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Parkinson associated protein LRRK2

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ZUSAMMENFASSUNG

Die Parkinsonsche Erkrankung (PD) ist eine chronisch, progressiv verlaufende, neurodegenerative Krankheit, die vor allem bei älteren Menschen vorkommt. Dabei kommt es in vielen Hirnregionen, vor allem aber in der Pars compacta der Substantia nigra, zum Absterben von dopaminergen Neuronen. Zu den damit verbundenen klinischen Symptomen zählt verlangsamte Bewegung, Muskelzittern, Haltungsinstabilität und Muskelsteifheit. Obwohl die Ursache für die Erkrankung noch unbekannt ist, wurden einige Faktoren entdeckt, die für den Ausbruch und Fortschritt der Erkrankung verantwortlich sind. Zu Diesen zählen synaptische Proteine, Chaperone sowie Kinasen, welche im mutierten oder amplifizierten Zustand zu frühzeitigem Ausbruch der Krankheit führen. LRRK2, eine Leucine-rich repeat Kinase, ist bei den meisten familiären PD Fällen mutiert. Deshalb scheint das LRRK2 Gen eine wesentliche Rolle bei der Entstehung der Krankheit zu spielen. Derzeit existiert nur ein hLRRK2 Tiermodell, das die wichtigsten Aspekte von PD aufzeigt, allerdings mit einer Mutation die nur in wenigen PD Fällen auftritt. Um die genauen Effekte von LRRK2 und dessen Rolle in PD zu studieren, wurde eine wildtyp-hLRRK2 Mauslinie entwickelt und charakterisiert.

hLRRK2 cDNA wurde erfolgreich in einen neuronenspezifischen Expressionsvektor kloniert, was durch Sequenzierung, verschiedene Restriktionsverdaue und Expressionsanalyse in eukaryotischen Zellen bestätigt wurde. Unter dem starken CMV Promoter konnte das Protein bei ca. 280 kD detektiert werden, wodurch die Funktionalität des cDNA Konstrukts bestätigt wurde. Der neuronenspezifische, schwächere Thy1.2 Promoter induziert ebenfalls die vollständige Expression von hLRRK2. Vier Founder Linien mit jeweils 1, 6, 20 und 60 Kopien des Transgens wurden charakterisiert. qPCR mit RNA aus Hirngewebe zeigte eine leichte Überexpression der hLRRK2-RNA, das Protein konnte aber weder durch WB noch IHC in den vier Monate alten Tieren nachgewiesen werden. Eine anfängliche Charakterisierung mittels Pole, Beam und Grip-Test konnte bei den transgenen Mäusen keine motorischen Defekte feststellen, was wahrscheinlich auf die niedrige Expression des Konstrukts in den jungen Tieren zurückzuführen ist.

Zusammenfassend zeigen unsere Ergebnisse, daß die Expression von LRRK2 unter dem neuronenspezifischen Thy1.2 Promoter lebensfähige Nachkommen

hervorbringt. Diese Tiere können, wenn sie PD typische Symptome im höheren Alter aufzeigen, als Modell für die Erkrankung verwendet werden. Dieses neuartige Tiermodell wäre einerseits geeignet, Funktion und Rolle von LRRK2 im Verlauf von PD zu studieren, andererseits könnte es zur Entwicklung und Testung neuartiger Medikamente und Behandlungsmethoden verwendet werden.

SUMMARY

Parkinson's disease (PD) is a chronic and progressive neurodegenerative disorder primarily found in elderly people. Neuronal loss of dopaminergic neurons in the brain, especially the pars compacta of the substantia nigra, leads to clinical symptoms such as slowing of movements, resting tremor, postural instability and rigidity. Although the cause of the disease is still unknown several factors were recently identified, which correlate with onset and progression of the disease. Such factors include synaptic proteins, chaperones as well as kinases, which were shown to lead to early onset of the disease when amplified or mutated. LRRK2 a leucine-rich repeat kinase is the protein most frequently found to be mutated in familial forms of PD. Therefore the LRRK2 gene seems to play an essential role in the onset of the disease. At present there is only one animal model for LRRK2 described, which mimics some aspects of the disease, but harbours a mutation which is found only in a minor percentage of all PD cases. To study the effect of LRRK2 and its role in PD a mouse model transgenic for the human LRRK2 protein was generated and initially characterized.

hLRRK2 cDNA constructs were successfully cloned into a neuron specific mammalian expression vector, which was verified by restriction digest, sequencing and expression analysis in eukaryotic cells. Under the strong CMV promoter, protein was detectable at around 280 kD, indicating functionality of the cDNA construct. The neuron specific weaker Thy1.2 promoter also induced expression of hLRRK2 in CHO or HEK cells. Four founder lines were characterised harbouring 1, 6, 20 and 60 copies of the transgene. Although qPCR from brain derived RNA resulted in moderate over-expression of the hLRRK2 RNA, hardly any LRRK2 protein was detectable in WB or IHC in 4 month old animals. An initial motor characterization of the mice failed to show detectable defects as tested by pole, beam or grip-test, probably due to the low expression of the construct in these young mice.

Taken together our results show that expression of LRRK2 under the neuron specific Thy1.2 promoter results in viable offspring, which might be a useful model for PD, when showing PD like symptoms at older age. Such a model should be highly valuable to obtain deeper insight into the role of LRRK2 in PD and will serve as a new tool for drug evaluation and development.

ABBREVIATIONS

aa	amino acid
AB	antibody
Amp ^r	ampicillin resistance
ANK	ankyrin domain
APS	ammonium persulfate
BAC	bacterial artificial chromosome
biot.	Biotinylated
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CDS	coding sequence
CHO	Chinese hamster ovary cells
CMV	cytomegalovirus
CNS	central nervous system
COMT	catechol-O-methyl transferase
COR	C-terminus of ROC domain
C-terminus	carboxy terminus
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
DH5 α	competent cells
diff.	differentiated
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
dsDNA	double strand deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid

FCS	foetal calf serum
FICC	fluorescence immunocytochemistry
Fig.	figure
fw	forward
GAPDH	glyceraldehyde-3-phosphatdehydr ogenase
gDNA	genomic deoxyribonucleic acid
GFP	green fluorescent protein
h	human
HEK	Human embryonic kidney cells
HRP	horse radish peroxidase
Hz	hertz
ICC	immunocytochemistry
IHC	immunohistochemistry
IL	interleukine
IMDM	Iscoe´s modified Dulbecco´s medium
kb	kilo base pairs
kD	kilo Dalton
LB	lysogeny broth
L-dopa	Levodopa, L-3,4-dihydroxyphenylalanine
LRR	leucine-rich repeat domain
LRRK2	Leucine-rich repeat kinase 2
m	mouse
MAO-B	monoamine oxidase B
MAPKKK	mitogen-activated protein kinase kinase kinase domain
MCS	multi cloning site
mRNA	messenger ribonucleic acid
NaOV	sodium ortho vanadate
NEAA	non-essential amino acid
neg.	negative
N-terminus	amino-terminus
NTP	nucleotide triphosphate
OD	optical density
Oligo dT	poly deoxythymidine
ORF	open reading frame

PBS	phosphate buffer ed saline
PBST	phosphate buffer ed saline tween
PCR	polymerase chain reaction
PD	Parkinson's disease
PFA	paraformaldehyde
qPCR	quantitative polymerase chain reaction
r	rabbit
RA	retinoic acid
rec.	recombinant
rev	reverse
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RNase	ribonuclease
ROC	Ras of complex domain, GTPase
Rpl32	ribosomal protein L32
RT	reverse transcriptase
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodiumdodecylsulphate
SH-SY5Y	neuroblastoma cells
sp.c.	spinal cord
sqPCR	semi-quantitative PCR
SS II RT	reverse transcriptase
stom.	Stomach
TAE	Tris acetic acid EDTA
tg	transgene
TH	tyrosine hydroxylase
Thy1.2	glycophosphatidylinositol linked surface protein
WB	Western Blotting
WD40	tryptophan-aspartic acid 40 dipeptide repeat
wt	wild type

1 INTRODUCTION

1.1 Parkinson's disease

Parkinson's disease (PD) is named after the physician James Parkinson who first described the disease in 1817 in his work titled "An Essay on the Shaking Palsy" in which he mentioned six patients with PD symptoms [1].

The disease is a chronic and progressive neurodegenerative disorder mainly affecting the extrapyramidal motor system. People affected by the disease show symptoms including slowing of movement (bradykinesia), resting tremor (4-6 Hz tremor, most noticeable at resting position), postural instability (loss of balance) and rigidity (stiffness with increased muscle tone). Secondary symptoms include cognitive dysfunction, depression and aphasia [2]. Symptoms are mainly caused by death of neurons producing the neurotransmitter dopamine in the pars compacta of the substantia nigra. Other regions of the brain stem including the locus coeruleus, raphe nuclei and others are also affected by cell loss [3].

The incidence of the disease increases with age, especially occurring with the age of 60 and older. Male persons have an 1.9 fold higher incidence to get PD than females. An exception is the Asian population where the risk to develop the disease is lower in men. Incidence rates concerning race/ethnicity are the highest for Hispanics, then Non-Hispanic Whites, Asians and lowest for Blacks [4]. In Austria ~ 30.000 people, in Germany ~250.000 people and in America more than 1.500.000 people (more than 60.000 new cases per year) are affected by the disease. The average duration from diagnosis to death is currently 15 years, with pneumonia as the most common cause for demise [1].

1.1.1 Classification of PD

There are four different subclasses of PD which are all characterized by bradykinesia, tremor, rigidity and postural instability which are classified as follows (classification is based on symptoms of the Parkinson's disease which are described in the EMEA Guideline on Clinical Investigation of Medicinal Products in the Treatment of Parkinson's Disease (CPMP/EWP/563/95 Rev.1)):

- Familial Parkinson syndromes originate from mutations in different proteins (eg. LRRK2, alpha-synuclein, parkin and others)
- Idiopathic Parkinson syndromes with unknown cause (~75% of PD cases) .
- Secondary PD syndromes due to drug misuse (e.g. MPTP) or different diseases which cause these symptoms (e.g. vascular disease, metabolic disease, inflammation or after brain injury).
- Other neurodegenerative diseases which cause Parkinsonism such as multiple system atrophy, progressive supranuclear palsy, corticobasal degeneration and dementia with lewy bodies are summarized as PD+ syndromes.

1.1.2 Pathophysiology

Parkinson symptoms are caused by decreased secretion of the neurotransmitter dopamine from neurons of the substantia nigra a substructure of the mesencephalon. Loss of these neurons leads to an imbalance of movement regulation, by blocking brain regions which facilitate movement and in contrast by stimulating regions which inhibit movement (both in the basal ganglia). The subsequently decreased dopamine levels lead to inhibition of excitatory projections from the ventral anterior nucleus of the thalamus to the motor cortex, leading to reduced motor activity (Fig.1 adapted from: http://en.wikipedia.org/wiki/Parkinson%27s_disease). Additionally, other dopaminergic pathways are affected which explain the neuropsychiatric pathology of the disease.

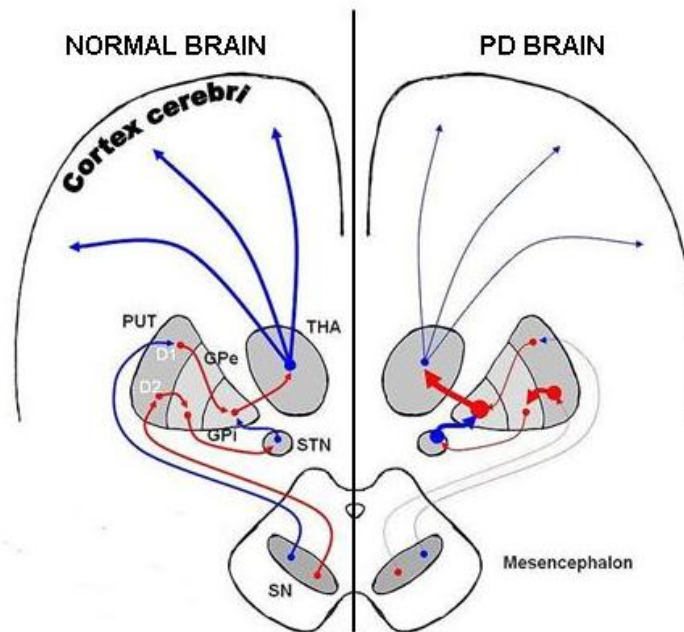


Fig.1 Dopamine pathway in normal brain (left side) and PD brain (right side). Red arrows indicate inhibiting pathways, blue arrows indicate stimulating pathways. In the normal brain, dopamine binds in equal amounts to dopamine receptors D1 (activating) and D2 (inactivating) in the putamen, which in consequence leads to stimulation of the cortex cerebri by the thalamus. In PD brain, reduced dopamine levels lead to excitation of the globus pallidus internus which inhibits excitatory activity of the thalamus. In consequence PD patients show hypokinesia.

GPe – Globus pallidus externus, GPi – Globus pallidus internus, PUT – Putamen, SN – Substantia nigra, STN – Nucleus subthalamicus, THA – Thalamus

1.1.3 Causes of PD

Although Parkinson's disease is a sporadic disorder of unknown cause, several factors have recently been discovered which correlate with the onset and progression of the disease. Several mutations in different genes causing PD symptoms have been identified.

Although the direct cause of the sporadic disease is currently still unknown, many factors are believed to be involved in the induction of neuronal death. These factors include the aggregation of α -synuclein (caused by mutations, multiplication or abnormal processing), substrate accumulation, abnormal ubiquitination, generation of reactive oxygen species, inappropriate phosphorylation, protein misfolding and loss of cellular protection [5], [6] (Fig.2).

