Aus dem Zentrum für Anatomie der Universität zu Köln
Geschäftsführender Direktor: Universitätsprofessor Dr. med. W. F. Neiss
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Expression der α4 Untereinheit des nikotinischen Acetylcholinrezeptors im temporalen Kortex bei Morbus Parkinson mit und ohne Demenz

Inaugural-Dissertation
zur Erlangung der Doktorwürde
der Hohen Medizinischen Fakultät
der Universität zu Köln

vorgelegt von
Andreas Plamper
aus Leverkusen

promoviert am 09. April 2014

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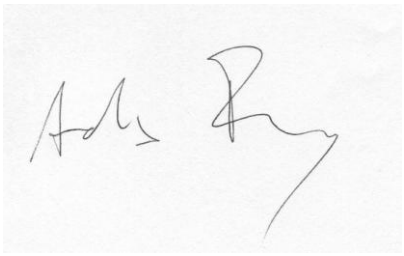

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Table of contents

Glossary

α-BTX	alpha – Bungarotoxin
ACh	Acetylcholine
AD	Alzheimer's disease
BCA	bicinchoninic acid
BSA	bovine serum albumine
C	control case
DAB	diaminobenzidine
DLB	Dementia with Lewy bodies
DTT	dithiothreitol
f	female
FCS	foetal calf serum
IHC	immunohistochemistry
LB	Lewy bodies
LBD	Lewy body disease
m	male
mAb	monoclonal antibody
MMSE	Mini Mental State Examination
nAChR	nicotinic acetylcholine receptor
n.s.	not significant
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PD	Parkinson's disease
PDD	Parkinson's disease with dementia
PMSF	phenylmethylsulfonylfluoride
p.m.	post mortem
SDS	sodium-dodecylsulfate
STG	superior temporal gyrus
TBS	tris – buffered saline
TBST	tris – buffered saline with Tween 20
WB	Western blot

1 Introduction

1.1 Neuronal nicotinic acetylcholine receptors

Among other ligand-gated ion channels such as $GABA_A$, $GABA_C$, glycine or 5HT-3 serotonin receptors, nicotinic acetylcholine receptors (nAChR) are an important member of this gene superfamily of neurotransmitter receptors [for review cf. 55, cf. 56, cf. 33, cf. 35, cf. 41, cf. 77]. On the structural level, nicotinic receptors are composed of five subunits forming an ion channel in their centre. The channel is gated via binding of the neurotransmitter acetylcholine (ACh) or other agonists such as nicotine, epibatidine, ABT-418, RJR-2403 or SIB-1765F. In addition, a variety of non-competitive (e.g. steroids or ethanol) and competitive antagonists such as d-Tubocurarine or the neuronal toxin, i.e. the venom of the snake *Bungarus multicinctus* α-Bungarotoxin (α-BTX) are known [for review cf. 37]. The receptors are permeable for Na^+, K^+ and Ca^{2+}, depending on their subunit composition [for review cf. 33]. Some structural characteristics are common for all subtypes: three hydrophobic transmembrane domains (M1-M3) are followed by a long intracellular loop (its length varying according to the subunit) and a fourth again hydrophobic transmembrane component (M4). This sequence is embedded in a large hydrophilic extracellular N-terminal portion and a short C-terminal domain [for review cf. 35, cf. 89, cf . 44].

As to the group of neuronal nAChRs, 12 subunits with a common ancestor (α2-α10 bearing the ligand-binding site and the structural components β2-β4) are currently known to organize this pentameric channel in different combinations so that a variety of combinations is feasible resulting in diverse functional and pharmacological properties [16, 25, 58; for review cf. 23, cf. 33-35, cf. 41, cf. 44, cf. 89]: While the subtypes α2-α6 combine with β2-β4 nAChRs to form a functional receptor of 2α and 3β subunits, α7-α9 are capable of assembling into functional homopentameric channels [58; , for review cf. 35, cf. 89]. Whereas the α7 nAChR, constituted homogeneously of five α7 subunits, is one of the main representatives of nAChRs that bind α-BTX, the predominant form of

receptors that bind agonists such as nicotine or acetylcholine with high affinity consists of two α4 and three β2 subunits and accounts for up to 90% of high affinity nicotine binding sites [30, 105, 115; for review cf. 34-35, cf. 56].
nAChRs are not only widely distributed in the central and peripheral nervous system [21, 111; for review cf. 36], but are also described (among others) in lymphatic, vascular or bronchial tissue [for review cf. 34]. Apart from being present on presynaptic axons on the cellular level, nAChRs can also be found peri-, extra- and postsynaptically as well as on extraneuronal cells [21, 22, 94; for review cf. 33, cf. 41, cf. 118]. Thus, a variety of functions such as presynaptical regulation of postsynaptic transmitter release, modulation of neuronal growth processes and more complex roles in the neural circuit of cognition, learning and memory ability have been ascribed to these widespread receptors [24, 110; for review cf. 5, cf. 29, cf. 35, cf. 36]. Moreover, nAChRs seem to have a pathogenetic key function in a number of neurological disorders such as autism, depression, schizophrenia, epilepsy as well as Alzheimer's disease and Parkinson's disease [70; for review cf. 19, cf. 36, cf. 41, cf. 55].

1.2 Parkinson's disease and cognitive impairment

Parkinson's disease (PD), a progressive neurodegenerative disease, is well known for its classical motor symptoms trias resting tremor, cogwheel rigidity and hypo- or akinesia [76; for review cf. 59]. Incidence rates of up to 3,5% and prevalence rates as high as 10 % among people older than 80 years put it in second place just behind Alzheimer's disease (AD) as the most common neurodegenerative disorder, a number still expected to rise with the increase of life expectancy especially in the industrial nations [104; for review cf. 86, cf. 97]. Apart from idiopathic Parkinson's disease, whose etiology still remains unknown, a row of other forms such as postencephalitic, neuroleptica-induced, metabolic or toxic Parkinsonism are known. Furthermore, causal mutations in several genes have been identified to induce PD in 2% of the European

population [for review cf. 54]. Moreover, clinical symptoms such as supranuclear palsy, orthostatic dysregulation or cerebellar and pyramidal symptoms point at a relationship to the entity of multi system atrophies [32; for review cf. 86]. A loss of dopaminergic neurons in the substantia nigra pars compacta is considered to be a pathogenetic key point for the development of the already mentioned motor symptoms [for review cf. 74, cf. 97]. Other neuropathological hallmarks of PD include the presence of cortical and/or brainstem Lewy bodies (LB), intracellular inclusions consisting, among others, of neurofilaments, ubiquitine, and α-synuclein [17, 120; for review cf. 9-10, cf. 74, cf. 97]. It has been proven that α-synuclein plays a key role in the pathogenesis of PD. An aggregation/overexpression of α-synuclein in protein complexes induces apoptosis in dopamine-containing neurons [28, 120; for review cf. 64]. Pathological markers such as LB, Lewy neurites (abnormally composed filaments similar to LB) and extraneuronal β amyloid deposits in the form of diffuse neuritic senile plaques as found in AD put PD in the group of the so-called Lewy body diseases (LBD) [for review cf. 64]. The group of LBD consists of PD with (PDD) or without dementia (PD) as well as dementia with Lewy bodies (DLB), a disease that is defined by cognitive impairment, extrapyramidal symptoms and prominent neuropsychiatric features [for review cf. 64-67, cf. 75]. Although consensus guidelines have been defined for the differential diagnosis of DLB and PDD, it is still difficult to distinguish between them [cf. 67]. It has however been defined that if dementia precedes or occurs within 12 months of the onset of parkinsonism, DLB is likely to be diagnosed whereas development of dementia later than 12 months after the beginning of parkinsonian symptoms is typical for PDD [63; for review cf. 54]. However, since the histopathological findings are quite similar in PDD and DLB, some authors have suggested that these two diseases represent two different stages of one spectrum of Lewy body diseases [7]. Also, the consensus guidelines have recently put importance on the development of dementia in PD, formerly neglected as a minor side symptom in contrast to the classic extrapyramidal features. It is described to have a prevalence of up to 45% [73, 91; for review cf. 15, cf. 26, cf. 54, cf. 64, cf. 85], its cumulative prevalence being reported to be

as high as 78% over 8 years of follow-up in a prospective study [1]. These numbers receive even more weight by the fact that PDD patients are proven to be impaired additionally by a higher disability rate, reduced quality of life and a two-fold increased mortality when compared to PD patients [73, 91; for review cf. 15]. Other clinical features in PDD include higher numbers in depression, rapid eye movement sleep behavioral disorder and repeated falls [7, 68, 91; for review cf. 15]. It is assumed that the development of dementia in PD is associated with older age, more severe neurologic symptoms, greater disability, past medical history of depression, poor L-DOPA response and male sex [45, 91; for review cf. 64]. Other risk factors such as duration of the disease or age at onset of PD are discussed controversially [45; for review cf. 64].

