This was not the case in our presymptomatic subject however. These findings are a valuable addition to neuropathological data within the FTLD spectrum.

Finally, in **chapter 5** the main findings of this thesis in light of the current knowledge about the disease are discussed and recommendations for future research are made.

## SAMENVATTING

Progressieve supranucleaire verlamming (PSP) is een neurodegeneratieve ziekte die gekenmerkt wordt door vroege houdingsinstabiliteit, supranucleaire blikverlamming en parkinsonisme. PSP werd altijd gezien als een vorm van atypisch parkinsonisme, wat de nadruk legt op de motorische verschijnselen van deze aandoening. Het is echter inmiddels duidelijk dat veel PSP patiënten prominente gedragsmatige en cognitieve symptomen ontwikkelen. In een deel van PSP patiënten zijn deze niet-motorische verschijnselen de belangrijkste manifestatie van de ziekte bij presentatie, gelijkend op de klinische presentatie van de gedragsmatige variant van frontotemporale dementie (FTD). De overlap tussen PSP en FTD wordt nog verder benadrukt door tau-positieve inclusies in de hersenen, die kenmerkend zijn voor PSP, maar die ook in veel hersenen van FTD patiënten worden gevonden. Om deze redenen kan PSP beschouwd worden als onderdeel van het klinisch-pathologische spectrum dat frontotemporale lobaire degeneratie (FTLD) genoemd wordt.



Het doel van dit proefschrift is het onderzoeken van klinische, neuropathologische en neurochemische aspecten binnen dit spectrum, met een focus op PSP.

Na een algemene inleiding van het proefschrift in **hoofdstuk 1.1**, verschaft **hoofdstuk 1.2** een algemeen overzicht over de klinische, genetische en pathologische aspecten van PSP. **Hoofdstuk 2** omvat nieuwe klinische aspecten van PSP. In **hoofdstuk 2.1** onderzoeken we de overleving en klinische voorspellers in 2 grote PSP en FTD patiënten cohorten. We vonden een kortere overleving bij PSP dan bij FTD patiënten, en dit verschil was nog meer uitgesproken tussen PSP en FTD-tau. Daarnaast verschillen PSP subgroepen in overleving, waarbij patiënten met het Richardson's syndroom een gemiddelde overleving van 6.8 jaar hebben en PSP-parkinsonisme patiënten van 10.9 jaar. Het mannelijk geslacht, oudere beginleeftijd en een hogere score op de PSP Rating Scale zijn onafhankelijk geassocieerd met een kortere overleving in PSP, terwijl in FTD een positieve familieanamnese en oudere beginleeftijd geassocieerd zijn met een slechtere prognose. Deze bevindingen kunnen clinici helpen de ziekteprogressie van verschillende fenotypen in te schatten, wat relevant is voor de voorlichting van patiënten.

In **hoofdstuk 2.2** trachten wij meer duidelijkheid te verkrijgen over de functionele rol van aangedane hersenregio's bij de cognitie van PSP door middel van het correleren van hersenperfusie metingen met neuropsychologische testscores. Hoewel algemeen wordt aangenomen dat vooral executieve dysfunctie is aangedaan bij PSP patiënten, tonen onze resultaten een meer globale cognitieve achteruitgang aan. Bovendien vonden wij een opmerkelijke mate van hypoperfusie in de cingulaire cortex in PSP, omdat de mate van hypoperfusie in de cingulaire cortex in PSP (wat van oudsher wordt gezien als een subcorticale aandoening) niet verschilt met die van FTD-tau (een corticale aandoening),

op het subgenuale deel na van de anterieure cingulaire cortex. De mate van hypoperfusie in de midcingulaire cortex (MCC) was gecorreleerd met executieve dysfunctie in beide aandoeningen.