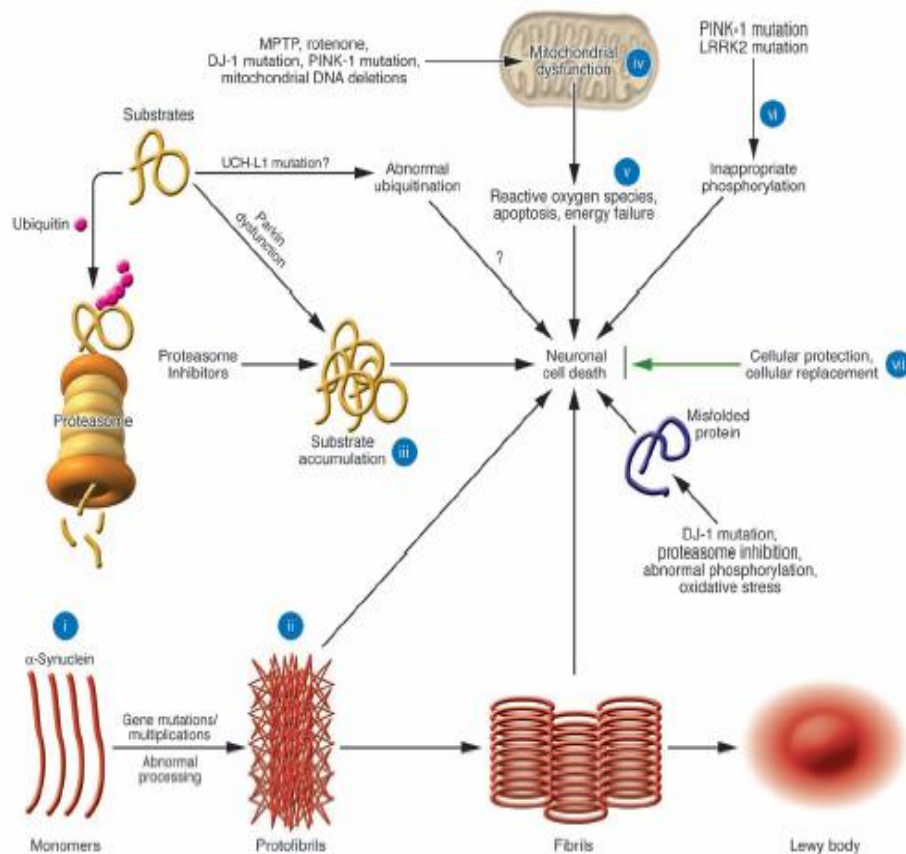


Fig.2 Parkinson's disease factors leading to neuronal death. Factors leading to dopaminergic cell death include alpha-Synuclein aggregation to protofibrils and fibrils, substrate accumulation, abnormal ubiquitination, mitochondrial dysfunction, inappropriate phosphorylation, misfolded proteins and loss of cellular protection. Potential targets for therapy (blue circles): gene silencing (i), prevention of aggregation (ii), down regulate toxic substrates (iii), enhance mitochondrial function (iv), radical scavengers and antioxidants (v), block LRRK2 kinase activity (vi), trophic factors or cell replacement (picture taken from Savitt et al. [5]).

One of the most frequently detected genes mutated in familial and sporadic PD is coding for the Leucine-rich repeat kinase 2 (LRRK2, PARKIN 8). So far six mutations in LRRK2 are associated with the disease causing autosomal dominant PD [7]. Another gene found to be mutated, duplicated or triplicated in PD is α -synuclein [8]. In addition, loss of function mutations in the genes Parkin, DJ-1, PINK1 and ATP13A2 cause recessive late onset PD. Heterozygous loss of function mutations in the glucocerebrosidase (GBA) gene increase the risk to develop PD [9].

Toxins including pesticides and metals like iron and manganese are causative for development of PD [10]. Finally factors like brain injury, rural living, lack of exercise, well-water ingestion and middle-age obesity are additional risk factors of PD [1], [11].

1.1.4 Diagnosis

A challenge for PD diagnosis is the overlap of symptoms with other neurodegenerative diseases (e.g. Alzheimer's disease) and the sub-classification of the disease. Clinically the most prominent symptom of PD is bradykinesia (slowness of initiation of voluntary movement and reduction in speed of repeated actions [1]; and should always be diagnosed if no specific other cause is present. Additionally resting tremor (4-6 Hz), muscular rigidity, postural instability and good response to L-dopa help to confirm diagnosis.

Finally combining traditional with novel molecular biological methods or PET scan techniques will help to properly diagnose the disease.

1.1.5 Therapy

Currently no drug is available for PD patients curing the cause of the disease. Only symptoms caused by the disease can be alleviated. At present L-dopa (Levodopa, L-3,4-dihydroxyphenylalanine) a precursor of dopamine, noradrenalin, adrenaline and melanin, combined with dopamine agonists and peripheral dopa decarboxylase inhibitors (benserazide or carbidopa; prevent metabolism of L-dopa) are used to compensate for the dopamine deficit thereby reducing PD symptoms. Usually patients get 300-600 mg/day in the first five years of the disease improving bradykinesia, rigidity, gait, swallowing, speech, postural instability and fatigue. Tremor symptoms can, if at all only be reduced after long time drug therapy [12]. Adverse effects of this drug therapy include nausea, anorexia and faintness. Dopamine-agonists (pramipexole, ropinirole, rotigotine and priribedil) activate dopamine receptors and substitute the function of L-Dopa causing the similar adverse events except of dyskinesias. Mainly patients under the age of 55 years receive this drug [1]. To reduce the early wearing-off of L-dopa activity patients additionally receive COMT inhibitors (Catechol-O-methyl transferase inhibitor; entacapone) and MAO-B inhibitors (Monoamine oxidase inhibitors; selegiline or rasagiline) [13]. COMT and MAO-B enzymes are usually responsible for degradation of dopamine, prevention of reaction leads to an increased half-life of dopamine in the synaptic cleft. Other therapeutic strategies include physical exercise and therapy,

logopedic therapy (to improve speech and swallowing) and occupational therapy (to improve everyday life).

As mentioned above mutations in the Leucine-rich repeat kinase 2 (LRRK2) gene are associated with most cases of familial autosomal dominant PD [14].

1.2 Leucine-rich repeat kinase 2 (LRRK2)

LRRK2 gene is located in the human genome on the long arm of chromosome 12 (12q12) composed of 51 exons (Entrez GeneID: 120892). The mRNA is 7 kb of size coding for a protein of 2527 amino acids. The LRRK2 protein is a multi-domain protein with approximately 280 kD.

People carrying mutations in this gene develop clinical symptoms that are indistinguishable from familial or sporadic late-onset PD. Until now there are several mutations in the LRRK2 protein known leading to pleomorphic pathology and autosomal dominant inheritance [15].

1.2.1 LRRK2 domains

The LRRK2 protein is composed of several domains with different functions. Starting with the N-terminus of the protein there is an ankyrin domain (ANK), the leucine-rich repeat (LRR), the Ras of complex (GTPase; ROC), the C-terminus of ROC domain (COR), the MAPKKK domain (mitogen-activated protein kinase kinase kinase; serine/threonine kinase) and the WD40 domain [16]. The ANK, LRR and the WD40 domains are responsible for protein/protein interactions with cytoskeleton proteins, transcriptions factors and proteins regulating signaling and cell cycle. The MAPKKK domain shows sequence homology to members of the serine/threonine kinase family which regulate cell survival and cell-death in response to intra- or extra-cellular signals [17], [18]. The ROC and COR domains have sequence homology with members of the ROCO family (GTPases).

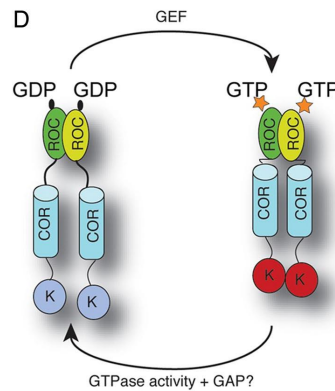


Fig.3 Kinase activity regulation by ROC domains. Dimeric formation of the ROC domains (green and yellow) bind GTP (star) which leads to further dimerisation of COR and kinase domain. Hydrolysis of the GTP activates kinase domains, the produced GDP induces conformational changes with resulting separation of the dimers. (picture taken from Deng et al. [19])

The function of the COR domains has to be clarified, the ROC GTPase domain seems to have regulatory functions in vesicle formation, vesicle movement on the cytoskeleton and membrane fusion [20]. The GTPase activity needs dimerisation of the two ROC domains for GTP hydrolysis and activation of the MAPKKK domain [19] [21] (Fig.3). Although the functions of the different domains are known, the cellular function of LRRK2 protein remains elusive.

1.2.2 Mutations

Several missense mutations were found in the LRRK2 gene accounting for up to 13% of familial PD and 5% of sporadic PD [22], [23] (Fig 4). This number is unexpectedly high for a disease that was thought not to be genetically determined. These autosomal dominant mutations are leading to the same phenotype in homozygous and heterozygous patients. PD relevant mutations are mainly located in the kinase (MAPKKK) and the GTPase domain (Roc-COR domain), leading to increased kinase activity [24].

The most prevalent mutation found to date in the Mediterranean countries is the G2019S mutation in the kinase domain, leading to increased enzymatic activity [25].

Of note not all mutations lead to increased activity. R1441C and R1441G mutations decrease GTPase activity and result in increased kinase activity [21]. The mutations R1941H, I2012T, I2020T and G2385R inhibit kinase activity including auto-phosphorylation and phosphorylation of MBP (myelin basic protein) and moesin. The mutations Y1699C and T2356I have no effect on catalytic activity [26]. In contrast Gloeckner and co-workers found that in their experiments the mutations Y1699C and I2012T are able to increase kinase activity [27]. Also other functional properties of the LRRK2 protein are affected by mutations including protein interaction domains which in consequence alter enzymatic activity [28].

Although a lot of studies were performed to clarify the function of LRRK2 domains still contradictory results remain in the literature.

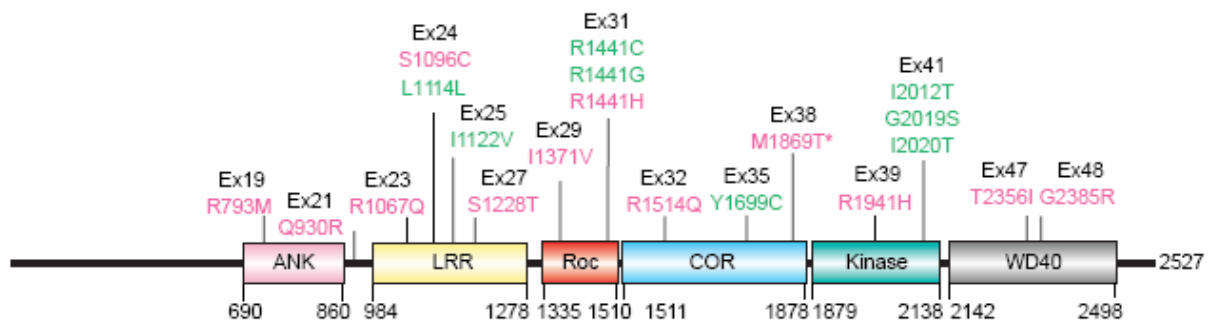


Fig.4 LRRK2 domains and mutations. Starting from the N-terminus the first domain is the ankyrin domain (ANK) followed by the leucine-rich repeat domain (LRR) (both protein/protein interaction domains), then the Ras of complex domain (Roc; GTPase), the C-terminus of Roc (COR), the MAPKKK domain and the protein/protein interaction domain WD40. Indicated are amino acid substitutions linked with the pathology (magenta), mutations that are not linked with the disease (green) and the corresponding exon location (black). Numbers beneath indicate the approximate boundaries of the domains. (picture from Mata et al., [16])

1.2.3 Interaction partners

As mentioned above, the LRRK2 protein shows enzymatic activity as a dimer. Interaction motives include the ROC domain, the LRR domain the WD40 motive and N-terminal regions (Deng et al.; PNAS 2008; 105(5):1499-504). LRRK2 interacts with the cytoskeleton (α -tubulin of microtubules and vimentin) and cytoskeleton associated proteins [29]. These proteins include moesin, ezrin and radixin, which link the actin-cytoskeleton to the plasma membrane [26].

Another LRRK2 interacting protein is clathrin, which is involved in vesicle formation [30]. Interaction could also be detected with the Rab5a protein involved in retrograde vesicle transport [31].

LRRK2 protein also seems to interact with chaperons like Hsp90 and other proteins involved in phosphorylation and protein translation [30].

Proteins involved in neurodegenerative diseases were also tested for interaction with LRRK2 protein. LRRK2 interacts with parkin, a protein of the E3 ubiquitin ligase complex, responsible for ligation of ubiquitin residues on proteins, targeting them for degradation. Interestingly LRRK2 does not interact with α -synuclein, tau or DJ-1, proteins found to be involved in fam. PD [32]. Recent studies could demonstrate that LRRK2 protein interacts with elements of the extrinsic cell death pathway including Fas-associated protein with death domain (FADD) and Caspase-8. Mutations in the LRRK2 gene were also shown to trigger this pathway leading to increased cell death after application of H_2O_2 as external stress signal [33].

1.2.4 Localization

In the brain, the LRRK2 protein is constitutively expressed in neurons, as well as in astrocytes and microglia cells, although at a lower level [34]. Cells expressing the LRRK2 protein are mainly located in the cerebral cortex, caudate-putamen, hippocampus and in dopaminergic neurons of the substantia nigra pars compacta [35], [36]. Paisán-Ruíz and coworkers (2004) could also detect LRRK2 expression by Northern Blot in other tissue such as liver, lung, heart and kidney [14].

In the cell, LRRK2 is mainly localized in the cytoplasm where it associates with lipid rafts, early endosomes, mitochondrial membranes, lysosomes, synaptic vesicles, cytoskeleton, golgi apparatus and the endoplasmic reticulum [37].

Colocalization of the LRRK2 protein with membranes and its association with cytoskeletal proteins implies that LRRK2 might play a role in membrane trafficking [37].

1.2.5 Cellular effects of LRRK2 mutations

The toxic effects of the LRRK2 protein in-vitro are generated by altered kinase activity [25]. Dopaminergic cell lines and primary neurons with increased kinase activity, resulting from LRRK2 mutations (e.g. G2019S), show increased apoptosis [38]. In contrast, a decreased kinase activity caused by altered ROCO and kinase domain (e.g. D1994N, DY2017-2018AL and K1906A), leads to reduced neuronal cell death.