The impairment of the cholinoceptive system has been shown within the course of Parkinson's disease: Reduced levels of nicotine, epibatidine or α - BTX binding sites [53, 60, 79, 87, 113; for review cf. 36] as well as decreased choline acetyltransferase activity have been demonstrated [53, 114]. Over the past few years, the levels of different nAChR subtypes came into the focus of a number of studies, emphasizing on the importance of the hypothesis that reduced levels of nAChRs might have an essential role in the development of cognitive impairment also in the course of PD [3,4, 13, 37]. These studies focused on the most abundant subtypes of nAChRs: the $\alpha4\beta2$ subtype and the $\alpha7$ subtype. Although this cholinergic hypothesis [for review cf. 5] is well-established in other neurologic diseases accompanied or dominated by dementia symptoms with AD being the most well-known and intensely studied disease of this group [38, 61-62, 79-80, 108-110], to the best of our knowledge, most of these investigations on PD have not distinguished explicitly between patients suffering additionally from clinically proven dementia (PDD) and patients that were not objectively demented (PD).

In healthy individuals, levels of $\alpha4\beta2$ - containing nAChRs are reported to be highest in the thalamus, distinct regions of the basal ganglia and the hippocampal formation, whereas moderate numbers in the cerebral cortex including the temporal region have been described [21; for review cf. 19, cf. 36]. Studies have focused in particular on the temporal cortex, since it is known that

the neurons of this region are among the first to be affected within the course of AD resulting in the most severe losses of cholinergic innervation (up to 80% depletion of cholinergic axons in advanced – stage AD) [7]. Furthermore, the neurons of the temporal region also reveal pathological lesions in fully developed PD [for review cf. 10]. These studies have successively demonstrated significant losses of nAChR binding in AD [4, 14, 38, 69] or PD [13, 37], with the superior temporal gyrus (STG) being the most important anatomical representative of this region [8; for review cf. 95].

The role of nicotinic receptors needs further elucidation in this context, since a distinction between PDD and PD could help to improve the understanding of the disease. This topic gains even more weight since cholinergic deficits are more marked in PDD than in PD as evidenced by greater neuronal loss in the nucleus basalis of Meynert [47]. It has already been shown in the early 1980s by Arendt *et al.* that the loss of neurons in the nucleus basalis of Meynert is more severe in PD than in AD [2]. Moreover, the majority of studies agree on the assumption that nicotine helps to improve symptoms in both AD and PD. Subtype specific drug agents might be of therapeutic effect for these patients, but more information concerning which subtype is to be targeted depending on the cognitive status is needed [20, 30, 69-70, 107; for review cf. 19, cf. 26, cf. 36, cf. 55, cf. 88].

2 Aim of the study

The importance of nicotinic acetylcholine receptors in the pathogenesis of cognitive impairment in neurodegenerative diseases has been demonstrated in a plethora of investigations. It is possible that cognitive decline in Parkinson's disease is also attributable to a reduced expression of distinct nAChR subtypes. A quite important question, since therapeutic approaches could target these subtypes specifically in order to improve symptoms such as motor or cognitive dysfunction. To the best of our knowledge however, studies examining these receptors in the context of dementia in Parkinson's disease have not made a clear distinction between demented and non-demented subjects.

In this study therefore patients patients were divided into these two groups according to the clinical diagnosis of dementia. Additional clinical information such as smoking behaviour was also documented resulting in a patient pool with well – documented past medical history.

The protein expression of the α4 nAChR subunit – being an important representative of high affinity nicotine binding sites – was analyzed in the temporal cortices of these patients and compared to age – matched controls by means of immunohistochemistry (IHC) and Western blot (WB) thereby dealing with the following questions:

1. Is the expression pattern of the α4 nicotinic acetylcholine receptor subunit protein in the temporal cortex of PDD and/or PD patients altered in comparison to age – matched controls?

2. Is the amount of α4 nicotinic acetylcholine receptor subunit protein in the temporal cortex of PDD and/or PD patients altered in comparison to age – matched controls?

3 Material and Methods

3.1 Tissue

Upon autopsy, tissue of the STG was acquired for immunohistochemistry from five individuals clinically diagnosed with PD and dementia (PDD; mean age: 77 ± 7 years, mean post mortem (p.m.) delay: 29 ± 7 hours). The mini mental status examination (MMSE, [31]) was used to determine dementia. A score of less than 20 resulted in the clinical diagnosis of dementia. Tissue samples were collected from three PD patients (MMSE $\geq$ 20; mean age: 70 ± 6 years, mean p.m. delay: 26 ± 17 hours) and five age-matched controls (C – mean age: 66 ± 4 years, mean p.m. delay: 20 ± 15 hours) respectively, who neither died of a neurological disease nor had any neuropathological relevant abnormalities such as Lewy bodies or amyloid plaques. Probes were fixed shortly after autopsy (for individual p.m. delays, cf. Table 1) in 4% buffered formaldehyde solution for at least 21 days followed by embedding in paraplast.

Analogously, for Western blot studies tissue of the corresponding brain area was obtained from each five PDD cases (mean age: 74 ± 6 years, mean p.m. delay: 25 ± 12 hours), PD patients (mean age: 70 ± 10 years, mean p.m. delay: 25 ± 7 hours) and control cases (mean age: 70 ± 4 years, mean p.m. delay: 22 ± 14 hours). All samples used for immunoblot analysis were stored immediately at –80°C until use.

All PD and PDD cases revealed cortical LB in the histological preparation. As given in Table 2 in detail, all of the cases presented minor histopathological changes as seen in AD (Braak stage $\leq$ II, with the exception of PDD 4: III, amyloid plaque amount was slight to moderate) [for review cf. 11]. None of the individuals taken for the study had a positive history of tobacco smoking. All samples including pathological examination data were kindly provided by Dr. R.A.I. de Vos (Laboratorium Pathologie Oost Nederland, Enschede, The Netherlands).

Clinical background information was made accessible by Dr. E.N.H. Jansen Steur (Medisch Spektrum Twente, Enschede, The Netherlands).

Table 1: Data of subjects investigated.

f = female, *m* = male., *p.m.* = post mortem delay in hours

	p.m.	**age**	**gender**	**MMSE**	**WB**	**IHC**
PDD1	28	73	m	17	X	X
PDD2	22	69	f	16	X	
PDD3	44	79	f	18	X	X
PDD4	12	83	m	14	X	
PDD5	18	68	f	15	X	X
PDD6	8	85	f	12		X
PDD7	48	80	f	18		X
PD1	22	68	m	26	X	
PD2	37	61	m	23	X	
PD3	18	87	m	23	X	
PD4	22	65	m	24	X	
PD5	26	71	f	24	X	X
PD6	9	75	f	24		X
PD7	42	64	m	24		X
C1	8	71	m	n.t.*	X	X
C2	22	66	f	n.t.	X	X
C3	44	67	f	n.t.	X	X
C4	14	75	m	n.t.	X	
C5	21	70	f	n.t.	X	
C6	9	66	m	n.t.		X
C7	18	61	f	n.t.		X

* n.t. : not tested

Table 2: Histopathological data of subjects investigated (Courtesy Dr. R.A.I. de Vos).