Een intrigerende vraag is of de midcingulaire hypoperfusie wordt vergezeld door veranderingen in de dichtheden van specifieke neurotransmitter receptoren. In **hoofdstuk 3** onderzoeken we dit door dichtheden van twintig verschillende receptoren van zeven neurotransmitter systemen te kwantificeren door middel van *in vitro* receptor autoradiografie op ongefixeerd hersenweefsel van de MCC en nucleus caudatus van 16 PSP patiënten en 14 controles. In de MCC bij PSP waren vooral GABAerge, glutamaterge en serotonerge receptoren gewijzigd, wat in scherp contrast staat met de veranderingen van vooral nicotinerge, cholinerge en adenosine receptoren in de nucleus caudatus. Dit toont de betrokkenheid aan van meerdere non-dopaminerge neurotransmitter systemen in de pathofysiologie van PSP. Daarnaast hebben we aangetoond dat PSP patiënten met een frontale presentatie en zonder een frontale presentatie postmortaal accuraat kunnen worden onderscheiden op basis van hun "receptor vingerafdrukken" van zowel de MCC als de nucleus caudatus, wat erop wijst dat PSP met een frontale presentatie niet alleen een klinische maar ook een neurochemische entiteit is.

De correlatie tussen executieve dysfunctie en hypoperfusie in de MCC en de significante veranderingen van neurotransmitter receptoren in deze regio suggereren dat de MCC een sleutelrol zou kunnen spelen bij de cognitie van PSP. Meer onderzoek hiernaar is gerechtvaardigd.

**Hoofdstuk 4** omvat onderzoek naar op PSP-gelijkende aandoeningen. In **hoofdstuk 4.1** beschrijven we drie generaties van een familie met 12 aangedane familieleden die zich presenteren met dementie en/of extrapiramidale verschijnselen. Door resultaten van koppelingsonderzoek, gehele exoom analyse en proteomics te combineren werd een enkele variant gevonden. Deze variant in het gen dat codeert voor de type I-β regulerende subeenheid van eiwitkinase A (*PRKAR1B*) ligt op het koppelingsgebied van de dimerisatie en docking domeinen van de subeenheid en wordt verondersteld de dimerisatie te veranderen of zelfs te verhinderen en zodoende de activatie van het kinase aan te tasten. Analyse van het *PRKAR1B* coderende gebied in autosomaal dominante ziekte van Parkinson en familiaire FTD met onbekend gen defect patiënten cohorten liet geen zeldzame, potentieel pathogene varianten zien die co-segregeren met de ziekte. Het neuropathologische fenotype is ook uniek, met veel α-internexin-positieve, maar FUS-negatieve neuronale inclusies. Het klinisch fenotype is opvallend omdat het ook in het late stadium heel aspecifiek is. De meeste neurodegeneratieve ziekten laten gedurende het beloop van de ziekte kenmerkende tekenen zien, zoals gestoorde oogbewegingen bij PSP, of hallucinaties bij LBD. Het fenotype van onze patiënten bleef zonder karakteristieke symptomen gedurende het leven. Deze bevindingen verbinden veranderde regulatie van eiwitkinase A door mutant PRKAR1B met late-onset neurodegeneratie.

**Hoofdstuk 4.2** verschaft de eerste beschrijving van neuropathologische veranderingen in een presymptomatische *MAPT* L315R draagster, die op 68-jarige leeftijd is overleden. De distributie van neurofibrillaire knopen is consistent met een Braak stage III. Echter, zijn de aanwezigheid van tau-positieve korrelcellen in de gyrus dentatus, tau-positieve gliale cellen in de witte stof en diffuse neuronale immunoreactiviteit in de temporale schors niet compatibel met vroege Braak stadia bij de ziekte van Alzheimer. De tau-immunoreactiviteit geeft waarschijnlijk het allereerste stadium van pathologie als gevolg van de *MAPT* mutatie weer. De bevestiging van abnormale tau deposities in onze presymptomatische mutatie draagster is van belang in het kader van de recente ontwikkeling van tau PET tracers. Ons onderzoek benadrukt het potentieel hiervan als presymptomatische biomarker. Daarnaast hebben wij de dichtheid van von Economo neuronen (VEN) onderzocht, omdat deze specifieke neuronen alleen voorkomen in de cingulaire cortex en frontale insula, wat een relatie met sociale cognitie suggereert. Deze neuronen lijken selectief aangedaan te zijn in vroeg stadium FTD. Dit was niet het geval in onze presymptomatische casus. Deze bevindingen zijn een waardevolle aanvulling op neuropathologische data binnen het FTLD spectrum.