Another cellular effect of the LRRK2 protein is the kinase dependent neurite outgrowth. MacLeod et al. [39] showed a reduced dendritic outgrowth and branching of axons and dendrites in primary neuronal cultures when LRRK2-kinase activity was increased. Cells expressing LRRK2 protein harboring mutations in the kinase domain (G2019S, I2020T) and ROC domain (R1441G) all showed this reduction, while cells expressing mutant proteins with mutations located within the COR domain like Y1699C or cells over-expressing wild-type LRRK2 showed no significant alterations [39].

1.2.6 Animal models

Until now just a few transgenic animals over-expressing wt and mutant LRRK2 are described.

Transgenic *Drosophila* were generated, expressing wt and mutant LRRK2 [40]. Flies with mutant LRRK2 show late onset loss of dopaminergic neurons and a reduced lifespan.

A transgenic mouse line with an N-terminal truncated form of human LRRK2 under Thy1.2 promoter was generated (E. Masliah, personal communication) but no obvious defect has been reported until now.

Recently, Li and coworkers developed a transgenic mouse-line over-expressing human LRRK2-R1441G from a bacterial artificial chromosome (BAC). Transgenic animals show some of the cardinal features of the disease, including age-dependent and L-dopa-responsive slowness of movement and reduced dopamine release in CNS. In contrast a mouse line over-expressing wild-type human LRRK2 did not develop age-dependent motor deficits [41].

The observed phenotypes and molecular alterations in these models are typical for PD-like neurodegeneration. Therefore a detailed analysis is required to evaluate whether LRRK2 is a target for potential novel treatment strategies against PD.

Therefore, a transgenic mouse line over-expressing human LRRK2 has been developed recently.

Aim of the diploma thesis was the expression analysis of the vector used for transgene production and an initial characterization of the transgenic animals over-expressing full length, wild type, human LRRK 2.

2 MATERIALS AND METHODS

2.1 Cloning and plasmid expression analysis

2.1.1 Eukaryotic expression vector pCMV6-XL5-LRRK2 (ORIGENE)

Human LRRK2 cDNA (7,7 kb) was cloned into the NotI site of the MCS of pCMV6-XL5 performed by ORIGENE. LRRK2 CDS is under control of the CMV promoter allowing high expression in eukaryotic cell types. This vector was used for transfection into CHO and HEK 293 cells to test LRRK2 expression.

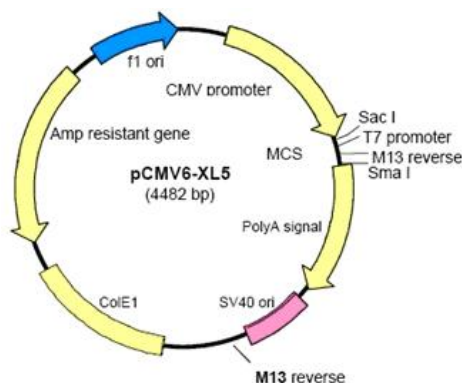


Fig.5 pCMV6-XL5 plasmid (taken from <http://www.origene.com/cdna/trueclone/vectors.msp>). Not I digestion and integration of LRRK2 gene into MCS for expression analysis. Ampr, selection marker in *E.coli*; CMV promoter, cytomegalovirus promoter for *in vivo* expression in mammalian cells; SV40 ori, replication in mammalian cells; ColE1, bacterial origin of replication; F1 ori, filamentous phage origin of replication; T7 promoter, *in vitro* transcription

2.1.2 Expression vector pUC18-Thy1.2-LRRK2 (Polygene)

Plasmid pUC18-Thy1.2-LRRK2 contains a Thy1.2 promoter normally coding for a glycoposphatidylinositol linked surface protein expressed in cells of thymus, nervous system and connective tissue. The modified Thy1.2 promoter with integrated LRRK2 CDS allows specific expression in nervous system and in the lung [42]. Cloning was performed by Polygene. This vector was used to test expression of LRRK2 in cells and for generation of hLRRK2 transgenic mice.

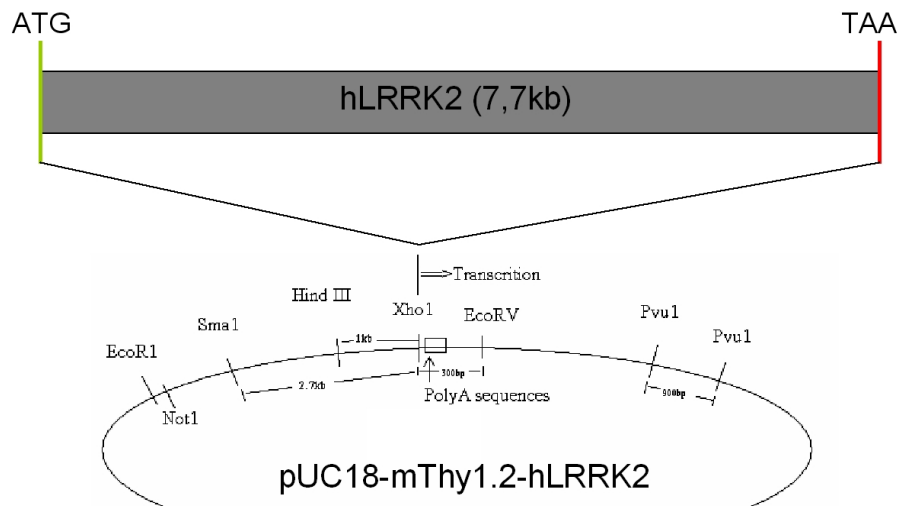


Fig.6 pUC18-Thy1.2-LRRK2 plasmid XhoI integration site of LRRK2 cDNA with intact Kozak consensus sequence and full length, non-interrupted ORF. Complete length of the plasmid with LRRK2 cDNA (7,7 kb), Thy1.2 cassette and pUC18 backbone is ~17kb. pUC18 vector contains *bla* gene, coding for β -lactamase providing ampicillin resistance and the pMB1 replicon *rep* for plasmid replication.

2.1.3 Transformation

The process for introducing plasmid DNA into bacteria (DH5 α) is called transformation.

20 μ l DH5 α competent cells (Invitrogen) were thawed on ice for 5 minutes and mixed with 1 μ l pCMV6-XL5-LRRK2 or Thy1.2-LRRK2 plasmid. Then DH5 α were placed back on ice for 30 minutes and then incubated 2 minutes at 42°C. After that, bacteria were placed 3 minutes on ice, LB medium (Roth) was added and was incubated 1 hour at 37°C. Then bacteria were pelleted (2000 rpm for 5 minutes) and resuspended in 100 μ l fresh LB medium (Roth), 20 μ l plated on LB agar containing ampicillin to select for transformed bacteria. Plates were incubated over night on 37°C to get single clones containing the plasmid.

2.1.4 Maxi prep - Plasmid DNA Purification with QUIAGEN Maxi Kit (Protocol from QIAprep Handbook)

For cloning and transfection high amounts of vector DNA were needed. Therefore large volumes of transformed bacteria were cultivated to obtain enough plasmid for the planned experiments. Isolation of big amounts of plasmid DNA from bacterial cultures is called maxi prep.

Bacterial culture, harvest, and lysis

100 ml overnight LB culture was pelleted with 6000 x g for 15 min at 4°C. The pellet was resuspended in 10 ml buffer P1 (containing RNase A), then 10 ml buffer P2 was added and the solution was inverted for 4-6 times and incubated at room temperature for 5 minutes. After that 10 ml buffer P3 was added and again inverted 4-6 times.

Bacterial lysate clearing

The lysate was transferred to a QIAfilter Cartridge and incubated at RT for 10 min. The cap was removed from the QIAfilter Cartridge outlet nozzle, the plunger inserted into the QIAfilter Maxi Cartridge and the cell lysate filtered into a 50 ml tube. After that 2,5 ml buffer ER was added to the filtered lysate, inverted 10 times and incubated 30 minutes on ice.

Binding, wash and elution of the plasmid DNA

The QUIAGEN-tip 50 was equilibrated by applying 10 ml Buffer QBT and allowed the column to empty by gravity flow. The filtered lysate was applied to the QUIAGEN-tip and allowed it to enter the resin by gravity flow. After that the QUIAGEN-tip was washed with 2 x 30 ml buffer QC. DNA was eluted with 15 ml Buffer QN.

Precipitation, wash and redissolving of the plasmid DNA

DNA was precipitated by adding 10,5 ml isopropanol to the eluted DNA and mixed. Then the solution was centrifuged at 15,000 g for 30 min at 4°C and the supernatant carefully decanted. The DNA pellet was washed with 5 ml of endotoxin-free room-temperature 70% ethanol and centrifuged at 15,000 g for 10 min. The supernatant was again decanted carefully and the pellet air dried for 5-10 minutes. DNA was redissolved in endotoxin-free buffer TE.

Determination of DNA concentration in the eluate was done by measurement of optical density (OD) with 260 nm wavelength (Photometer , Perkin Elmer Lambda 2).

$$\text{OD}_{260 \text{ nm}} 1 = 50 \mu\text{g} / \text{ml} = 50 \text{ ng} / \mu\text{l}$$

2.1.5 Restriction enzyme digestion

Restriction enzyme digestion was done from Maxi-prep DNA eluates. Enzyme NotI (Fermentas) was used for plasmid pCMV6-XL5-LRRK2 to cut out the LRRK2 gene (7kb) from the vector. For plasmid pUC18-Thy1.2-LRRK2 endonucleases Pvu I (Fermentas), Xho I (Fermentas), EcoR V (New England BioLab), EcoR I (Fermentas) and Hind III (Fermentas) were used independently for analysis. 1 μg plasmid DNA was incubated for 1 hour at 37°C with 10 u of enzyme. Then samples were loaded on 1% Agarosegel with SYBRsafe (Invitrogen) and run with 100V for 2 hours. Imaging was done with UV-trans-illuminator to detect DNA bands.

2.1.6 PCR

In the polymerase chain reaction sequence specific primers bind to DNA and allow amplification by Taq polymerase (Promega 5u/ μl). Different templates were used, including DNA from bacteria, DNA from cell culture and genomic DNA from mice. Primer pairs used for analysis are listed below.

Reagents used for one PCR reactions were mixed in 200 μl thin wall PCR tubes:

Reagents	volumes
Template DNA	1,00 μl
10x reaction mix (Roche)	2,50 μl
Taq polymerase	0,25 μl
10mM dNTPs (Promega)	0,50 μl
MgCl ₂ (50 mM)	1,00 μl
Forward primer	0,50 μl
Reverse primer	0,50 μl
MiliQ water	18,75 μl

25,00 μl / reaction

Tubes were placed into Biometra T3 Thermocycler and PCR was performed as follows:

- 90°C for 2 min. (DNA-denaturation step)
- PCR amplification: 35 cycles of:
 - 90°C for 15 sec.
 - 57°C for 30 sec.
 - 72°C for 1 min. 30 sec.
- Final extension:
 - 72°C 5 min.
 - 4°C hold

2.1.7 Semi-quantitative PCR

For determination of the approximate amount of LRRK2 transgene in genomic DNA samples, semi-quantitative polymerase chain reaction (sqPCR) was done. Primers used for internal control were specific for the control gene mGAPDH, LRRK2 specific primer 4/5 were used for transgene detection (details below). PCR conditions used were the same as mentioned above, but an aliquot for subsequent analysis was taken after 16, 18, 21, 24, 27, 30, 33 and 36 cycles.

2.1.8 Quantitative PCR with iQ SYBR green Supermix (BIO RAD)

Quantitative PCR (qPCR) was either used to determine the amount of LRRK2 transgenes in gDNA of transgenic mice, or to analyse LRRK2 RNA expression compared to an internal control gene. This method detects the amount of synthesised dsDNA in a PCR reaction in real time. We used the fluorochrome SYBR green (BIO RAD) which intercalates with dsDNA only to detect increasing amounts of dsDNA during synthesis.

Samples were combined with primers and iQ SYBR green Supermix (BIO RAD) in tubes and analysed in the MyiQ Cyclor (BIO RAD).

2x iQ SYBR green Super mix (BIO RAD)

100 mM	KCl
40 mM	Tris-HCl; pH8
0,4 mM	of each dNTP
50 U/ml	iTaq DNA Pol.
6 mM	MgCl ₂
20 nM	fluoresceine SYBR Green I
Stabilizers	

PCR conditions

3 minutes	95°C (initial denaturation step)
40 repeats of:	
10 sec.	95°C
20 sec.	57°C (anneal)
20 sec.	72°C (PCR)
6 sec.	80°C (Real time)
Melting curve (57 steps)	
6 sec.	55,0-94,2°C (0,7°C temperature steps)

After PCR, results were analysed with the MyiQ cyclor software (BIO RAD) and additionally by conventional gel electrophoresis.

2.1.9 Primer

Primers used for PCR, RT-PCR, sqPCR and qPCR were synthesised by Microsynth.

Primers specific for control genes:

- IL2 primer (gDNA)
forward: 5'-CTA GGC CAC AGA ATT GAA AGA TCT-3'
reverse: 5'-GTA GGT GGA AAT TCT AGC ATC ATC-3'

- Rpl32 primer (cDNA)
forward: 5'-CCA TCT GTT TTA CGG CAT CA-3'
reverse: 5'-ATT GTG GAC CAG GAA CTT GC-3'
- mGAPDH primer (mouse, NM_001001978) (cDNA)
forward: 5'-GAG TAT GTC GTG GAG TCT ACT GG-3'
reverse: 5'-CCA TCC ACA GTC TTC TGG GTG GCA-3'

Transgene specific primers used bind to the human LRRK2 coding sequence (cDNA) in the plasmid and to the expressed LRRK2 mRNA. These primers are able to detect presence of the transgene on DNA and RNA level. Two different primers were used for analysis, one binding to the N-terminus and one to the C-terminus of the transgene. Hence, it is possible to detect full length hLRRK2 DNA and mRNA.