(- no findings, + mild findings, ++ moderate findings)

	LB	Braak	Amyloid	Tau
PDD1	X	I	+	-
PDD2	X	II	+	-
PDD3	X	I	-	-
PDD4	X	III	++	-
PDD5	X	I	+	-
PDD6	X	I-II	+	-
PDD7	X	I	-	-
PD1	X	II	++	-
PD2	X	I	-	-
PD3	X	I	+	-
PD4	X	II	-	-
PD5	X	II	-	-
PD6	X	II	+	+
PD7	X	0	-	-

3.2 Antibodies

For immunohistochemistry, mAb 299, a monoclonal antibody directed against the α4 nAChR subunit, was used. This antibody, generated using purified rat brain neuronal nAChR, was kindly provided by Prof. J. Lindstrom (University of Pennsylvania, School of Medicine, Philadelphia, USA). It recognizes epitopes within the extracellular domain of the α4 subunit [78, 115]. Detailed characterisation and specificity testing are reported elsewhere [78, 115].

For Western blotting, a guinea pig polyclonal antibody against α4 (AB5590, Chemicon, Hofheim, Germany) was applied as primary antibody. Its immunogen is a synthetic peptide which corresponds to the amino acid sequence 568-588 of the rat nAChR α4 protein.
Please refer to chapter 5.1 for recent findings about the specificity of the applied antibodies.

3.3 Immunohistochemistry

7µm thick sections of each sample were cut with a microtome (HM350, Microm, Walldorf, Germany) and mounted onto adhesion slides (Histoslides adhäsiv, Shandon, Frankfurt, Germany). Three sections from each brain sample were subjected to IHC. Adjacent sections were taken for cresyl – violet – staining (Nissl) in order to determine the total count of neuronal cells.
A standard ABC-technique was used for the immunohistochemical detection of the α4 subunit. After deparaffinization, rehydration and equilibration in Tris-buffered saline (TBS: 0.05M Tris pH 7.6, 0.15M NaCl), sections were incubated in TBS containing 0.05% trypsin (20min, 37°C), followed by rinsing in tap water for 10 minutes and washing as before. The samples were treated with 0.5% hydrogen peroxide (H_2O_2) for 20min, in order to block the endogenous peroxidase. Subsequent to washing as before, sections were pre-incubated in 30% foetal calf serum (FCS) diluted in TBS and incubated with the primary antibody mAb299 (diluted 1:150 [33µg/ml] in TBS containing 1% bovine serum albumine (BSA)) overnight at 4°C. The next day the sections were rinsed in TBS (3x 10min), and a biotinylated anti-rat IgG antibody (Jackson, distributed by Dianova, Hamburg, Germany) was added for 40min (diluted 1:400 in TBS with 1% BSA). After repeated washes, the sections were incubated with a streptavidin-peroxidase-complex (Vectastain ABC-kit; Alexis, Grünberg, Germany) for up to 40min according to manufacturer's instructions. Subsequently, the sections were rinsed once more and then incubated with nickel-enhanced diaminobenzidine (DAB) in order to visualize the

immunoreaction [99]. After detection, sections were dehydrated and coverslipped with DePeX (Serva, Heidelberg, Germany).

All steps were performed at room temperature, unless indicated otherwise. All essays were carried out in triplicate for each sample.

Omission of the primary and/or the secondary antibody yielded no signal.

For semiquantitative analysis, camera lucida drawings with a drawing tube-equipped microscope (BH-2, Olympus, Hamburg, Germany) were assessed by counting positively immunolabelled neurons, regardless of their labelling intensity, in 250µm wide perpendicular stripes as described in detail elsewhere [93]. Corresponding stripes of Nissl-stained sections were evaluated for total neuron number count in the cortex. The lower cell count in Nissl-stained sections is due to the fact that only Nissl-stained cells with a nucleolus were counted as neurons, while all immunopositive cells regardless of this criterion were included in their count. Numerical densities were received by correcting the resulting counts of both the immunolabelled and the cresyl-violet stained neurons for the individual height of the cortical stripe. The respective numerical densities were then put in relation to each other and compared with the respective ratios of the other groups. A mean value was received from three independently performed counts for each specimen and used for statistical evaluation by means of the t-test (SPSS version 10.0). Photo documentation was obtained with an Olympus Vanox AHBT3 photomicroscope. Photographs were proceeded onto Kodak Ektachrome 64T film (Kodak, Rochester, USA).

3.4 Western blot

30mg of each brain tissue were homogenized by ultrasonication in 16 volumes of ice-cold lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 5mM EDTA, 1mM phenylmethylsulfonylfluoride [PMSF]) and subjected to centrifugation at 1,000 *g* for 10min at 4°C. Protein content in the supernatants was quantified using the bicinchoninic acid (BCA) kit (PIERCE, Perbio Science, Bonn, Germany) [100]. Brain extracts were stored in aliquots at -80°C until further use.

Protein samples were mixed with two volumes of a sodium-dodecylsulfate (SDS)/ Dithiothreitol (DTT) mix (80% SDS-mix [0.1 mol/l TBS [pH 6.8], 4% SDS, 0.05% bromophenolblue, 20% glycerol], 20% DTT) and heated to 97°C for three minutes.

The protein samples (10µg per lane) were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) (100V, 110min; electrophoresis buffer: 25mM Tris, 192mM glycine, 0.1% SDS) and subsequently blotted onto nitrocellulose membranes (350mA, 60min; transfer buffer: 25mM Tris pH 8.3, 192mM glycine, 20% MeOH) using a transfer unit (Mini Protean 3, Bio-Rad, Munich, Germany) [52, 103].

In a first step, blots were blocked with 5% non-fat dried milk in TBST (Tris buffered saline with Tween 20 (Sigma-Aldrich, Schnelldorf, Germany): 50mM Tris pH 7.5, 150mM NaCl, 0.1% Tween 20) for 60min followed by overnight incubation with the primary antibody AB5590 (diluted 1:2,000 in 1% non-fat dried milk in TBST). After thorough washing in TBST (4x15min), the membranes were incubated with biotinylated anti-guinea pig IgG for 60 minutes (Vector Laboratories, distributed by Alexis, Grünberg, Germany; diluted 1:20,000 in 1% non-fat dried milk in TBST). The blots were washed as before, incubated with a streptavidin-biotinylated-HRP-complex for 60 minutes (Amersham Bioscience, Freiburg, Germany; diluted 1:10,000 in 5% non-fat dried milk in TBST). Following the rinse in TBST, the blots were developed using enhanced chemiluminiscence (ECL-Lumi-Light Plus, Roche Diagnostics, Mannheim, Germany) for 5 to 40min and exposed onto standard X-ray films (Kodak) for visualization of the signals.

All steps, except when indicated, were performed at room temperature. All essays were carried out in triplicate and quadruplicate, respectively.

Omission of the primary and/or the secondary antibody yielded no signal.

Quantitative analysis of the band intensities was carried out by means of a GS-690 densitometer and the Multi-Analyst software (version 1.1; Bio-Rad, Munich, Germany). Three and four independent trials, respectively, were assessed for statistical evaluation by determining the integral of the optical density (OD) of each band and calculating the average value for each specimen. These values

were set in proportion to the average value of all control cases. A t-test was performed to determine statistical significance (SPSS version 10.0).