Tot slot worden in **hoofdstuk 5** de belangrijkste bevindingen van dit proefschrift besproken in het licht van de huidige kennis over de ziekte en worden suggesties gedaan voor toekomstig onderzoek.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 3R | Tau isoforms with three repeat microtubule binding sites |
| 4R | Tau isoforms with four repeat microtubule binding sites |
| AD | Alzheimer's disease |
| AKAP | A-kinase anchoring protein |
| CB | Coiled bodies |
| CBD | Corticobasal degeneration |
| CBS | Corticobasal syndrome |
| CI | Cytoplasmic inclusions |
| CSF | Cerebrospinal fluid |
| D/D | Dimerization/docking |
| FAB | Frontal assessment battery |
| FTD | Frontotemporal dementia |
| FTD-MND | Frontotemporal dementia with motor neuron disease |
| FTLD | Frontotemporal lobar degeneration |
| FTDP-17 | Frontotemporal dementia with parkinsonism linked to chromosome 17 |
| FUS | Fused in sarcoma |
| *GRN* | *Progranulin* |
| H&Y | Hoehn and Yahr |
| LBD | Dementia with Lewy bodies |
| *LRRK2* | *Leucine-rich repeat kinase 2* |
| *MAPT* | *Microtubule associated protein tau* |
| MCC | Midcingulate cortex |
| MMSE | Mini-Mental state examination |
| MSA | Multiple system atrophy |
| NFT | Neurofibrillary tangles |
| NIFID | Neuronal intermediate filament inclusion disease |
| NII | Neuronal intranuclear inclusions |
| NINDS-SPSP | National Institute for Neurological Diseases and Stroke and Society for PSP |
| NT | Neuropil threads |
| PD | Parkinson's disease |
| PET | Positron emission tomography |
| PGAF | Pure akinesia with gait freezing |
| PKA | Protein kinase A |
| PNFA | Progressive nonfluent aphasia |
| PSP | Progressive supranuclear palsy |
| PSP-P | Progressive supranuclear palsy -parkinsonism |

| | |
|---|---|
| PSPRS | Progressive supranuclear palsy rating scale |
| SNP | Single nucleotide polymorphism |
| SPECT | Single-photon emission computed tomography |
| TA | Tufted astrocytes |
| TARDBP | Transactive response DNA-binding protein (previously known as TDP-43) |
| UPDRS | Unified Parkinson disease rating scale |

# Appendices

**PSPRS**
**FAB**

## PROGRESSIVE SUPRANUCLEAR PALSY RATING SCALE

(Golbe *et al*. Brain 2007 Jun;130(Pt 6):1552–65)

### I. HISTORY (from patient or other informant)

**1. Withdrawal** (relative to baseline personality) **0 1 2**

0 None
1 Follows conversation in a group, may respond spontaneously, but rarely if ever initiates exchanges.
2 Rarely or never follows conversation in a group.

**2. Aggressiveness** (relative to baseline personality) **0 1 2**

0 No increase in aggressiveness
1 Increased, but not interfering with family interactions
2 Interfering with family interactions

**3. Dysphagia for solids** **0 1 2 3 4**

0 Normal; no difficulty with full range of food textures
1 Tough foods must be cut up into small pieces
2 Requires soft solid diet
3 Requires pureed or liquid diet
4 Tube feeding required for some or all feeding

**4. Using knife and fork, buttoning clothes, washing hands and face** (rate the worst) **0 1 2 3 4**

0 Normal
1 Somewhat slow but no help required
2 Extremely slow; or occasional help needed
3 Considerable help needed but can do some things alone
4 Requires total assistance

**5. Falls** (average frequency if patient attempted to walk unaided) **0 1 2 3 4**

0 None in the past year
1 < 1 per month; gait may otherwise be normal
2 1–4 per month
3 5–30 per month
4 > 30 per month (or chairbound)

**6. Urinary incontinence** **0 1 2 3 4**

0 None or a few drops less than daily
1 A few drops staining clothes daily
2 Large amounts, but only when asleep; no pad required during day
3 Occasional large amounts in daytime; pad required
4 Consistent, requiring diaper or catheter awake and asleep