Primer binding to the N-terminus of the LRRK2 gene:

LRRK2 fw1: 5'-TCATTTCCAGCCAATGATGA-3'
LRRK2 rev1: 5'-TGGAGCAAACAGAACTCAC-3'

Primer binding to the C-terminus of the LRRK2 gene:

LRRK2 fw4: 5'-ATTCAACGGAAAGAAATGTAATG-3'
LRRK2 rev5: 5'-TTCTGCTTTTGTGTACCTTCAGTAT-3'

2.1.10 Gel electrophoresis

Gel electrophoresis was used to separate DNA or RNA in an agarose gel with respect to different size. A 1% agarose (Invitrogen) gel in 1x TAE was used for analysis. For detection of the nucleic acid the intercalating reagent SYBR safe (Invitrogen) was added to liquid agarose before the gel was casted. Samples for gel electrophoresis were mixed with loading buffer and separated by 10V/cm of the gel.

DNA ladder used as standard was FastRuler DNA ladder low range (Fermentas).
The UVP UV-Transilluminator was used for detection of DNA/RNA signals.

50 x TAE: (1 liter)

242 g	TRIS (Roth)
57,1 ml	Acetic acid
100 ml 0,5 M	EDTA (pH 8,0) (Roth)

Loading buffer (6x)

10mM	Tris-HCL (pH 7,6) (Sigma)
0,15 %	Orange G (Sigma)
0,03 %	Xylene Cyanol (Sigma)
60 %	Glycerol (Roth)
60 mM	EDTA (pH 8,0) (Sigma)

2.1.11 SuperScript™ One-Step RT-PCR with Platinum *Taq* (Invitrogen)

This method combines reverse transcription and PCR in a single reaction catalysed by SuperScript™ II Reverse Transcriptase and Platinum *Taq* DNA Polymerase (Invitrogen).

cDNA synthesis and pre-denaturation:

50°C for 30 min
94°C for 2 min

PCR amplification: 35 cycles repeat

94°C for 15 sec (denature),
54°C (fw1/rev1) or 52°C (fw4/rev5) for 30 sec (anneal)
72°C for 30 sec

Final extension:

72°C for 5 min
Hold 4°C

PCR samples were loaded on 1 % agarose gel to detect amplified products.

2.1.12 Cell culture

Cell lines:

Three different cell lines were used for analysis of plasmid expression on RNA and protein level. All cells were cultivated in cell culture flasks (Greiner bio-one 75 cm²) at 37°C in a humidified atmosphere with 5% CO₂ (Tissue culture incubator, Salvis).

Cells and medium:

- Chinese hamster ovary cells (CHO cells)

Medium (500 ml):

- Iscove's Modified Dulbecco's Medium (IMDM, Gibco)
- 10% foetal calf serum (FCS, Gibco)
- 100 U/ml penicillin (Invitrogen)
- 100 µg/ml streptomycin (Invitrogen)
- 5 ml non-essential-amino acid (NEAA) 100x (Invitrogen)
- 5 ml Na-pyruvate 100 mM (Sigma)
- 2 ml gentamycin (Invitrogen)

- Human embryonic kidney cells (HEK 293)

Medium (500 ml):

- Dulbecco's Modified Eagle Medium (DMEM, Gibco)
- 10% FCS (Gibco)
- 2mM L-glutamine (PAA)
- +10 ml Pen/Strep (Invitrogen)

- SH-SY5Y cells (Neuroblastoma cell line) [43]

Medium (500 ml):

- 1:1 F12 nutrient mixture (Gibco) and IMDM
- 10% FCS (Gibco)
- +10 ml Pen/Strep (Invitrogen)
- +5 ml NEAA

2.1.13 Cell thawing

For thawing of cells, incompletely thawed cells were added to 10 ml pre-cooled medium. Then, cells were centrifuged 5 minutes with 800 g (Heraeus SEPATECH Magafuge 1.0), supernatant was removed and the pellet dissolved in 20 ml medium and transferred into cell culture flasks (Greiner bio-one 75 cm²) and grown at 37°C in a humidified atmosphere with 5% CO₂ (Tissue culture incubator, Salvis).

2.1.14 Cell splitting

Medium was removed from approximate 90% confluent cells (nearly 3 times/week) and washed with 1xPBS (Gibco). Then cell dissociation solution (Sigma) was added to the cells and briefly incubated at 37°C in the incubator. When cells detached from the surface, medium was added and transferred to fresh 50 ml tubes. Cells were centrifuged with 800 g for 5 minutes and the supernatant removed. The pellet was dissolved in fresh medium and cells were diluted in 1:1, 1:5 and 1:10 dilutions in cell culture flasks (Greiner bio-one 75 cm²).

2.1.15 Cell cryoconservation

Cells were detached with 2 ml cell dissociation solution (Sigma) then 3 ml medium was added. Cells were pelleted by 5 minutes centrifugation with 800 g and dissolved in 1800 µl medium/10 % DMSO (Fluka). For storage, cells were placed in cryotubes at -80°C.

2.1.16 Lipofectamine Transfection (Invitrogen)

For transfection cells were grown to 60- 80 % confluence in a 6-well plate (Greiner bio one; 9,5 cm² growth area, Ø 34,8 mm).

Before transfection, growth medium was replaced by 1 ml of serum free medium. DNA was pre-complexed with the PLUS-Reagent by dilution of 1,6 µg DNA in 125 µl

serum-free medium (without antibiotics), 20 µl PLUS-Reagent and incubation for 15 minutes at room temperature. 10 µl Lipofectamine Reagent was diluted in 125 µl serum-free medium, added to the pre-complexed DNA and incubated for additional 15 minutes at room temperature. Then the DNA-PLUS-Lipofectamine Reagent complexes were added to the cells, mixed gently and incubated at 37°C in the incubator for 4 hours. After 4 hours 1 ml serum containing medium was added to the cells and incubated for 24 hours at 37°C. After that cells were used for further analysis.

2.1.17 Immunocytochemistry

For immunocytochemistry, cells were grown on culture slides (VWR) until 80-90 % confluence and transfected as described above. Medium was removed and cells were washed 2 times carefully with 1xPBS. For fixation, pre-cooled methanol (Roth) was added to the cells and incubated at -20°C for 10 minutes. Then, cells were washed 3x with 1xPBS and blocked with 10 % normal goat serum in 1xPBS/0,25 % Triton X-100 (Fluka). Primary antibody incubation (diluted in blocking solution) was done over night on 4°C. The following day, cells were washed 5 x with 1xPBS and incubated for 1 hour with secondary antibody (1:250 diluted in blocking solution). For fluorescence staining cells were mounted in vectashield DAPI-mounting medium (Vector). For DAB staining, cells were incubated with ABC solution (Vector) for 30 minutes, washed and incubated with DAB substrate (Vector), and counterstained with haematoxylin. Cells were covered by Cytomation Glycergel mounting medium (Dako) and mounted. Finally slides were evaluated under a microscope (Leitz DIAPLAN) with 40x magnification.

1st antibody (diluted in 10 % normal goat serum in PBS/0,25 % Triton X-100)

- NB 300-268 rabbit polyclonal anti-LRRK2 antibody (Novus Biologicals)

2nd antibody (diluted in 10 % normal goat serum in PBS/0,25 % Triton X-100)

- Biotinylated goat anti-rabbit antibody (Vektor)
- Goat anti-rabbit antibody Texas red (Vektor)

2.1.18 RNA-Extraction from cell culture with RNeasy Kit from QUIAGEN (protocol from QUIAGEN RNeasy Handbook)

Cells used for RNA extraction were cultivated in 6-well plates until ~90% confluence. When starting with purification of total RNA cell culture medium was replaced by 350 µl RLT buffer (10 µl 14,3 M β-mercaptoethanol per 1ml RLT). A rubber polishmen was used to detach the cells from the surface and then cells were transferred into a fresh RNase-free tube. After that 350 µl 70% ethanol was added and the solution was mixed by pipetting. 700 µl suspension was transferred to the RNeasy spin column. The homogenate was centrifuged 15 sec. with 10.000 g to bind RNA to the column, the flow through was discarded. Then 700 µl RW1 buffer was pipetted onto the column and again centrifuged 15 sec. with 10.000 g. After that two times 500 µl RPE buffer was loaded and centrifuged under the same conditions. For elution, the column was placed in a fresh tube and 50 µl RNase-free water was added to the column and centrifuged 1 min. with 10.000 g.

RNA concentration was determined by measurement of optical density (OD) at 260 nm wavelength (photometer, Perkin Elmer Lambda 2).

$$\text{OD}_{260 \text{ nm}} 1 = 40 \text{ } \mu\text{g} / \text{ml} = 40 \text{ ng} / \mu\text{l}$$

2.1.19 DNase treatment (Promega)

RNA from cell culture and animal tissue was treated with RQ1 RNase-free DNase I (Promega) to remove genomic and plasmid DNA from the samples. DNA contamination had to be eliminated to get RNA specific signals in PCR and qPCR.

To assess the correct amount of DNase for the used samples (1 unit DNase per 1 microgram RNA), RNA concentration was determined by NanoDrop (Thermo Scientific). DNase treatment was done for 30 minutes at 37°C in DNase Reaction Buffer (400 mM Tris-HCl pH 8, 100 mM MgSO₄, 10 mM CaCl₂; Promega). Reaction was stopped by adding 1 µl RQ1 DNase Stop Solution (20 mM EGTA; Promega) to the samples and incubation at 65°C for 10 minutes to inactivate the DNase.

2.1.20 cDNA synthesis with Super Script First-Strand Synthesis System for qPCR (Invitrogen)

DNase treated and untreated RNA samples were used for cDNA synthesis. To proof the purity of RNA samples and exclude DNA contamination, samples were run in duplicates whereas in only one sample reverse transcriptase was added. Synthesised cDNA was used as template for PCR and Q-PCR.

For RT reaction, RNA was added to the Oligo dT synthesis mix:

total RNA (5 µg)	x µl
dNTP (10 mM)	1 µl
oligo dT primer	1 µl
DEPC water	up to 10 µl

The RNA/oligo dT mixture was incubated for 5 minutes at 65°C in the T3 thermocycler (Biometra) and then placed on ice. Afterwards, 9 µl reaction mix was added to the samples:

10x RT buffer	2 µl
25 mM MgCl ₂	2 µl
0,1 M DTT	2 µl
RNase OUT (40 U/µl)	1 µl
<u>DEPC water</u>	<u>2 µl</u>
Total volume	9 µl

Samples were placed in the thermocycler (Biometra T3) for 2 minutes at 42°C and 1 µl Super Script II RT (Invitrogen) was added to one tube of the duplicates. Then all samples were incubated for 50 minutes at 42°C for cDNA synthesis and 15 minutes at 70°C to terminate the reaction. After cooling 1 µl RNase H (Invitrogen) was added and incubated for 20 minutes at 37°C for degradation of the RNA.

2.1.21 Protein extraction from eukaryotic cells

For protein extraction, medium was removed and cells were washed two times with 1xPBS (Gibco). Then 100 µl RIPA buffer was added to each well, cells were detached with a cell scraper and transferred to a fresh tube. Cells were vortexed thoroughly and incubated on ice for 20 minutes. After that, lysate was centrifuged 10 minutes at 4°C with 13.000 g. The supernatant was collected and stored at -80°C (quick frozen in liquid nitrogen).

RIPA buffer (radio immunoprecipitation assay buffer)

50 mM	Tris pH 7,4
150 mM	NaCl (Roth)
1 %	Triton-X100 (Sigma)
0,5 %	Sodium-deoxycholate
5 mM	EDTA (Sigma)

Working solution:

9 ml	RIPA buffer
1 ml	NaF (Sigma)
100 µl	100x NaOV (Sigma)

2.1.22 Bradford protein assay

Determination of protein concentration in a homogenate was done by Bradford assay. The dye reagent changes from red to blue by cationic and hydrophobic protein binding with an absorbance maximum at 595 nm wavelength.

For analysis the dye reagent concentrate (BioRad) was diluted 1:5 with miliQ-Water (Millipore) and filtered through a Whatman filter to remove precipitates. BSA (2 mg/ml; Pierce) serial dilutions were used as a standard (concentrations see below). Samples were diluted 1:10 and 1:20 and used in duplicates. 10 µl BSA standard or protein samples were pipetted on micotiterplates (Nunc) and 200 µl dye reagent was added and mixed. Plates were incubated at room temperature for 5 minutes and then

absorbance was measured at 595 nm wavelength with a plate reader (Tecan Sunrise).

Standard	Protein conc.	Volume H ₂ O	volume BSA standard
Original	2,00 mg/ml	-	.1 ml glass vial
St 1.....	0,50 mg/ml.....	150 µl	50 µl original
St 2.....	0,40 mg/ml.....	10 µl	40 µl St 1
St 3.....	0,30 mg/ml.....	20 µl	30 µl St 1
St 4.....	0,20 mg/ml.....	30 µl	20 µl St 1
St 5.....	0,10 mg/ml.....	40 µl	10 µl St 1
St 6.....	0,05 mg/ml.....	45 µl	5 µl St 1
Blank	0,00 mg/ml.....	50 µl	-

2.1.23 SDS polyacrylamide gelelectrophoresis (SDS PAGE)

For the separation of proteins a polyacrylamide gel containing SDS was used, separating the molecules according to their molecular size.

For analysis a 4% acrylamide stacking gel and 7% acrylamide running gel was casted.

7% running gel

MiliQ H ₂ O (Millipore)	15.150 ml
1.5 M Tris-HCl, pH 8.8 (Sigma)	7.500 ml
10% (w/v) SDS (Fluka)	0.300 ml
30% Acrylamide (Roth)	6.900 ml
10% (w/v) ammonium persulphate (Roth)	0.150 ml
TEMED (Roth)	0.020 ml

4% stacking gel

MiliQ H ₂ O (Millipore)	3.050 ml
0.5 M Tris-HCl, pH 6.8 (Sigma)	1.250 ml
10% (w/v) SDS (Roth)	0.050 ml
30% Acrylamide (Roth)	0.670 ml
10% (w/v) ammonium persulphate (Roth)	0.025 ml
TEMED (Roth)	0.005 ml

1x running buffer

25 mM	Tris-HCl (Sigma)
200 mM	Glycine (VWR)
0.1% (w/v)	SDS (Fluka)

Polymerized gels were placed in western gel gadget and filled with 1x running buffer. Samples from cell culture or animal tissue were mixed with Sample buffer, incubated at room temperature (not heated!) for 10 minutes and 20 µg loaded on the SDS gel. As positive control 5 µg recombinant LRRK2 protein (205 kD, aa 970-2527, Invitrogen) was loaded on the gel.