4 Results

4.1 Expression pattern of α4 nAChR subunit protein in Parkinson's disease with and without dementia

α4 nAChR subunit expressing neurons could be located in all laminae of the superior temporal gyrus in all individuals, demonstrating a similar distribution pattern when comparing the two patient groups to the controls (Fig. 1 & 2).

In all control cases, positive staining was observed in the cytoplasm and the apical dendrites, whereas the nucleus remained unlabelled (Fig. 3a-b). Most PD cases demonstrated a similar immunoreaction of the cytoplasm and the apical dendrites (Fig. 3c-d). However, in some cases of the PDD group, antibody reaction in the dendrites was missing whereas perikarya were stained more intensely, resulting in a difficult distinction between nucleus and cytoplasm. Moreover, perikarya revealed signs of cellular decay such as atrophy of the perikarya or the apical dendrites (3e-f) and also did not present the comparatively stronger immunolabel of layers II/III and V as seen in most of the other specimens studied (Fig. 1).

The α4 subunit nAChR protein was also detected in non – pyramidal cells showing characteristics of astrocytes which were mainly found on the pial surface, layer I and the white substance (data not shown).

By assessing the camera lucida drawings (Fig. 2), a mean value of 302 ± 30 total neurons in comparison to a mean value of 351 ± 28 α4 immunoreactive cells was calculated for the control cases. The mean values of the PD patients varied only slightly (301 ± 31 and 350 ± 56, respectively). Considering the PDD cases, the counts were assessed to 302 ± 29 total neurons and 314 ± 38 α4 immunopositive cells. The decrease of the mean value of α4 nAChR immunopositive cells proved to be statistically not significant as did the corresponding ratios of immunopositive neurons / Nissl – stained neurons (Controls versus PD versus PDD: 1.18 ± 0.13 versus 1.18 ± 0.10 versus 1.05 ± 0.08, Table 3 and Fig. 4).

Table 3: Numerical densities of Nissl – stained and α4 nAChR immunoreactive neurons in the superior temporal gyrus of controls and Parkinson's desease patients with and without dementia. Data are presented as means ± standard deviations. *n.s.*: difference statistically not significant.

Neurons per mm^2	**Total (Nissl)**	**α4 positive**	**α4 / Nissl**
Controls (C1-3, 6-7)	302 ± 30	351 ± 28	1.18 ± 0.13
PD patients (PD5-7)	301 ± 31	350 ± 56	1.18 ± 0.10
C vs. PD	*n.s.*	*n.s.*	*n.s.*
PDD patients (PDD1, 3, 5-7)	302 ± 29	314 ± 38	1.05 ± 0.08
C vs. PDD	*n.s.*	*n.s.*	*n.s.*

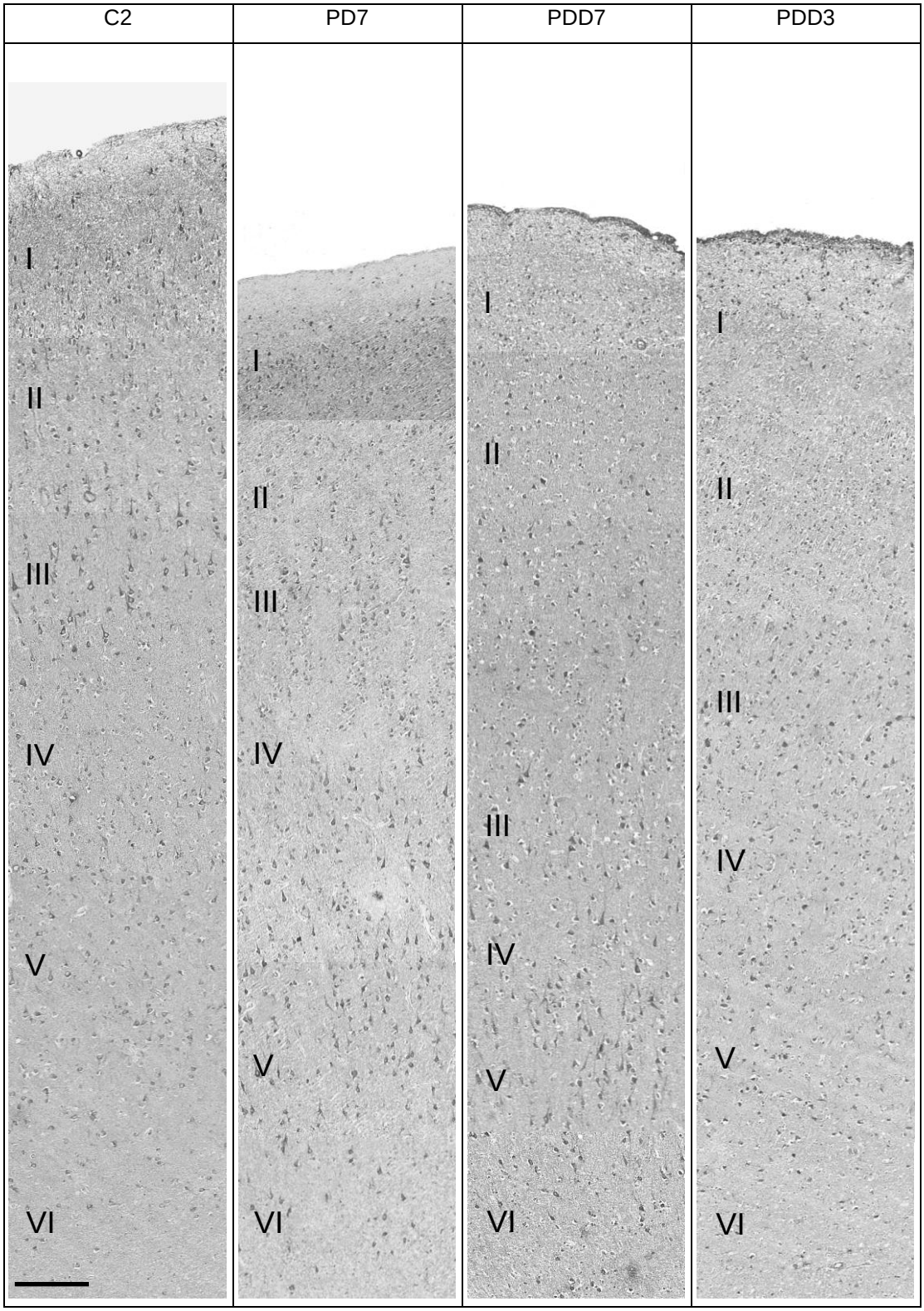


Fig. 1: Photo images showing the neuronal profiles of representative cases. Note that all PD cases and most PDD cases (PDD 7) revealed almost the same regular histoarchitecture of the superior temporal gyrus as the controls, whereas in some PDD cases (PDD 3) the distinction between the different layers became difficult. Layers are indicated. Scale bar: 200µm.