**7. Sleep difficulty** **0 1 2 3 4**

0 Neither 1° nor 2° insomnia (i.e., falls asleep easily and stays asleep)
1 Either 1° or 2° insomnia; averages > 5 hours sleep nightly
2 Both 1° and 2° insomnia; averages > 5 hours sleep nightly
3 Either 1° or 2° insomnia; averages < 5 hours sleep nightly
4 Both 1° and 2° insomnia; averages < 5 hours sleep nightly

## II. MENTAL EXAM

Items 8–11 use this scale

0 Clearly absent
1 Equivocal or minimal
2 Clearly present, but not interfering with activities of daily living (ADL)
3 Interfering mildly with ADL
4 Interfering markedly with ADL

**8. Disorientation** **0 1 2 3 4**

**9. Bradyphrenia** **0 1 2 3 4**

**10. Emotional incontinence** **0 1 2 3 4**

**11. Grasping/imitative/utilizing behaviour** **0 1 2 3 4**

## III. BULBAR EXAM

**12. Dysarthria** (ignoring palilalia) **0 1 2 3 4**

0 None
1 Minimal; all or nearly all words easily comprehensible (to examiner, not family)
2 Definite, moderate; most words comprehensible

3 Severe; may be fluent but most words incomprehensible
4 Mute; or a few poorly comprehensible words

**13. Dysphagia** **0 1 2 3 4**

(for 30–50 cc of water from a cup, if safe)

0 None
1 Fluid pools in mouth or pharynx, or swallows slowly, but no choking/coughing
2 Occasionally coughs to clear fluid; no frank aspiration
3 Frequently coughs to clear fluid; may aspirate slightly; may expectorate frequently rather than swallow secretions
4 Requires artificial measures (oral suctioning, tracheostomy or feeding gastrostomy) to avoid aspiration

## IV. SUPRANUCLEAR OCULAR MOTOR EXAM

Items 14–16 use this scale. Rate by inspection of saccades on command from the primary position of gaze to a stationary target.

0 Not slow or hypometric; 86–100% of normal amplitude
1 Slow or hypometric; 86–100% of normal amplitude
2 51–85% of normal amplitude
3 16–50% of normal amplitude
4 15% of normal amplitude or worse

**14. Voluntary upward saccades** **0 1 2 3 4**

**15. Voluntary downward saccades** **0 1 2 3 4**

**16. Voluntary left and right saccades** **0 1 2 3 4**

**17. Eyelid dysfunction** **0 1 2 3 4**

0 None
1 Blink rate decreased (< 15/minute) but no other abnormalities
2 Mild inhibition of opening or closing or mild blepharospasm; no visual disability
3 Moderate lid-opening inhibition or blepharospasm causing partial visual disability
4 Functional blindness or near-blindness because of involuntary eyelid closure

## V. LIMB EXAM

**18. Limb rigidity** (rate the worst of the four) **0 1 2 3 4**

0 Absent
1 Slight or detectable only on activation
2 Definitely abnormal, but full range of motion possible
3 Only partial range of motion possible
4 Little or no passive motion possible

**19. Limb dystonia** **0 1 2 3 4**

(rate worst of the four; ignore neck and face)

0 Absent
1 Subtle or present only when activated by other movement
2 Obvious but not continuous
3 Continuous but not disabling
4 Continuous and disabling

**20. Finger tapping** (if asymmetric, rate worse side) **0 1 2**

0 Normal (> 14 taps/5 sec with maximal amplitude)
1 Impaired (6–14 taps/5 sec or moderate loss of amplitude
2 Barely able to perform (0–5 taps/5 sec or severe loss of amplitude)

**21. Toe tapping** (if asymmetric, rate worse side) **0 1 2**

0 Normal (> 14 taps/5 sec with maximal amplitude)
1 Impaired (6–14 taps/5 sec or moderate loss of amplitude
2 Barely able to perform (0–5 taps/5 sec or severe loss of amplitude)

**22. Apraxia of hand movement** **0 1 2**

0 Absent
1 Present, not impairing most functions
2 Impairing most functions

**23. Tremor in any part** **0 1 2**

0 Absent
1 Present, not impairing most functions
2 Impairing most functions

## VI. GAIT/MIDLINE EXAM

**24. Neck rigidity or dystonia** **0 1 2 3 4**

0 Absent
1 Slight or detectable only when activated by other movement
2 Definitely abnormal, but full range of motion possible
3 Only partial range of motion possible
4 Little or no passive motion possible