5x sample buffer

10% w/v	SDS (Fluka)
10 mM	β-mercaptoethanol (Sigma)
20 % v/v	Glycerol (Roth)
0.2 M	Tris-HCl, pH 6.8 (Sigma)
0.05% w/v	Bromophenolblue (Sigma)

5 µl Protein ladder (PageRuler Plus prestained protein ladder / Fermentas, HiMark prestained protein standard / Invitrogen) was loaded to indicate the size. Electrophoresis was performed in the gel chamber (Biorad, MGV-202) 1 hour with 150 V constant (Power supply - Biorad, Power Pac).

2.1.24 Western Blotting (WB)

The samples were separated by SDS gel electrophoresis according to molecular size. Then proteins got transferred voltage-dependent on a 0,45 µm pore-size nitrocellulose membrane (Amersham Bioscience Hybond-C Extra) (Blot chamber ñ Mini Trans-Blot cell, Biorad). Fig.7 shows the sandwich model of the protein transfer.

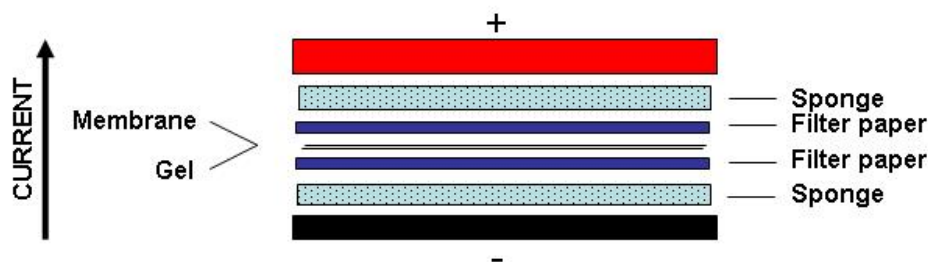


Fig.7 Sandwich model for protein transfer. Negatively charged proteins migrate to the plus pole and bind to the membrane.

Western Blotting was performed in transfer buffer over night at 4°C with 30V constant.

10x transfer buffer (pH should be 8,3; d o not adjust)

30.3 g	Trizma base (= 0.25 M) (Roth)
144 g	Glycine (= 1.92 M) (VWR)

2 L 1x transfer buffer

400 ml	Methanol (Roth)
200 ml	10x transfer buffer
1400 ml	MiliQ H ₂ O (Millipore)

Ponceau staining was done to confirm complete protein transfer to the membrane. The membrane was used directly for further experiments or stored at 4°C until further use.

Membrane was washed in PBST (1x PBS + 0,01 % Tween20 (Roth)) and blocked 1 hour with 5% (w/v) milk in PBST. Then the membrane was incubated with the primary antibody diluted in 5 % milk/PBST for 1 hour at room temperature [44, 45], followed by 3 times 5 minutes washing with PBST to remove unspecific binding of the antibody. After that, membrane was incubated with the HRP conjugated secondary antibody diluted in 5 % milk/PBST for 30 minutes at room temperature and washed three times 5 minutes with PBST. ECL+ detection kit (GE Healthcare) was used for antibody detection. Signal was evaluated with UVP Bioimaging system.

After evaluation, the membrane was stripped with stripping buffer for 20 minutes at room temperature to remove primary antibody, washed briefly in PBST and used for another antibody incubation.

Stripping buffer: 0.5 L

0.2 M	Glycine, pH 2.5 (VWR)
0.05%	Tween 20 (Roth)

Primary antibody [44, 45]

- Mouse 16H7 -A5 anti-LRRK2 monoclonal antibody (AFFiRiS AG) (1:50)
- NB 300-268 rabbit polyclonal anti-LRRK2 antibody (Novus Biologicals) 1:1.000
- Rabbit polyclonal anti LRRK2 antibody AB 9682 (Chemicon, Millipore)
- Anti-TH (tyrosine hydroxylase) antibody 1:2.000
- Rabbit polyclonal anti- β actin antibody (Novus Biologicals) 1:1.000

Secondary antibody

- Goat anti-mouse-HRP IgG+IgM (H+L) (Jackson) 1:20.000
- Anti-rabbit IgG-HRP (GE Healthcare) 1:10.000

2.2 Animal characterization

2.2.1 Transgenic mouse line

Transgenic animals were generated and bred by PolyGene according to their standardized method. Briefly, the isolated fragment was injected into zygotes and transferred into IVC-held foster mothers. C57Bl/6N offsprings were screened and verified by tail biopsy. Finally four transgenic founders, three male and one female animal paired with wildtype C57Bl/6N animals were received. Progeny of the founder animals was genotyped and analysed by behavioural tests and biochemical analysis for transgene expression.

2.2.2 Behavioural analysis

Behavioural analysis was done with 6 animals derived from crossing LRRK2-founder animals and wild type littermates (C57Bl/6N). Animals (3 wt, 3 tg) were tested on 4 consecutive days. Four different tests were performed in this experiment. These tests are intended to analyse the motor function of these animals.

Grooming

The fur and the overall shape of the animals were inspected routinely. Reduced grooming activity might be indicative for motor or mental impairment.

Challenging beam

With this test, motor function (walking and balance) of the mice was tested. A plastic beam with 1m length and 5-1,5 cm narrowing diameter covered with a grid was used. Mice had 2 training days (beam without grid) with 5 trials each. On the third day, mice performed the probe trial with the grid, which was filmed for later evaluation. The total number of steps and the number of errors per steps (front and hind limbs) were counted.



Fig.8 Beam test



Fig.9 Beam with grid

Pole test

With this test the grip strength and climbing skills were monitored. A wooden pole of 50 cm length and 1 cm diameter was wrapped with a gauze bandage. The time to turn around (U-turn-time), the time to descent (runtime) and the total time (maximum 120 seconds) was reported. Behavioural test data from five repetitions per mouse were collected over three days.

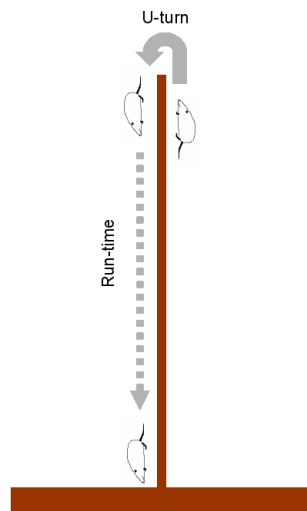


Fig.10 Pole test

Coordination and muscle function were tested.

Grip test

The muscular strength was tested by placing the mice on the metal lid of the cage. The lid was inverted and placed on the top of two cylinders. Mice had to hang on the lid. The time until mice fell off was measured and after 2 minutes the test was stopped. The test was repeated three times for each mouse and the data was collected over three days.



Fig.11 Grip test Endurance and muscle function were tested

2.2.3 Genomic DNA purification (Wizard® Genomic DNA Purification Kit, Promega)

For genotyping mouse tail tips were used for genomic DNA purification (genomic DNA purification system; Promega). Mouse tail tip (2-3 mm) was transferred into a 1,5 ml tube. 275 µl digestion solution was added to each sample and incubated over night (16-18 hours) at 55 °C. The next day, 250 µl SV lysis buffer was added and vortexed. Mixture was transferred to the minicolumn and spinned for 3 minutes with 13.000 g. The flow-through was discarded, 650 µl wash solution added to the column and centrifuged for 1 minute with 13.000 g. Washing was repeated for 3 times followed by centrifugation for 2 minutes at 13.000 g. Then matrix was dried by 2 minutes centrifugation with 13.000 g. For elution, column was placed in a new 1,5 ml microcentrifuge tube and 250 µl nuclease-free water was added on the matrix. After 2 minutes incubation the column was centrifuged 1 minute with 13.000 g. This step

was repeated once using the eluate from the previous step, to increase DNA concentration.

Digestion solution:

Nuclei Lysis solution	200 µl
0,5 M EDTA (pH 8)	50 µl
Proteinase K 20 mg/ml (Roche)	20 µl
<u>RNase A solution</u>	<u>5 µl</u>
	275 µl

2.2.4 Genotyping PCR

Genomic DNA from mouse tail tips was used for genotyping PCR to detect the presence of the transgene. Internal control primers used, were the IL2 primer (see above). For LRRK2 detection, LRRK2 primer 1 and LRRK2 primer 4/5 were used (see above). PCR conditions were the same for all primer combinations (see above).

2.2.5 Brain fixation and dehydration

After preparation of mouse brains one hemisphere was used for biochemical analysis (RNA and protein extraction) and the other hemisphere was dehydrated and embedded in paraffin for microtome (Leitz 1516) sectioning.

For tissue fixation, the brains were incubated in 4% PFA (paraformaldehyde) (VWR) over night on 4°C. The next morning:

tissue was placed for 30 minutes in 1xPBS followed by stepwise dehydration:

- 2 hours in 30 % ethanol (Merck)
- 1 hour in 30 % ethanol
- 2 x 2 hours in 50 % ethanol
- 70 % ethanol for 2 days

Two days later:

- Fresh 70 % ethanol 1 hour at room temperature
- 2 x 1,5 hours in 90 % ethanol
- 2 x 1,5 hours in abs. ethanol
- 10 minutes in Xylene (beaker glass) (Roth)
- Over night in liquid paraffin (incubator 60°C)

The next day:

- Change paraffin in the morning
- Sagittal embedding at midday

Tissue sections of 7 µm thickness were made with microtome (Leitz 1516) and placed on glass slides.

2.2.6 Immunohistochemistry

PFA fixed brain sections (see above) were placed in xylene 2 x for 10 minutes to remove the paraffin. After that tissue had to be rehydrated:

- 2 x 10 minutes in abs. ethanol
- 2 minutes in 70 % ethanol
- 2 minutes in 50 % ethanol
- 2 minutes in 30 % ethanol
- 2 minutes in miliQ H₂O (Millipore)

Antigen-retrieval was done with Citra solution (DAKO) in the microwave for 3 minutes at maximum power and 10 minutes with 360 W. Slides were cooled down to room temperature for 20 minutes and were then washed two times with PBS. Peroxidase was blocked by incubation of the slides 10 minutes in 3 % H₂O₂ (Merck) diluted in PBS and then washed again 2 x in PBS. Brain sections were blocked with MOM blocking solution (MOM Kit, Vector Labs)(in PBS) in a humidified chamber for 1 hour. Then, tissue was incubated with the primary antibody, diluted in protein concentrate (MOM Kit, Vector Labs) in PBST (PBS + 0,01 % Tween20 (Roth)) over night at 4°C

in a humidified chamber. Slides were then washed 3 x 3 minutes in PBST and incubated 30 minutes with the second biotinylated antibody (diluted in protein concentrate/PBST; MOM Kit). Following, slides were 3 x 3 minutes washed with PBST and incubated 30 minutes with ABC solution (Vector). After wash with PBST and miliQ water tissue was stained with DAB (Vector). Reaction was stopped in miliQ water, counterstain was done with haematoxylin (Sigma) for ~1 minute and again washed with miliQ water. Subsequently, brain sections were dehydrated:

- 2 minutes in 30 % ethanol
- 2 minutes in 50 % ethanol
- 2 minutes in 70 % ethanol
- 2 x 2 minutes in abs. ethanol
- 2 x 2 minutes in xylene

Tissue was embedded in Entellan mounting medium (Merck). The following day, dried slides were scanned and evaluated with Mirax Scan (Zeiss).

1st antibodies (diluted in protein concentrate (MOM Kit, Vector)/PBST)

- NB 300-268 (Novus Biologicals) polyclonal rabbit antibody, binding to human LRRK2 protein (1:300)
- 16H7-A5 monoclonal anti-LRRK2 antibody (1:50) (AFFiRiS AG)
- Neuron specific mouse NeuN antibody (Millipore) (1:500) [46]

2nd antibodies (1:250 diluted in protein concentrate (MOM Kit, Vector) /PBST)

- Biotinylated anti-mouse antibody (MOM Kit, Vector)
- Biotinylated goat anti-rabbit antibody (Vector)

2.2.7 RNA-Extraction from mouse tissue with RNeasy Kit from QUIAGEN (protocol from QUIAGEN RNeasy Handbook)

A cube of 3mm diameter was cut out of fresh tissue and used for RNA isolation. Tissue-cubes were disrupted in 600 µl RLT buffer with micropistilles and homogenized with QUIAshredder. 600 µl of 70% ethanol was added to the homogenates and transferred to the RNeasy spin columns. Samples were centrifuged 15 seconds with 10.000 rpm, the flow-through was discarded. Then 700 µl RW1 buffer was added and centrifuged, followed by 2 washing steps with 500 µl RPE buffer. After a final centrifugation of 1 minute at full speed, to dry the pellet, the column was placed in a fresh tube and RNA was eluted with 50 µl RNase-free water (1 min. at 10.000 rpm).

2.2.8 Protein extraction from mouse tissue with RIPA buffer

For protein extraction, 1ml RIPA buffer (see above) was added to the tissue, disruption and homogenisation was done with a glass mortar. Homogenate was then incubated at 4°C rotating for 30 minutes. Samples were centrifuged (4 minutes, 4°C with 12.000 rpm) and supernatant was transferred into a fresh tube. Samples were stored at -80°C until further use.

2.2.9 NuPAGE Protein Electrophoresis System (Invitrogen)

For separation of high molecular-weight proteins the NuPAGE Protein Electrophoresis System with 3-8 % Tris-Acetate gels (Invitrogen) was used. Western Blotting conditions were modified, to obtain clear separation of high molecular weight proteins.