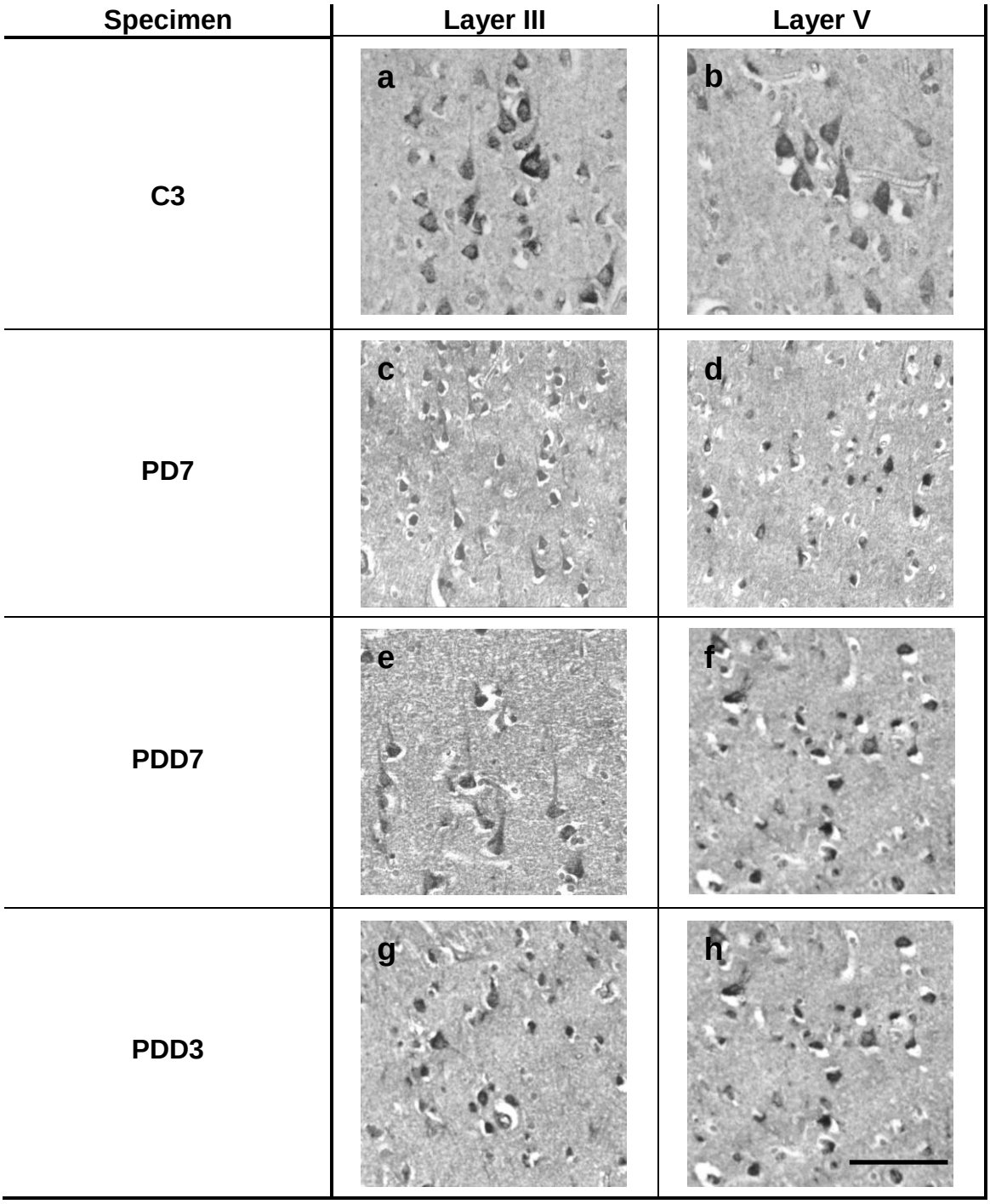


Fig. 2: Microphotographical details of the α4 nAChR protein expression in layers III and V of the STG. Some cases diagnosed with Parkinson's disease with dementia (PDD) bore an intense immunosignal of their perikarya and apical dendrites (e, f) comparable to that of the age – matched controls (a, b), whilst other PDD – cases lacked these labelling patterns and presented signs of cellular decay (g, h). PD cases showed similar characteristics as the controls (c, d). Scale bar: 100µm (i.e. neuron size in control case, e.g. C3 = 20µm).

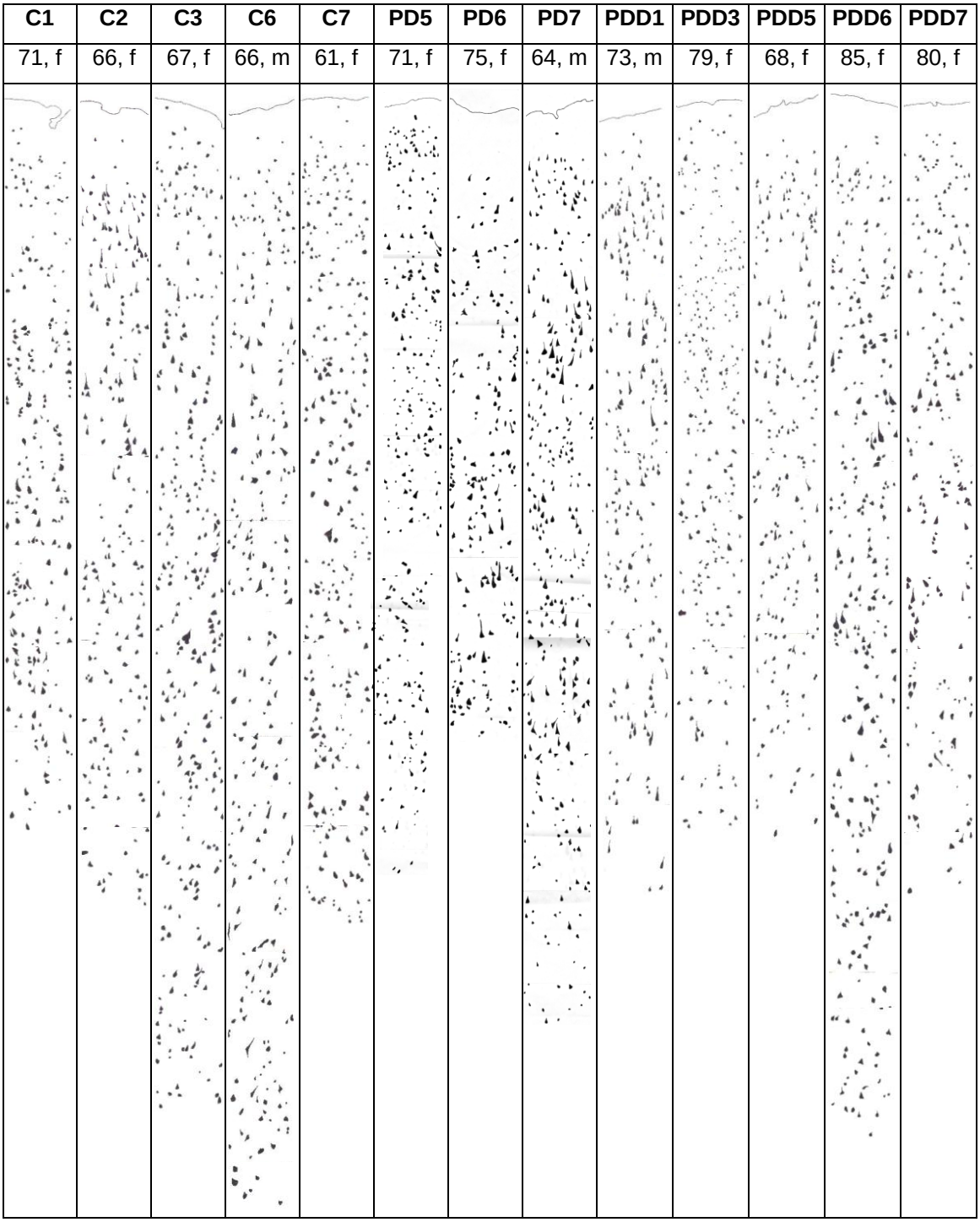


Fig. 3: Camera lucida drawings demonstrating the neuronal profiles of α4 immunopositive nAChRs in the STG of demented Parkinson patients (PDD), non – demented patients (PD) and age – matched controls (C). Case number as well as age and gender are given for each column. Width of cortical stripe: 250µm. *f* = female, *m* = male.