**25. Arising from chair** **0 1 2 3 4**

0 Normal
1 Slow but arises on first attempt
2 Requires more than one attempt, but arises without using hands
3 Requires use of hands
4 Unable to arise without assistance

**26. Gait** **0 1 2 3 4**

0 Normal
1 Slightly wide-based or irregular or slight pulsion on turns
2 Must walk slowly or occasionally use walls or helper to avoid falling, especially on turns
3 Must use assistance all or almost all the time
4 Unable to walk, even with walker; may be able to transfer

**27. Postural stability** (on backward pull) **0 1 2 3 4**

0 Normal (shifts neither foot or one foot)
1 Must shift each foot at least once but recovers unaided
2 Shifts feet and must be caught by examiner
3 Unable to shift feet; must be caught, but does not require assistance to stand still
4 Tends to fall without a pull; requires assistance to stand still

**28. Sitting down** **0 1 2 3 4**

(may touch seat or back but not arms of chair)

0 Normal
1 Slightly stiff or awkward
2 Easily positions self before chair, but descent into chair is uncontrolled
3 Has difficulty finding chair behind him/her and descent is uncontrolled
4 Unable to test because of severe postural instability

## FRONTAL ASSESSMENT BATTERY

Dubois *et al.* Neurology 2000. Dec 12;55(11)1621–6

**1. Similarities (conceptualization)**

"In what way are they alike?"

- A banana and an orange
  (In the event of total failure: "they are not alike" or partial failure: "both have peel," help the patient by saying: "both a banana and an orange are fruit"; but credit 0 for the item; do not help the patient for the two following items)
- A table and a chair
- A tulip, a rose and a daisy

**Score**

(only category responses [fruits, furniture, flowers] are considered correct)
Three correct: 3
Two correct: 2
One correct: 1
None correct: 0

**2. Lexical fluency (mental flexibility)**

"Say as many words as you can beginning with the letter 'S,' any words except surnames or proper nouns."
If the patient gives no response during the first 5 seconds, say: "for instance, snake." If the patient pauses 10 seconds, stimulate him by saying: "any word beginning with the letter 'S.' The time allowed is 60 seconds.

**Score**

(word repetitions or variations [shoe, shoemaker], surnames, or proper nouns are not counted as correct responses)
> 9 words: 3
6–9 words: 2
3–5 words: 1
< 3 words: 0

**3. Motor series "Luria" test (programming)**

"Look carefully at what I'm doing."
The examiner, seated in front of the patient, performs alone three times with his left hand the series of "fist–edge–palm."

"Now, with your right hand do the same series, first with me, then alone."
The examiner performs the series three times with the patient, then says to him/her: "Now, do it on your own."

**Score**
Patient performs six correct consecutive series alone: 3
Patient performs at least three correct consecutive series alone: 2
Patient fails alone, but performs three correct consecutive series with the examiner: 1
Patient cannot perform three correct consecutive series even with the examiner: 0

**4. Conflicting instructions (sensitivity to interference)**
"Tap twice when I tap once."
To ensure that the patient has understood the instruction, a series of 3 trials is run: 1–1-1.
"Tap once when I tap twice."
To ensure that the patient has understood the instruction, a series of 3 trials is run: 2–2-2.
The examiner then performs the following series: 1–1-2–1-2–2-2–1-1–2.

**Score** No errors: 3
1–2 errors: 2
> 2 errors: 1
Patient taps like the examiner at least four consecutive times: 0

**5. Go–No Go (inhibitory control)**
"Tap once when I tap once."
To ensure that the patient has understood the instruction, a series of 3 trials is run: 1–1-1.
"Do not tap when I tap twice."
To ensure that the patient has understood the instruction, a series of 3 trials is run: 2–2-2.
The examiner then performs the following series: 1–1-2–1-2–2-2–1-1–2.

**Score** No errors: 3
1–2 errors: 2
> 2 errors: 1
Patient taps like the examiner at least four consecutive times: 0

**6. Prehension behaviour (environmental autonomy)**
"Do not take my hands."
The examiner is seated in front of the patient. Place the patient's hands palm up on his knees. Without saying anything or looking at the patient, the examiner brings his own hands close to the patient's hands and touches the palms of both the patient's hands,

to see if he will spontaneously take them. If the patient takes the examiner's hands, try again after asking the patient: "Now, do not take my hands."