Formulation of the NuPAGE Tris-Acetate Gels (Invitrogen)

Precasted gels were used

NuPAGE® LDS Sample Buffer (4X) (Invitrogen)

106 mM	Tris HCl
141 mM	Tris base
2%	LDS
10%	Glycerol
0.51 mM	EDTA
0.22 mM	SERVA® Blue G250
0.175 mM	Phenol Red
pH 8.5	

Sample preparation for gel loading

Sample	x µl
NuPAGE® LDS Sample Buffer (4X)	2.5 µl
NuPAGE® Reducing Agent (10X)	1.0 µl
Deionized Water	to 6.5 µl
Total Volume 10 µl	

NuPAGE® Tris-Acetate SDS Running Buffer (20X) (Invitrogen)

50 mM	Tricine
50 mM	Tris base
0.1%	SDS
pH 8.24	

Equal amounts of proteins for each sample were diluted in sample buffer and incubated at room temperature for 10 minutes until loaded on the gel. Electrophoresis conditions were 1 hour with 150 V.

After separation of the samples in the Tris-Acetate Gel, proteins were transferred to a nitrocellulose membrane, as described above (transfer 1hour 30 V constant).

NuPAGE® Transfer Buffer (20X) (Invitrogen)

25 mM Bicine (Sigma)

25 mM Bis-Tris (free base)

1 mM EDTA (Sigma)

pH 7.2

Working solution:

NuPAGE® Transfer Buffer (20X)	50 ml
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NuPAGE® Antioxidant	1 ml
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Methanol	200 ml
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<u>Deionized Water</u>	<u>749 ml</u>
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Total Volume	1000 ml
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Western Blotting and subsequent analysis was performed as described above.

3 RESULTS

A transgenic mouse model for PD was generated and initially characterized. The plasmid used for transgene production carrying the human LRRK2 gene (hLRRK2), was cloned and tested *in vitro*. Subsequently transgenic hLRRK2 animals were tested using behavioural and biochemical methods for LRRK2 transgene expression.

3.1 Plasmid Characterization

As a first step to create a novel transgenic mouse line, a full length cDNA clone containing the complete coding sequence of human LRRK2 was obtained from Origene and subsequently characterized.

3.1.1 pCMV6-XL5-LRRK2 (Origene) expression analysis

Before production of the transgenic mouse line transcription and translation of full length human LRRK2 was analyzed by testing pCMV6-XL5-hLRRK2 transfected cells. Then the complete CDS was cloned in pUC18-Thy1.2 (Polygene) backbone for transgene production.

First *in-vitro* analysis explored expression of human LRRK2 gene inserted in the pCMV6-XL5 backbone (Origene) on RNA and protein level. Detection of full length LRRK2 RNA was done by PCR and qPCR using N-terminal and C-terminal specific primers. Western Blotting (WB) and immunocytochemistry (ICC) was done to detect LRRK2 protein *in-vitro*.

3.1.1.1 Restriction enzyme analysis of the LRRK2 plasmid

In order to detect LRRK2 CDS in the pCMV6-XL5 plasmid DNA was treated with NotI restriction enzyme leading to cleavage on both borders of LRRK2 gene. The LRRK2 insert was detected by gel electrophoresis on an agarose gel. The restriction digest resulted in a 4,5 kb band (pCMV6-XL5 backbone) and a 7 kb LRRK2 band (Fig.12). As control untreated DNA was loaded additionally.



Fig.12 pCMV6-XL5-LRRK2 digestion with NotI enzyme. Three maxi prep samples treated with NotI enzyme to test LRRK2 gene presence. Line with Ø show uncut plasmid DNA with 11,5kb band size. NotI treated DNA show LRRK2 band (7kb) and pCMV-backbone (4,5kb).

In addition to this restriction digest, the LRRK2 CDS was sequenced (Microsynth). Sequencing resulted in a single reading frame of 7,7 kb as expected (data not shown).

3.1.1.2 Transcription analysis of LRRK2 plasmid

As a second step, expression of hLRRK2 was tested *in-vitro*. Two different cell lines (CHO, HEK) were used for detection of LRRK2 RNA expression. Therefore cells were transfected using Lipofectamine (Invitrogen) with pCMV6-XL5-hLRRK2 vector (pCMV6-LRRK2) and incubated for 48 hours after transfection. Cells were disrupted and the protein homogenates were analysed by WB. Additionally total RNA was extracted with RNeasy spin columns (Quiagen) and tested by PCR/qPCR.

RNA samples were treated with RQ1 DNase (Promega) to avoid DNA contamination. Subsequently we used treated and untreated RNA for cDNA synthesis with oligo dT primers (Invitrogen). For evaluation of DNA contamination, cDNA synthesis was done in parallel without reverse transcriptase. cDNA samples were used for PCR with primers specific for the LRRK2 N-terminus (primer 1) and C-terminus (primer 4/5) and primers specific for the housekeeping gene GAPDH. H₂O peripheral dopa decarboxylase inhibitor (benserazide or carbidopa; prevents metabolism of L-dopa) was also used in PCR reaction as negative control as well as plasmid DNA (pCMV6-LRRK2) as positive control for LRRK2.

Analysis showed that CHO and HEK cells transfected with hLRRK2 express hLRRK2 whereas untransfected cells do not show LRRK2 expression (Fig.13, 14). As positive control, GAPDH expression was detected in both transfected and untransfected cells.

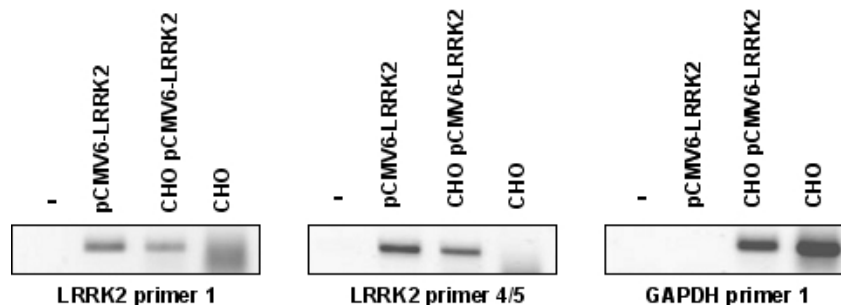


Fig.13 LRRK2 RNA detection from pCMV6-XL5-LRRK2 transfected CHO cells by PCR. RNA extracts from pCMV6-XL5-LRRK2 transfected/untransfected CHO cells used for PCR with LRRK2 specific primer (1, 4/5) showed presence of LRRK2 RNA in transfected but not in untransfected cells. Primer dimers in lane 4 of left and middle gel are present. In contrast GAPDH specific bands can be detected in both transfected and untransfected CHO cells. Water control in all PCR reactions was used and loaded in lane one. Plasmid DNA pCMV6-LRRK2 (second lane) was used as positive control.
-..neg. control

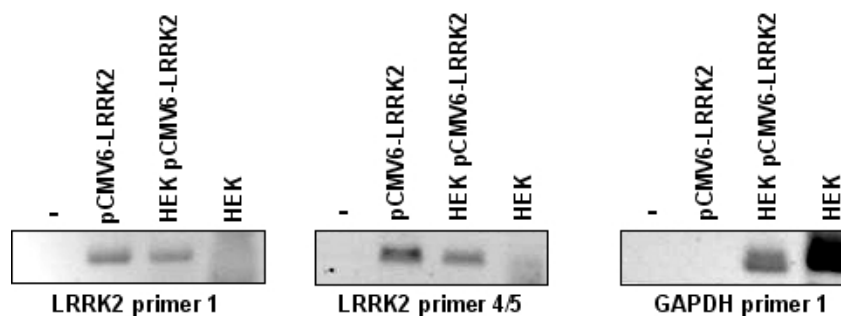


Fig.14 LRRK2 RNA detection from pCMV6-XL5-LRRK2 transfected HEK cells by PCR. PCR with LRRK2 specific primer (1, 4/5) display the presence of LRRK2 RNA in extracts of pCMV6-LRRK2 transfected HEK cells. In contrast, GAPDH specific bands are detected in transfected and untransfected cell extracts. Water control. was loaded in the first lane. Plasmid DNA pCMV6-LRRK2 (second lane) was used as positive control.
-..neg. control

3.1.1.3 Analysis of LRRK2 expression by Western Blotting

Protein homogenates from transfected and untransfected CHO and HEK cell extracts were analysed for LRRK2 protein expression detected by Western Blotting. Total protein concentration was determined by Bradford assay. Thus equal amounts of protein were loaded and analysed by WB. Recombinant LRRK2 protein was used as positive control. LRRK2 (280 kD) was detected by two LRRK2 specific antibodies, NB300-268 and 16H7-A5, in protein extracts from transfected but not in untransfected cells (Fig.15). The recombinant LRRK2 protein with 205 kD was also detected by both antibodies.

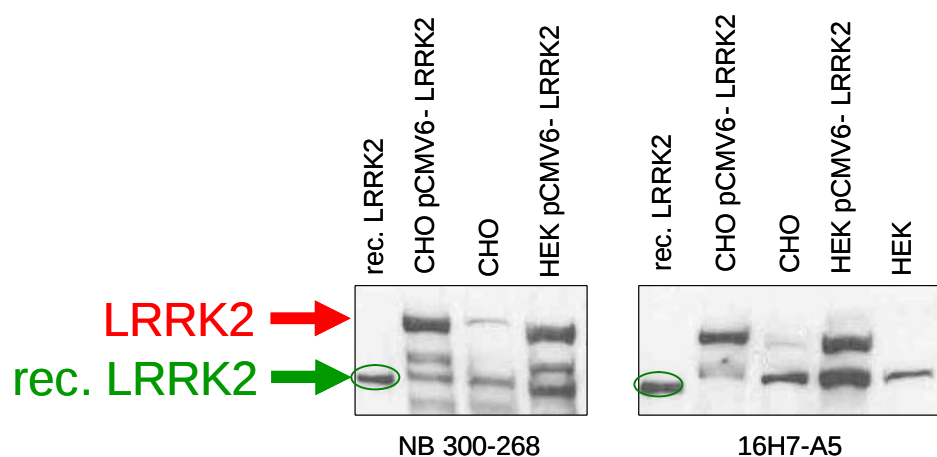


Fig.15 Western Blotting with cell culture protein extracts. pCMV6-XL5-LRRK2 transfected cells tested for expression of LRRK2 protein by LRRK2 specific antibodies NB300-268 and 16H7-A5. NB300-268 showed a band with the expected size of LRRK2 protein (~280 kD) in transfected but not in untransfected cells. Rec. LRRK2 protein was used as control for Western Blotting with a size of ~205 kD leading to different migration of rec. and endogenous LRRK2 protein. 16H7-A5 antibody also detects LRRK2 protein in transfected but not in untransfected cells.

green cycle...rec. LRRK2 protein

3.1.1.4 Fluorescence-immunocytochemistry (FICC) and Immunocytochemistry (ICC) of pCMV6-LRRK2 transfected cells

The presence of LRRK2 protein in WB prompted us to determine the cellular localization of LRRK2 by FICC/ICC. Therefore CHO and HEK cells were transfected with pEGFP (plasmid expressing green fluorescent protein), as transfection control, and pCMV6-LRRK2. As showed in Fig.16 and 17 LRRK2 protein expression was detected by NB300-268 antibody (Novus Biologicals). We observed structured cytoplasmic localization of LRRK2 protein only in transfected CHO and HEK cells (red). GFP was also detected in the cytosol (green). Nuclear staining was done with

DAPI mounting medium (blue fluorescent), Vector) or haematoxylin (blue). Additionally LRRK2 shows punctuate staining pattern in transfected cells. These results were expected as Hatano et al [37] could show that LRRK2 protein associates with lipid rafts, early endosomes, mitochondrial membrane, lysosomes, synaptic vesicles, cytoskeleton, golgi apparatus and the endoplasmic reticulum. Unspecific binding of NB300-268 or secondary antibody was not detected in untransfected cells or staining with secondary antibody only.

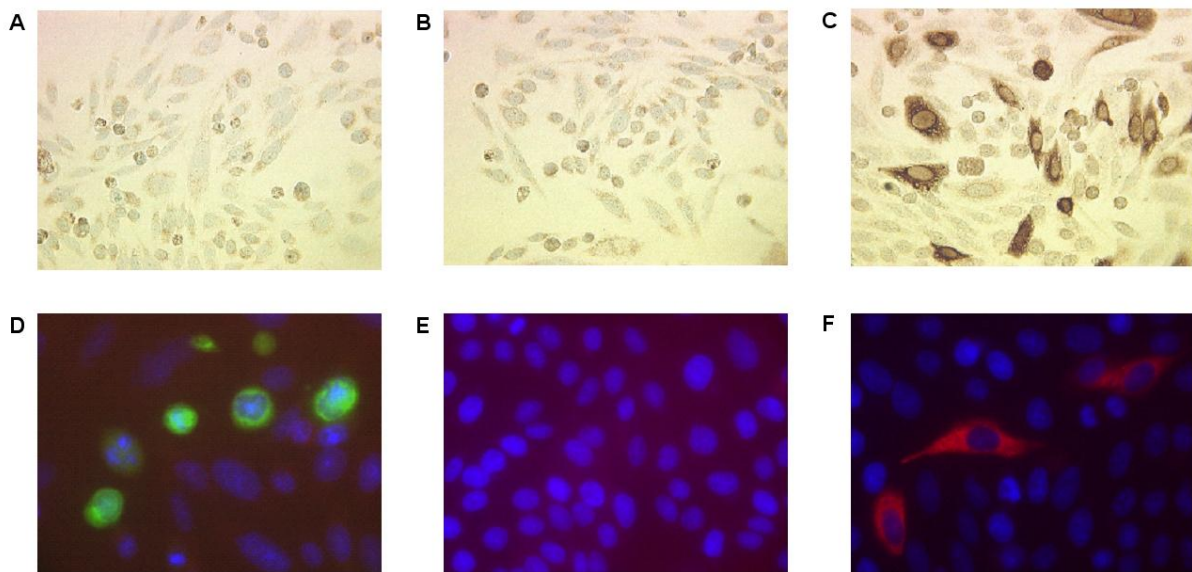


Fig.16 Immunocytochemistry with CHO cells transfected/untransfected with pCMV6-XL5-LRRK2. (A-C) DAB staining with biotinylated secondary antibody, (D-F) fluorescence immunocytochemistry (FICC) with texas red conjugated secondary antibody. (A) Untransfected CHO cells stained with LRRK2 specific antibody NB300-268 show no background signal of staining. (B) pCMV-LRRK2 transfected CHO cells incubated with biotinylated secondary antibody alone do not show unspecific binding of the antibody. (C) LRRK2 protein staining in pCMV-LRRK2 transfected CHO cells with NB300-268 indicating cytoplasmic localisation of the protein. (D) FICC with pEGFP transfected CHO cells show GFP expression but fail to show NB 300-268 signals. (E) pCMV-LRRK2 transfected CHO cells incubated with secondary antibody alone does not indicate unspecific binding. (F) Cytoplasmic localisation of LRRK2 protein in pCMV-LRRK2 transfected CHO cells (red).