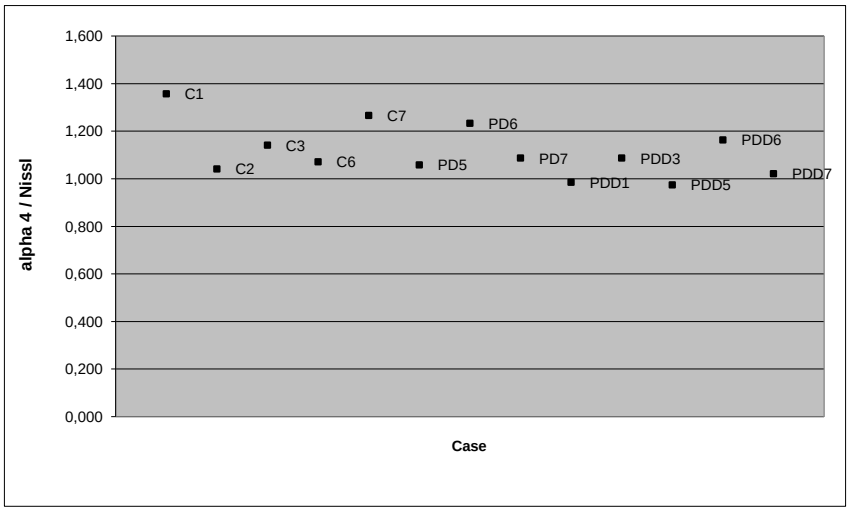
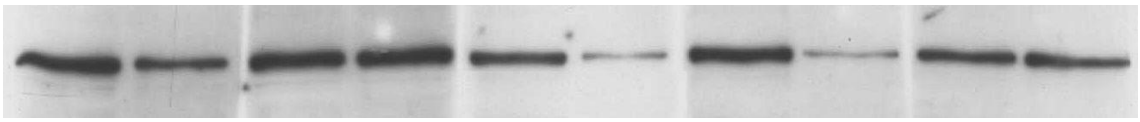


Fig. 4:
Ratio of α4 immuno-reactive neurons per mm^2 / total number of neurons per mm^2 (Nissl). Represented are the mean values for each specimen.

4.2 Expression of α4 nAChR subunit protein in Parkinson's disease with and without dementia in Western blot analysis

The α4 nAChR subunit protein was detected as a single band at 100 kDa in the STG – samples by Western blot analysis. The signal intensity was reduced in some cases compared to controls. Statistical evaluation of the mean optical densities, however, revealed no significant differences (PDD versus C: 8.15 $\pm$ 2.7 versus 10.34 $\pm$ 2.3). Similar results showing no statistical significant difference were found when comparing the mean optical densities of the PD cases to that of the controls (PD versus C: 10.46 $\pm$ 2.5 versus 10.57 $\pm$ 2.0).



C1 C2 C3 C4 C5 PDD1 PDD2 PDD3 PDD4 PDD5

Fig. 5: Representative X – ray film demonstrating the protein expression of the α4 nAChR subunit. Controls (C1-C5) were compared to demented Parkinson patients (PDD1-PDD5).

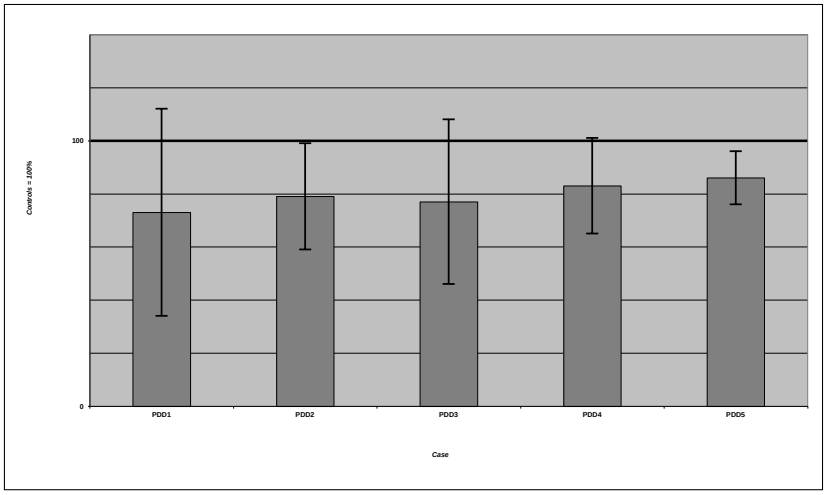
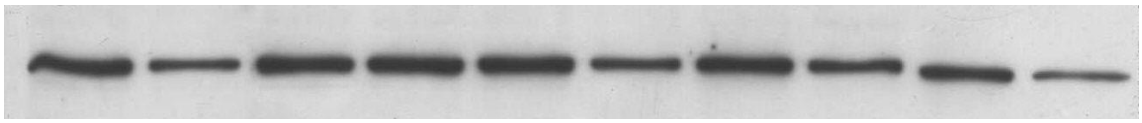


Fig. 6:
Protein quantification of the α4 nAChR subunit in the STG of demented Parkinson's disease patients. Protein amount is expressed as percentage of the protein amount found in age – matched controls which has been scaled to 100%. Represented are the mean values and standard deviations of four independently performed trials.



C1 C2 C3 C4 C5 PD1 PD2 PD3 PD4 PD5

Fig. 7: Representative X – ray film demonstrating the protein expression of the α4 nAChR subunit. Controls (C1-C5) were compared to Parkinson's disease patients (PD1-PD5).

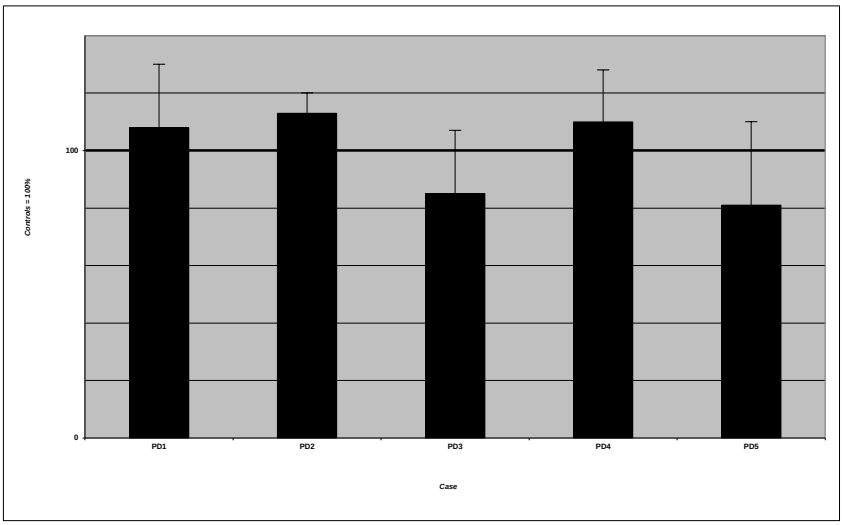


Fig. 8:
Protein quantification of the α4 nAChR subunit in the STG of Parkinson's disease patients. Protein amount is expressed as percentage of the protein amount found in age – matched controls which has been scaled to 100%. Represented are the mean values and standard deviations of three independently performed trials.

	Controls (C1-5)	**PD patients (PD1-5)**
OD	10.57 $\pm$ 2.0	10.46 $\pm$ 2.5
Percentage	100	99
C vs. PD	*n.s.*	
	Controls (C1-5)	**PDD patients (PDD1-5)**
OD	10.34 $\pm$ 2.3	8.15 $\pm$ 2.7
Percentage	100	79
C vs. PDD	*n.s.*	

Table 4: Statistical analysis of the optical densities (OD) in mm^2. Data are presented as means $\pm$ standard deviatons of three (PD) / four (PDD) independent trials.
n.s.: difference statistically not significant.

5 Discussion

5.1 Methods

Standard protocols were applied for both the immunohistochemical and the Western blotting analyses.