**Score**

Patient does not take the examiner's hands: 3
Patient hesitates and asks what he/she has to do: 2
Patient takes the hands without hesitation: 1
Patient takes the examiner's hand even after he/she has been told not to do so: 0

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## ABOUT THE AUTHOR

Wang Zheng Chiu was born on October 3rd, 1980 in Groningen, the Netherlands. He graduated from the Nienoord College in Leek in 1999 and studied Business Administration at the Rijksuniversiteit Groningen before being accepted into Medical School in 2001. He obtained his medical degree at the Erasmus University Medical Center in Rotterdam in 2008. In that year he started his PhD project on progressive supranuclear palsy under the supervision of prof. dr. J.C. van Swieten. In 2012 he started his residency at the Neurology department of the Erasmus University Medical Center in Rotterdam (head: prof. dr. P.A.E. Sillevis Smitt), while finishing his thesis. Currently he lives in Rotterdam with his girlfriend Nina. They are the proud parents of Noa.

## LIST OF PUBLICATIONS

**Chiu WZ**, Seelaar H, Kamphorst W, Seeley WW, van Swieten JC. Tau pathology in a presymptomatic *MAPT* L315R mutation carrier. *In preparation*

**Chiu WZ**, Donker Kaat L, Boon AJW, Kamphorst W, Schleicher A, Zilles K, van Swieten JC, Palomero-Gallagher N. Multireceptor fingerprints in progressive supranuclear palsy. *Alzheimers Res Ther. 2017 Apr 17;9(1):28.*

Respondek G, Stamelou M, Kurz C, Ferguson LW, Rajput A, **Chiu WZ**, van Swieten JC, Troakes C, Al Sarraj S, Gelpi E, Gaig C, Tolosa E, Oertel WH, Giese A, Roeber S, Arzberger T, Wagenpfeil S, Höglinger GU; Movement Disorder Society-endorsed PSP Study Group. The phenotypic spectrum of progressive supranuclear palsy: a retrospective multicenter study of 100 definite cases. *Mov Disord. 2014 Dec;29(14):1758–66.*

Wong TH*, **Chiu WZ***, Breedveld GJ, Li KW, Verkerk AJ, Hondius D, Hukema RK, Seelaar H, Frick P, Severijnen LA, Lammers GJ, Lebbink JH, van Duinen SG, Kamphorst W, Rozemuller AJ; Netherlands Brain Bank., Bakker EB; International Parkinsonism Genetics Network., Neumann M, Willemsen R, Bonifati V, Smit AB, van Swieten J. PRKAR1B mutation associated with a new neurodegenerative disorder with unique pathology. *Brain. 2014 May;137(Pt 5):13611–73.*

*These authors contributed equally to this work

Respondek G, Roeber S, Kretzschmar H, Troakes C, Al-Sarraj S, Gelpi E, Gaig C, **Chiu WZ**, van Swieten JC, Oertel WH, Höglinger GU. Accuracy of the National Institute for Neurological Disorders and Stroke/Society for Progressive Supranuclear Palsy and neuroprotection and natural history in Parkinson plus syndromes criteria for the diagnosis of progressive supranuclear palsy. *Mov Disord. 2013 Apr;28(4):504–9.*

Luk C, Compta Y, Magdalinou N, Martí MJ, Hondhamuni G, Zetterberg H, Blennow K, Constantinescu R, Pijnenburg Y, Mollenhauer B, Trenkwalder C, Van Swieten J, **Chiu WZ**, Borroni B, Cámara A, Cheshire P, Williams DR, Lees AJ, de Silva R. Development and assessment of sensitive immune-PCR assays for the quantification of cerebrospinal fluid three- and four-repeat tau isoforms in tauopathies. *J Neurochem. 2012 Nov;123(3):396–405.*

**Chiu WZ**, Papma JM, de Koning I, Donker Kaat L, Seelaar H, Reijs AE, Valkema R, Hasan D, Boon AJ, van Swieten JC. Midcingulate involvement in progressive supranuclear

palsy and tau-positive frontotemporal dementia. *J Neurol Neurosurg Psychiatry. 2012 Sep;83(9):910–5.*