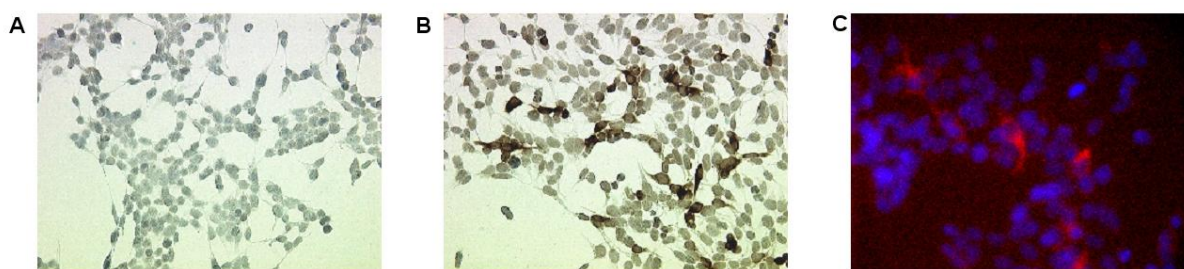


Fig.17 Immunocytochemistry with HEK cells transfected/untransfected with pCMV6-XL5-LRRK2. (A, B) ICC (C) FICC. (A) Untransfected HEK cells stained for background detection show no signal. (B) pCMV6-LRRK2 HEK cells stained with NB300-268 antibody detect LRRK2 protein in the cytoplasm. (C) Similar staining was obtained with FICC.

Summarizing LRRK2 RNA was detected in CHO and HEK derived RNA extracts by PCR. Also by WB and immunocytochemistry LRRK2 protein could be detected in cell extracts.

Plasmid DNA was sent to Polygene for transgene production.

3.1.2 pUC18-Thy1.2-LRRK2 (Polygene) expression analysis

In order to generate a novel transgenic mouse over-expressing hLRRK2 in neurons, the hLRRK2 cDNA was cloned into a pUC18 vector containing the neuron specific Thy1.2 promoter cassette. Cloning was performed at Polygene. After successful cloning, the insert was verified by DNA sequencing.

The pUC18-Thy1.2-hLRRK2 plasmid was injected into zygotes and transferred into IVC-held foster mothers. For *in-vitro* analysis of pUC18-Thy1.2-hLRRK2 expression, Polygene delivered plasmid DNA to AFFiRiS AG.

The new plasmid pUC18-Thy1.2-hLRRK2 (Thy1.2-LRRK2) was checked for LRRK2 expression. Therefore LRRK2 RNA was detected by PCR and LRRK2 protein by WB and FICC/ICC, as shown for pCMV6-XL5-LRRK2 plasmid.

3.1.2.1 Restriction enzyme digestion

Determination of human LRRK2 CDS in the Thy1.2-LRRK2 vector was done by restriction enzyme digestion. Plasmid DNA was cut with PvuI, XhoI, EcoRV, EcoRI or HindIII enzymes to get different cleavage fragments, which were separated by gel

electrophoresis for evaluation. As expected PvuI produced 2 fragments, XhoI cut once leading to one fragment, EcoRV produced 3 fragments, EcoRI produced 5 fragments and HindIII 5 fragments (Fig.18).

This analysis confirmed correct insertion of the hLRRK2 sequence into pUC18-Thy1.2 plasmid.

Enzyme	Fragments	Fragment size in kb
Pvu I	2	16, 1
Xho I	1	17
EcoR V	3	12, 3, 2
EcoR I	5	6, 5, 3.4, 2.2, 0.24
Hind III	5	9, 4.5, 1.7, 1.5, 0.3

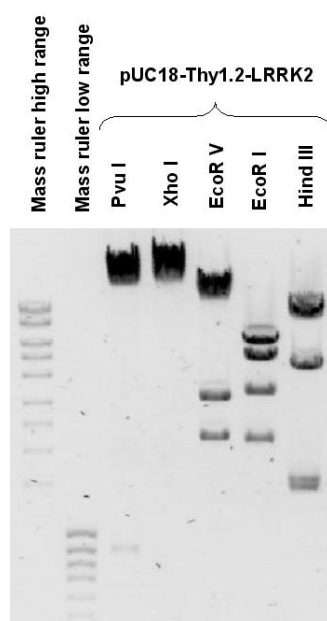


Fig.18 Restriction enzyme treatment of pUC18-Thy1.2-LRRK2. First two lanes show DNA ladder high and low range (Fermentas). Pvu I enzyme led to 16 kb and 1 kb fragments. Xho I cut once in the vector leading to 17 kb band. EcoR V produced 12 kb, 3 kbp and 2 kb. EcoR I led to 6 kb, 5 kb, 3.4 kb, 2.2 kb, and 0,24 kb fragments. Enzyme Hind III produced 5 DNA brands with the size of 9 kb, 4.5 kb, 1.7 kb, 1.5 kb and 0,3 kb. Short fragments were detected because of long separation time. Restriction enzyme treatment showed the presence of LRRK2 in the plasmid.

3.1.2.2 Reverse transcription and qPCR

Detection of LRRK2 RNA expressed from the pUC18-Thy1.2 plasmid, was done by quantitative PCR (qPCR) using LRRK2 specific primers in different transfected cell lines. An internal control (ribosomal protein L32) was used as reference.

Two different cell lines, HEK and SH-SY5Y were used for expression analysis by qPCR.

As described above, RNA samples were treated with RQ1 DNase (Promega) to avoid DNA contamination and tested for DNA contamination. For qPCR LRRK2

specific primers (primer 1) and housekeeping gene primers were used (Rpl32 primer 1).

Amplification of primer targets is illustrated by increasing fluorescence signal in Fig.19.A. After PCR reaction, the specificity of the primers was controlled by measurement of a single melting peak for each primer pair (Fig.19.B). The table shows the numbers of cycles when fluorescence threshold is reached. The lower the number of cycles the more template DNA is present at the beginning of the PCR reaction. Samples were used in duplicates and the average calculated. Values from samples without reverse transcriptase (-RT; background signal) were subtracted to get an approximate estimation of RNA expression.

Analysis confirmed expression of LRRK2 RNA in transfected HEK cells but not in SH-SY5Y cells. As expected Rpl32 expression was detected in all cell lines (Fig.19.C).

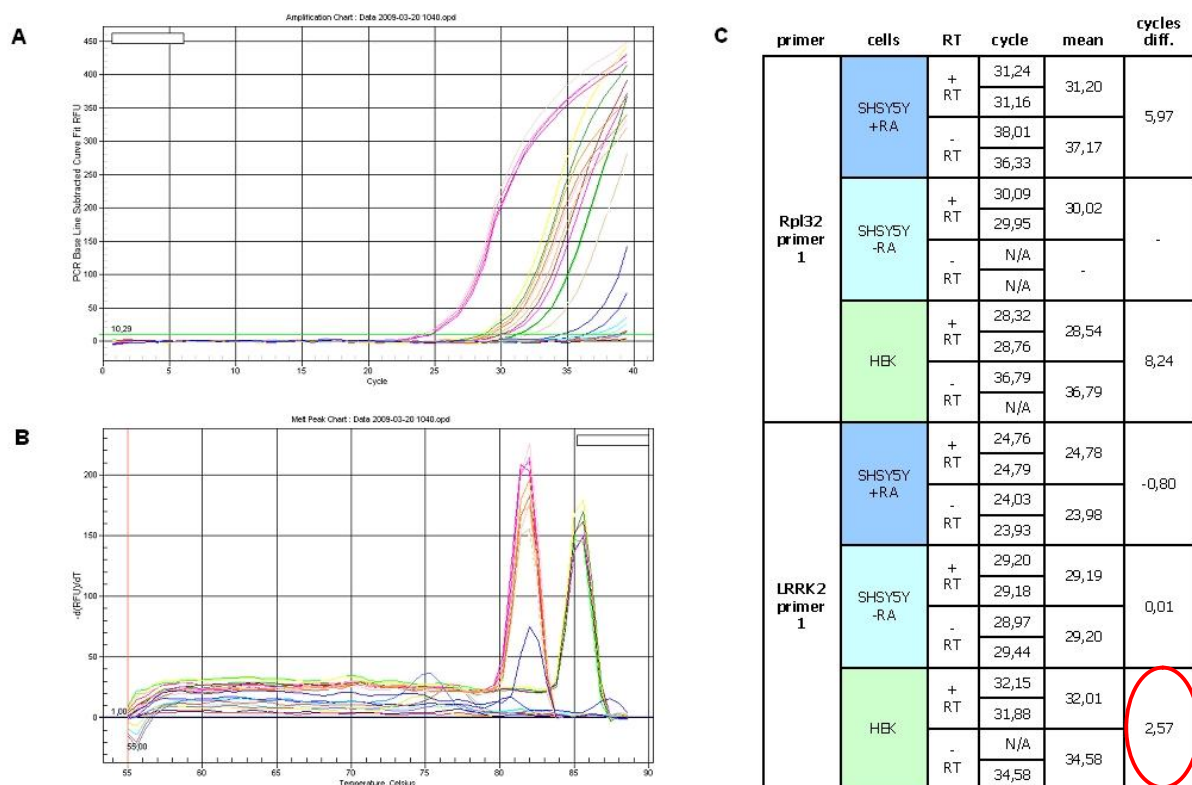


Fig.19 qPCR with cDNA from different cell lines transfected with pUC18-Thy1.2-LRRK2. qPCR reaction graph from two primer pairs, showing increasing fluorescence signal with progressing cycle number (A). The melting peak chart shows melting points of the fragments after PCR reaction. One temperature maximum for each primer indicates amplification of a single target sequence (B). Table of results illustrates the presence of LRRK2 cDNA in HEK cells indicated by the red circle (C).

Samples obtained from qPCR were additionally analysed by gel electrophoresis (Fig.20). Results showed the expected bands for both primer pairs. Unspecific amplification was not detected. Rpl32 bands showed similar results as obtained by qPCR, indicating expression in all cells. LRRK2 bands in SH-SY5Y samples without reverse transcriptase (- SS II RT) display plasmid DNA contamination. This contamination did not exist in HEK cell samples. Therefore LRRK2 RNA expression was detected in transfected HEK cell s, supporting the qPCR results.

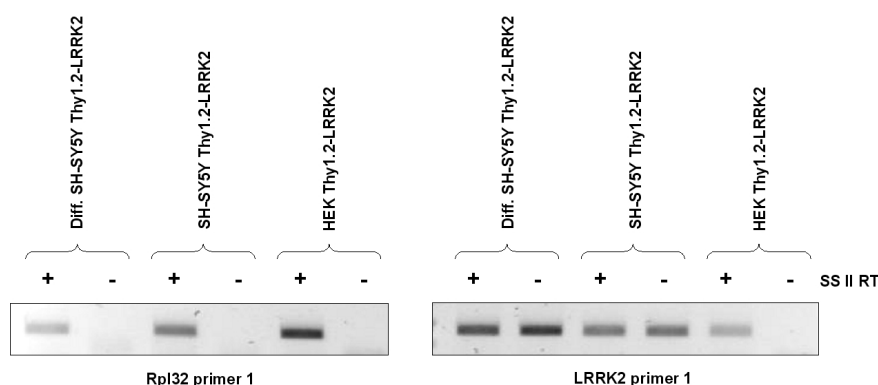


Fig.20 qPCR gel from different Thy1.2-LRRK2 transfected cell lines to detect LRRK2 RNA expression. Left picture indicates cDNA from Rpl32 house-keeping gene in different Thy1.2-LRRK2 transfected cell lines. Signal in lines with plus are from reverse transcription and contain cDNA (+ SS II RT); in lines with minus there is no signal because of absence of reverse transcription (- SS II RT) to exclude the contamination with plasmid DNA. Right picture shows LRRK2 specific bands, in SH-SY5Y samples the signals (in minus lanes) indicate plasmid DNA contamination. Only in HEK samples contamination can be excluded, bands are LRRK2 cDNA specific.