The post mortem stability of high affinity nicotine binding sites, represented mainly by the $\alpha4\beta2$ nicotinic acetylcholine receptor has been proven elsewhere [6, 30].

mAb 299 is an $\alpha4$ subunit specific antibody that has found wide – spread acceptance and application in nAChR research. Its specifity has been characterized repeatedly [78, 115]. Several studies demonstrated that subunit specific antibodies such as mAb 299 do not show any cross – reaction during Western blot analyses of these proteins in oocytes and transfected fibroblasts [92, 106]. Furthermore, the antibody was used in human cell lines such as SH – SY5Y as well as mouse fibroblast cell lines (M10) showing specific immunoreactivity in cell cultures when expressing the $\alpha4\beta2$ receptor, but not when transfected [38, 62].

These are some of the reasons why mAb 299 has been applied repeatedly for comparable study aims, i.e. immunolocalization of the subunit in tissue from healthy and diseased individuals [e.g.: 13-14, 37-38].

For detection of the $\alpha4$ nAChR protein on blots, the polyclonal antibody AB5590 was applied, as mAb 299 showed many unspecific bands. Failure in the attempt of performing Western blots with mAb 299 could be explained by the application of different antibody lots. AB5590 was able to detect a single reproducible band at 100 kDa being higher than the expected molecular weight of 70kDa [Elliott *et al.*, 1996], but could be confirmed by the use of goat polyclonal anti-$\alpha4$-nAChR subunit sc-1772 (Santa Cruz Biotechnology, Heidelberg, Germany) which revealed a band at the same height (data not shown, compare to 4.2.1).

However, recent discoveries have put into focus questions on the specificity of several nAChR subunit specific antibodies: applying different $\alpha7$ nAChR specific antibodies (mAb 306, mAb 319 and H-302), Herber *et al.* 2004 have

demonstrated comparable staining patterns between brain tissue of α7 knockout mice and wild type mice using immunohistochemistry, resulting in multiple unspecific bands in both genotypes when using Western blotting [40]. These results are supported by similar findings of another study detecting equal labelling patterns in immunohistochemistry and Western blot analysis when using α7 nAChR specific antibodies on brain tissue of α7 subunit – deficient knock – out mice [71]. It was confirmed by PCR that the corresponding allele of the α7 subunit was no longer detectable in the knock out specimen, so that immunoblotting of α-BTX affinity purified brain extracts yielded no signal. However, significant differences could neither be seen in the signalling pattern of immunohistochemistry nor in immunoblotting comparing the knock – out specimen to the wild type.

These findings gain even more importance for the present study, since a joint paper of various cooperating groups published in 2007 and presented previously at a meeting has demonstrated similar immunohistochemistry and immunoblot results in corresponding knock out and wild type mice for antibodies against the α3-, α4-, α7-, β2- and β4- nAChR subunits using standard protocols [71-72]. Among the tested antibodies were mAb 299 and AB5590. The monoclonal antibody showed a widespread staining pattern in all brain regions without difference in the distribution pattern or labelling intensity between $\alpha4^{-/-}$ and $\alpha4^{+/+}$ mice. The application of AB5590 in IHC resulted in an insufficient, non – evaluable quality of the immunoreactions, whereas in Western blot analysis, a single band at 100 kDa was detected on both genotypes.

A discussion of possible explanations for this surprising finding would be beyond the scope of this work, but modified antigenic determinants, certain (murine) proteins that might cross react with the antibodies due to structural relation to the epitope sequence or incomplete characterization of certain polyclonal antisera raised in rabbit or goat are some of the topics that have to be discussed and investigated further [27; for review cf. 48]. An extensive discussion can be found in Moser *et al.*, 2007 [71].

Future studies should include the investigation of other nAChR subunits also determining the mRNA levels. Additionally, high affinity agonists/antagonists

such as epibatidine or α-BTX should be applied in corresponding knock out specimen. Furthermore, the use of affinitiy purified antibodies for Western blot analyses should be considered.

The presently available knock out studies and their conclusions might be limited to the species of the tissue used, since there are possibly to be differences in epitope or overall protein expression profiles in other species including human tissue. However, for the time being, conclusions of studies working with nAChR subunit specific antibodies, including the results of the present investigation, should be interpreted with caution. Standard methods like the ones applied in this study have to be augmented, as some works have e.g. suggested the application of iodinated monoclonal antibodies on unfixed cryostat sections for IHC as reliable [112].

5.2 The α4 nicotinic acetylcholine receptor in Parkinson's disease with and without dementia

The present investigation was designed to assess the expression pattern and the protein amount of the nAChR α4 subunit in the temporal cortices of patients suffering from Parkinson's disease with dementia or without using immuno-histochemistry and Western blotting. Our data did not show statistically significant differences in the immunohistochemistry or the immunoblotting trials in both patient groups when comparing them to age – matched controls.

On the cellular level, labelling of α4 immunoreactive structures was found in pyramidal neurons, non – pyramidal neurons and a few glial cells in all three groups studied. These findings are in accordance with those of other studies [50, 96, 109; for review cf. 36].

Inconsistent results are found when reviewing the currently available data on studies investigating the nAChR α4 subunit in PD: Burghaus *et al.* demonstrated a significant reduction of nAChR α4 subunit protein in the STG of PD patients by means of Western blot [13]. In this study however, no data about the cognitive status (i.e. MMSE scores or other diagnostic criteria for dementia)

were taken into consideration. A study by Guan *et al.* (2002) found no differences in the amount of nAChR α4 subunit protein in several cortical regions of PD patients as compared to age – matched controls [37]. Although details on the cognitive status of the patients recruited for this study were available by means of the DSM III R criteria, these patients were not subdivided into groups with no or mild to moderate dementia. A decrease of [^{3}H]-epibatidine binding sites could be revealed in these patients, whereas no data on the α4 mRNA levels are given. The findings about the protein levels of the α4 subunit are in accordance with the present study [37].

Results are also heterogeneous in studies investigating the α7 subunit: There are studies that showed a significant reduction of nAChR α7 protein in PD patients with unknown cognitive status [13] as well as in PD patients that had MMSE scores varying from 8-26 (meaning that demented as well as non – demented patients were put into the same group) [4]. There are also studies that demonstrated a parallel increase of nAChR α7 subunit protein and of [^{125}I]-α-BTX binding sites in PD patients [37]. Our group was not able to find statistically significant alterations in the protein amount nor in the immunohistochemical expression of this subunit, reaching only significant reduction when determining the ratio of α7 immunoreactive neurons compared to all (i.e. Nissl – stained) neurons [82-83, 116-117]. These studies also had distinctive information concerning the cognitive status of the subjects investigated and thus were able to subdivide the patients into PD and PDD groups.

Due to the missing subdivision into PD and PDD groups in most of these studies, it becomes obvious that detailed information about the cognitive status and other anamnestic information from the patients' history such as smoking behaviour or other nicotine application have to be taken into account when nicotinic acetylcholine receptors are to be investigated in PD. This fact appears to be even more notable since an influence on nAChR expression by chronic application of nicotine has been demonstrated repeatedly [20, 30, 113-114; for review cf. 12, cf. 69-70, cf. 119] as well as the fact that patients suffering from neurodegenerative diseases such as AD benefit from nicotine administration

[for review cf. 42, cf. 88]. An additional clinical characteristic to be involved could be the patient's handedness, as some studies have pointed out the effect of left or right handedness on the anatomy and histoarchitecture of the temporal cortex [for review cf. 95]. It might thus be of interest to include information on the hemisphere taken at autopsy for the investigation.

In contrast to the diverging conclusions about the expression of the nAChR α4 subunit in the studies quoted above, the majority of binding – studies in this field agree on the fact that the number of [^{3}H]-epibatidine-binding sites is markedly decreased in several neurodegenerative diseases such as AD and PD [3, 37, 60, 87, 113-114; for review cf. 36]. 5-[^{125}I]-A-85380 is a recently developed ligand for the nAChR α4β2 subtype, enabling both in vitro and in vivo binding studies by means of SPECT (single photon emission computed tomography) [for review cf. 43]. Studies using this ligand confirmed reduced nicotinic binding sites in several cortical, cerebellar as well as brainstem regions in PD patients [39, 68, 81]. 2-[^{18}F]F-A85380 is another ligand with high affinity to the α4β2 nAChR protein and was also applied in autoradiography studies demonstrating reduced binding sites in patients with PD, AD and DLB [18, 90].