Dopper EG, Seelaar H, **Chiu WZ**, de Koning I, van Minkelen R, Baker MC, Rozemuller AJ, Rademakers R, van Swieten JC. Symmetrical corticobasal syndrome caused by a novel C.314dup progranulin mutation. *J Mol Neurosci. 2011 Nov;45(3):354–8.*

Hoglinger GU, Melhem NM, Dickson DW, Sleiman PM, Wang LS, Klei L, Rademakers R, de Silva R, Litvan I, Riley DE, van Swieten JC, Heutink P, Wszolek ZK, Uitti RJ, Vandrovcova J, Hurtig HI, Gross RG, Maetzler W, Goldwurm S, Tolosa E, Borroni B, Pastor P; PSP Genetics Study Group, Cantwell LB, Han MR, Dillman A, van der Brug MP, Gibbs JR, Cookson MR, Hernandez DG, Singleton AB, Farrer MJ, Yu CE, Golbe LI, Revesz T, Hardy J, Lees AJ, Devlin B, Hakonarson H, Muller U, Schellenberg GD. Identification of common variants influencing risk of the tauopathy progressive supranuclear palsy. *Nat Genet. 2011 Jun 19;43(7):699–705.*

Donker Kaat L, **Chiu WZ**, Boon AJ, van Swieten JC. Recent advances in progressive supranuclear palsy: a review. *Curr Alzheimer Res. 2011 May;8(3):295–302.*

**Chiu WZ**, Donker Kaat L, Seelaar H, Rosso SM, Boon AJ, Kamphorst W, van Swieten JC. Survival in progressive supranuclear palsy and frontotemporal dementia. *J Neurol Neurosurg Psychiatry. 2010 Apr;81(4):441–5*

Seelaar H, Klijnsma KY, de Koning I, van der Lugt A, **Chiu WZ**, Azmani A, Rozemuller AJ, van Swieten JC. Frequency of ubiquitin and FUS-positive, TDP-43-negative frontotemporal lobar degeneration. *J Neurol. 2010 May;257(5):747–53.*

# PHD PORTFOLIO

## 1. PHD TRAINING

| | Year | Workload (Hours/ECTS) |
|---|---|---|
| **Courses** | | |
| - Classical Methods for Data-analysis | 2008 | 5.5 |
| - SNP course V | 2008 | 2 |
| - Autumn School on Cognitive, Affective and Nociceptive Functioning of the Anterior Cingulate Cortex, Oppurg, Germany | 2010 | 1.5 |
| - Stereological quantification of von Economo Neurons, San Francisco, USA | 2010 | 4 |
| - Biomedical English Writing and Communication | 2012 | 4 |
| **(Inter)national conferences/seminars** | | |
| - 6th International conference on Frontotemporal Dementias, Rotterdam, the Netherlands, Poster | 2008 | 1 |
| - PSP International Medical Workshop, London, UK | 2009 | 1 |
| - American Academy of Neurology, Seattle, USA, Poster | 2009 | 1 |
| - 7th International conference on Frontotemporal Dementias, Indianapolis, USA, Poster | 2010 | 1 |
| - 8th International conference on Frontotemporal dementias, Manchester, UK, Poster | 2012 | 1 |
| **Other** | | |
| - Wetenschappelijke vergadering NVN, Garderen, the Netherlands, Poster | 2008 | 0.5 |
| - Wetenschappelijke vergadering NVN, Amsterdam, the Netherlands, Oral presentation | 2010 | 1 |
| - Mix and Match meeting Alzheimer Nederland, Utrecht, the Netherlands | 2011 | 0.5 |
| - Department research meetings | 2008–8012 | 2 |

## 2. TEACHING ACTIVITIES

| | Year | Workload (Hours/ECTS) |
|---|---|---|
| **Lecturing** | | |
| - Lecture about survival in PSP and FTD, Rotterdam, the Netherlands | 2009 | 1 |
| - Lecture about multireceptor fingerprints in PSP, Tilburg, the Netherlands | 2014 | 1 |
| **Supervising Master's theses** | | |
| - 2 Medical students | 2009–9011 | 4 |
| **Total** | | **32** |