Binding studies that investigated the cholinergic function in other neurodegenerative diseases such as progressive supranuclear palsy or multi system atrophy revealed moderate to severe loss of nicotinic binding sites in subcortical regions as well [summarized in 7].

These concordant findings in the majority of binding studies are striking, since nAChRs composed of the α4β2 subtype are supposed to represent the molecular equivalent of epibatidine binding sites and for the ligands 5-[^{125}I]-A-85380 and 2-[^{18}F]F-A85380. However, Guan *et al.* (2002) have demonstrated a significant loss of β2 on both the protein and mRNA level in PD, pointing at the possibility that the reduction of β2 subunit could be responsible for the decreased binding sites [37].

Concluding from these results, it appears that the role of nAChRs in the interaction of acetylcholine and high-affinity nicotinic binding sites as well as α-BTX binding sites conceals a higher complexity than expected. This aspect is interesting, since the role of nAChRs seems to be clearer in Alzheimer's

disease: the majority of studies that have investigated the role of nAChRs in AD have come to the conclusion that decreased nAChR subunit expression accompanies cognitive decline [4, 14, 38, 62, 108-110].

The present findings cannot support the hope that nAChR subunit – specific pharmacological agents could be easily found for treatment of dementia in Parkinson's disease. PD remains a multi – system degeneration. A recent in vivo study has presented data showing that (non – demented) PD patients with longer durations of the disease can have less cholinergic impairment as well as less severe motor symptoms in contrast to early pharmaco – resistant PD patients [98]. Obviously, the clinical phenotype of the motor symptoms (for example tremor – predominant disease versus postural instability and gait disturbance) plays a role in the development of cholinergic denervation [102]. The data indicated that cortical and subcortical cholinergic denervation show a certain heterogeneity in this disorder that involves multiple CNS systems. Thus, depending on which region/system of the CNS is affected to a certain extent, different clinical symptoms can prevail [7].

However, evidence exists that stimulation of the nicotinic cholinergic system ameliorates both motor and cognitive symptoms. Chronic nicotine application has been shown to improve the classic trias of motor symptoms and to have a protective effect against nigrostriatal damage in experimental models [46, 49; for review, cf. 84]. Quik and colleagues have accumulated evidence that some striatal nAChR subtypes are particularly susceptible to nigrostriatal damage and have therefore proposed to search for specific agonists or antagonists to target these receptors [for review, cf. 84]. Examples of subtype – specific agonists are ABT-418 or SIB-1508Y for the $\alpha 4\beta 2$ subunit, SIB-1553A for the $\beta 4$ subunit or GTS – 21 for the $\alpha 7$ subunit [51, 70, 110; for review cf. 19, cf. 55, cf. 57, cf. 101].

Finding options for treatment of Parkinson's disease remains a challenge for the scientific community, as the impact of PD on the public health system and socio – economic structures will remain high as prevalence rates are still expected to incline with growing life expectancy especially in the industrialized countries.

It becomes evident that additional studies are necessary to elucidate the role of nAChRs in Parkinson's disease with and without dementia. These studies should pay attention to detailed patient history such as mental/cognitive status, smoking behavior, medication, handedness or the clinical phenotype. Studies that investigate different nAChR subtypes in different cortical as well as subcortical brain regions by means of different both in vitro and vivo techniques would be of remarkable help to shed light into this still unclear situation.

6 Summary

Nicotinic acetylcholine receptors are an important representative of neurotransmitter receptors and are widely distributed in the nervous system. Due to their important roles within a variety of neuronal circuits, these receptors seem to have a pathogenetic key function in neurodegenerative diseases. Reduced levels of cortical nicotinic binding sites have been repeatedly described in Parkinson's disease. These results point at the possibility that the development of dementia – an important additional symptom that occurs in nearly every second patient affected by Parkinson's disease – is associated with a loss of cortical nicotinic acetylcholine receptor expression, as described in Alzheimer's disease.

Although studies have already been performed on the dysfunction of the cholinergic system in Parkinson's disease, to our knowledge, no study has distinguished between demented and non – demented individuals. Therefore, the expression and the protein amount of the α4 nicotinic acetylcholine receptor subtype was assessed by immunohistochemistry and Western blot in the temporal cortex of patients suffering from Parkinson's disease with and without dementia and compared to controls.

No statistical significant changes in the α4 subunit expression could be found in the receptor expression or the protein amount in both patient groups compared to the control group.

Recent studies on k. o. mice have questioned the specifity of a number of subunit specific antibodies including the two antibodies applied in this study thus demanding for a cautious interpretation of this study's findings. It is, however, still likely that alterations in the cholinergic circuit are responsible for cognitive decline in Parkinson's disease and therefore this neuronal system might represent a potential, therapeutic target. Further studies including in vivo binding studies should shed light on this still unclear situation.

7 Zusammenfassung

Nikotinische Acetylcholinrezeptoren stellen einen bedeutenden Vertreter der Neurotransmitterrezeptoren dar und sind im Nervensystem weit verbreitet. Aufgrund ihrer wichtigen Funktionen innerhalb einer Vielzahl neuronaler Schaltkreise scheinen diese Rezeptoren eine pathogenetische Schlüsselrolle bei neurodegenerativen Erkrankungen zu haben. Es wurde wiederholt eine Reduktion nikotinischer Bindungsstellen bei Morbus Parkinson beschrieben. Diese Ergebnisse weisen auf die Möglichkeit hin, dass die Entwicklung von Demenz – ein wichtiges, zusätzliches Symptom, das bei beinahe jedem zweiten an Morbus Parkinson erkranktem Patienten auftritt – mit einem Verlust kortikaler, nikotinischer Acetylcholinrezeptorbindungsstellen einhergeht, wie auch bei Morbus Alzheimer beschrieben.

Gleichwohl Untersuchungen zur Dysfunktion des cholinergen Systems bei Morbus Parkinson durchgeführt wurden, hat nach unserem Kenntnisstand keine Studie zwischen dementen und nicht – dementen Patienten unterschieden. Daher wurde hier die Expression und die Proteinmenge der α4 Untereinheit des nikotinischen Acetylcholinrezeptors mittels Immunhistochemie und Western blot im Temporalkortex von Patienten, die an Morbus Parkinson mit und ohne Demenz litten, untersucht und mit einer Kontrollgruppe verglichen.

Es konnten hierbei keine statistisch signifikanten Unterschiede in der Rezeptorexpression oder der Proteinmenge in beiden Patientengruppen im Vergleich zu der Kontrollgruppe festgestellt werden.

Aktuelle Untersuchungen an nAChR knock out Mäusen haben die Spezifität diverser, für Untereinheiten spezifische Antikörper in Frage gestellt. Dies beinhaltet die beiden im Rahmen dieser Studie verwendeten Antikörper, so dass eine vorsichtige Interpretation der Ergebnisse dieser Studie zu fordern ist. Es ist jedoch dennoch wahrscheinlich, dass Veränderungen des cholinergen Schaltkreises für kognitive Störungen bei Morbus Parkinson verantwortlich sind und daher dieses neuronale System ein potentielles, therapeutisches Ziel darstellt. Zusätzliche z.B. in vivo Bindungsstudien sollten diese unklare Situation weiter erhellen.

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10 Lebenslauf

Mein Lebenslauf wird aus Gründen des Datenschutzes in der elektronischen Fassung meiner Arbeit nicht veröffentlicht.