In addition LRRK2 RNA expression from pCMV6-LRRK2 and Thy1.2-LRRK2 plasmids was compared in HEK cells following transfection. pCMV6-LRRK2 transfection efficiency was much higher compared to plasmid Thy1.2-LRRK2, therefore providing a positive control for further analysis. Again qPCR was used to assess Rpl32 and LRRK2 cDNA, synthesized from total cell RNA extracts (Fig. 21).

primer	cells	SS II RT	cycle	mean	diff. RT	diff. +RT
Rpl 32 primer 1	HEK pCMV LRRK2	+ RT	29,31	29,31	0	4,30
			29,32			
		- RT	N/A	-		
			N/A			
	HEK	+ RT	33,84	33,61	0	
			33,39			
		- RT	N/A	-		
			N/A			
	HEK Thy1.2 LRRK2	+ RT	29,01	29,06	8,00	
			29,10			
		- RT	N/A	37,06		
			37,06			
	HEK	+ RT	30,02	29,95	0	
			29,88			
		- RT	N/A	-		
			N/A			
LRRK2 primer 1	HEK pCMV LRRK2	+ RT	23,64	23,69	6,99	9,06
			23,73			
		- RT	30,83	30,67		
			30,51			
	HEK	+ RT	32,98	32,74	0	
			32,50			
		- RT	N/A	-		
			N/A			
	HEK Thy1.2 LRRK2	+ RT	31,62	31,77	2,53	
			31,92			
		- RT	N/A	34,29		
			34,29			
	HEK	+ RT	N/A	33,46	0	
			33,46			
		- RT	N/A	-		
			N/A			

Fig.21 qPCR with RNA from pCMV-LRRK2 or Thy1.2-LRRK2 transfected HEK cells. LRRK2 RNA expression from two different plasmids was tested by qPCR. Rpl32 primer for house keeping gene should be equally expressed in all cells which was not the case for HEK-pCMV-LRRK2/HEK cells (diff. +RT 4,3) indicating different template concentrations. This difference must be considered, leading to 4,76 cycles difference of LRRK2 RNA between pCMV-LRRK2 transfected and untransfected cells. LRRK2 RNA expression was detected in HEK Thy1.2-LRRK2 transfected cells (1,69).

As shown in Fig.21 Rpl32 gene expression was present in all samples tested (middle lane). Unexpectedly, Rpl32 expression was not equal in pCMV6-LRRK2 transfected and untransfected HEK cells indicated by 4,3 PCR cycles difference. LRRK2 RNA expression was detected in pCMV6-LRRK2 and Thy1.2-LRRK2 transfected HEK cells. pCMV6-LRRK2 transfected HEK cells showed higher LRRK2 RNA levels (4,76 PCR cycles difference in pCMV6-LRRK2 transfected HEK cells compared to 1,69 cycles in Thy1.2-LRRK2 transfected cells). Thus, pCMV6-LRRK2 transfection efficiency seems to be much higher compared to plasmid Thy1.2-LRRK2.

Again qPCR samples were loaded on an agarose gel (Fig.22). Obtained bands showed the correct size and specificity of the primers. Empty Rpl32 primer minus lanes indicated absence of genomic DNA contamination. In HEK pCMV6-LRRK2 cells plasmid DNA contamination was present, which was not the case in Thy1.2-LRRK2 transfected cells (no band in lanes without reverse transcriptase; - SS II RT). Thus, the DNase treatment was not sufficient to remove all DNA from the HEK pCMV6-LRRK2 samples.

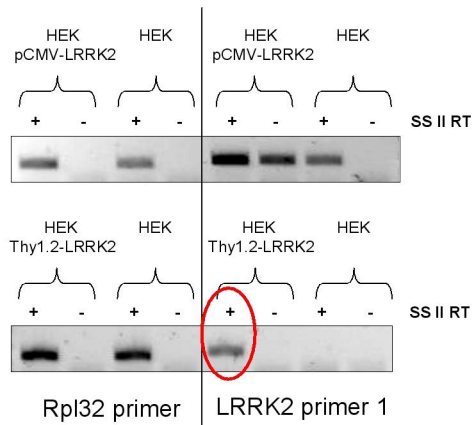


Fig.22 qPCR samples separated on a gel.

Upper picture shows expression of Rpl32 RNA in both cell samples but only in + SS II RT lane on the left side, indicating absence of gDNA contamination. Right side shows pCMV-LRRK2 contamination in - SS II RT lane and a weak signal in untransfected HEK cells. High intensity of positive signal indicated presence of LRRK2 RNA in pCMV-LRRK2 transfected HEK cells. Rpl32 bands in the lower picture indicate absence of gDNA contamination. LRRK2 RNA could only be detected in Thy1.2-LRRK2 transfected HEK cells.

As expected from qPCR, LRRK2 expression was confirmed. In Thy1.2-LRRK2 transfected HEK cells a signal solely derived from LRRK2 RNA was obtained. In contrast in pCMV6-LRRK2 transfected HEK cells the signal was contaminated by plasmid DNA. Nevertheless, the stronger gel band in HEK pCMV6-LRRK2 +RT lanes compared to -RT lanes indicated LRRK2 RNA expression.

Overall, LRRK2 expression was detected from both plasmids.

3.1.2.3 Analysis of LRRK2 expression by Western Blotting

As mentioned above, proteins from transfected and untransfected HEK cell extracts were analysed for the presence of LRRK2 by SDS Page and WB. Blots were incubated with three different LRRK2 specific antibodies (NB300-268, AB 9682 and 16H7-A5) to detect protein expression (Fig.23). As positive control additionally recombinant LRRK2 protein was loaded on the gel.

NB300-268 antibody detected rec. LRRK2 protein and a weak LRRK2 protein band in HEK Thy1.2-LRRK2 cell extracts, but not in untransfected HEK cells. Recent results showed an increased cell death induced by the Thy1.2-LRRK2 plasmid, making analysis difficult (C.Lahsnig, pers. communication). In addition unspecific bands were detected as well.

Antibody AB 9682 failed to detect rec. LRRK2 protein (pos. control) and showed only weak unspecific binding.

Monoclonal antibody 16H7-A5 recognized rec. LRRK2 protein only, but proteins from cell extracts were not detected.

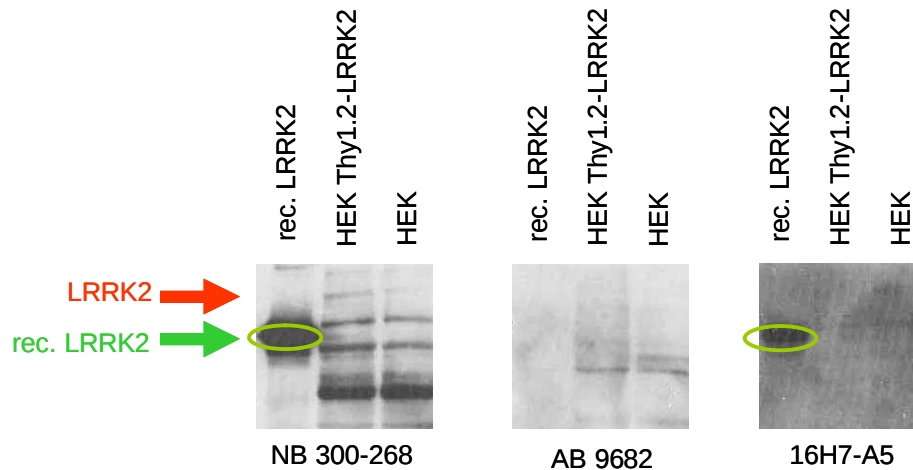


Fig.23 Western blot with HEK protein extracts. In the left panel recombinant LRRK2 protein (~ 205 kD) was detected. A weak signal of LRRK2 protein (~280 kD) was detected in HEK cells, showing a small difference in band intensity between transfected and untransfected HEK cells. In the middle, AB 9682 failed to detect rec. LRRK2 protein and over-expressed LRRK2. Similar results to AB 9682 were achieved by 16H7-A5, though rec. LRRK2 protein was detected (right panel).

Additionally, LRRK2 expression was tested in Thy1.2-LRRK2 transfected and differentiated SH-SY5Y cells (differentiation has been done using retinoic acid). NB300-268 and 16H7-A5 antibody incubation resulted in highly unspecific signals, therefore LRRK2 expression could not be determined (data not shown).

3.1.2.4 Fluorescence-immunocytochemistry (FICC) and Immunocytochemistry (ICC) of Thy1.2-LRRK2 transfected cells

The presence of LRRK2 protein in WB prompted us to determine the cellular localization of LRRK2 by FICC/ICC in CHO and SH-SY5Y cells. Cells were transfected with Thy1.2-LRRK2 vector. The NB300-268 antibody specific for LRRK2 was used. Protein detection was done by DAB or fluorescence staining.

Staining of CHO cells transfected with Thy1.2-LRRK2 failed to show LRRK2 specific signal with DAB staining (Fig.24). Fluorescence staining revealed a signal indicative of LRRK2 expression.

In addition, SH-SY5Y cells transfected with both plasmids were stained using NB300-268 antibody. Cytoplasmic staining was detected but there was no difference in the staining pattern of transfected and untransfected cells (data not shown).

LRRK2 expression was detected in cells transfected with pUC18-Thy1.2-LRRK2 plasmid by PCR and qPCR. LRRK2 protein was only weakly detected in transfected HEK cells by WB and in CHO cells by FICC. In other cell lines LRRK2 protein expression could not be demonstrated. Again this might be due to the increased apoptosis detected in the presence of Thy1.2-hLRRK2 leading to a very low number of hLRRK2 positive cells (C.Lahsnig, per s. communication).

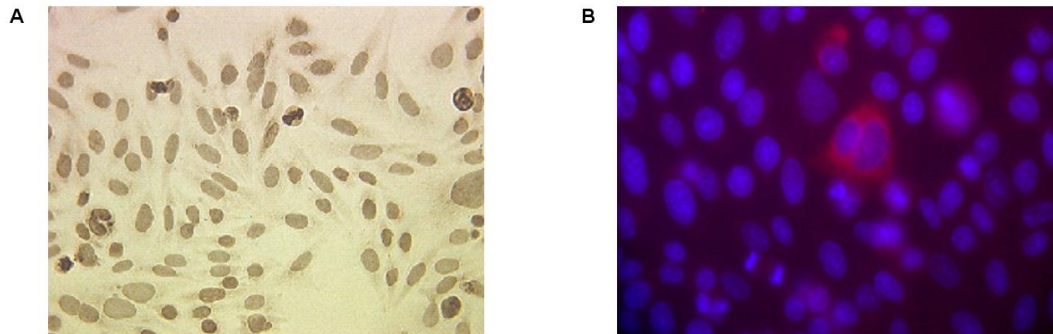


Fig.24 Immunocytochemistry with CHO cells transfected with Thy1.2-LRRK2. (A) Thy1.2-LRRK2 transfected CHO cells stained with NB300-268 antibody failed to show LRRK2 specific staining. (B) FICC staining with these cells incubated by the same antibody detected LRRK2 protein in the cytoplasm.

3.2 Characterization of LRRK2 transgenic founder animals

Transgenic animals were generated and bred by PolyGene according to standardized methods. Transgenic animals were genotyped and analysed by behavioural tests and biochemical analysis.

3.2.1 Genotyping

Breeding cages with transgenic (tg) animals and wild-type littermates (wt; C57Bl/6N) of four months of age were delivered to AFFiRiS AG. First, presence of the LRRK2 transgene was detected by genotyping PCR. The number of inserts in the genome was determined by semi-quantitative PCR and qPCR. Subsequently, characterization of founders including observation of normal animal behaviour was initiated. Wt as well as tg mice were showing no differences in normal behaviour.

3.2.1.1 Genotyping PCR

For genotyping, genomic DNA (gDNA) was used with human LRRK2 specific primers 1 and 4/5 for transgene detection and mouse specific GAPDH primers as positive control (housekeeping gene primer) (Fig.25). H₂O was used as negative control (minus), Thy1.2-LRRK2 plasmid DNA as positive control (plus).

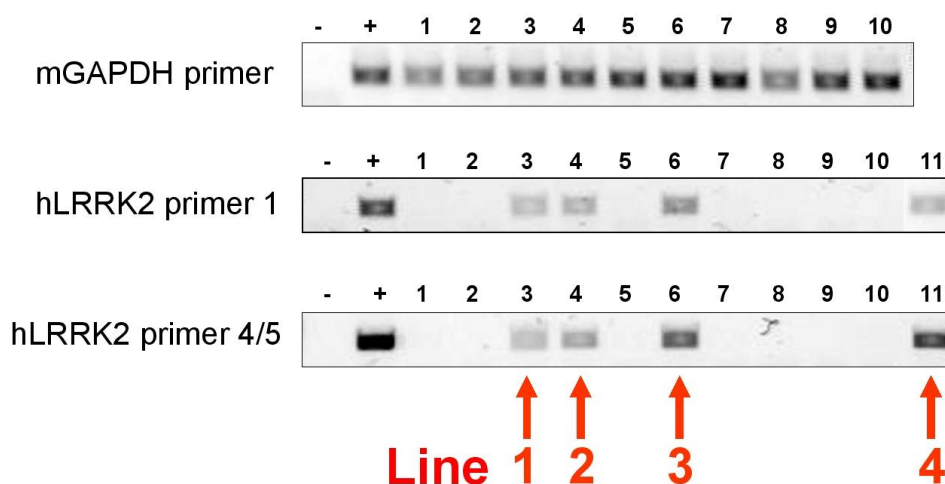


Fig.25 Genotyping PCR with founder DNA. House-keeping gene specific primer mGAPDH shows specific bands in all animals. LRRK2 specific primer 1 and 4/5 indicate transgenic animals 3,4,6 and 11 leading to 4 different founder lines (in red). Water as neg. control (minus) and Thy1.2-LRRK2 plasmid DNA as pos. control (plus).

After PCR, samples were separated by agarose gel electrophoresis. As expected, results from genotyping PCR reproduced the data from Polygene (four transgenic animals (4 lines) and corresponding wt animals). The negative control showed no PCR signal, positive control displayed the same band as in transgenic mouse samples. Taken together three male and one female founder (line 2) was obtained.

3.2.1.2 Semi-quantitative PCR

After genotyping, the relative number of LRRK2 insertions in the tg founder animals was determined. Therefore semi-quantitative PCR (sqPCR) was done with gDNA from the four founder animals. As a positive PCR control, control gene primers (mGAPDH) were used and LRRK2 primers for transgene detection. PCR reaction was stopped after 16, 18, 21, 24, 27, 30, 33 and 36 cycles and loaded on an agarose gel (Fig.17).

Amplicon size from both primers showed in all lines the expected results. The bands obtained using GAPDH primers lead to similar intensity of the signal in all animals and correlated to the respective LRRK2 signals. The ratio indicates the amount of transgene as compared to the housekeeping gene. According to the results displayed in Fig.26, lane 2 showed the lowest sqPCR signal, however results did not allow to determine whether line 1,3 and 4 show higher copy numbers. Thus qPCR was performed as well.

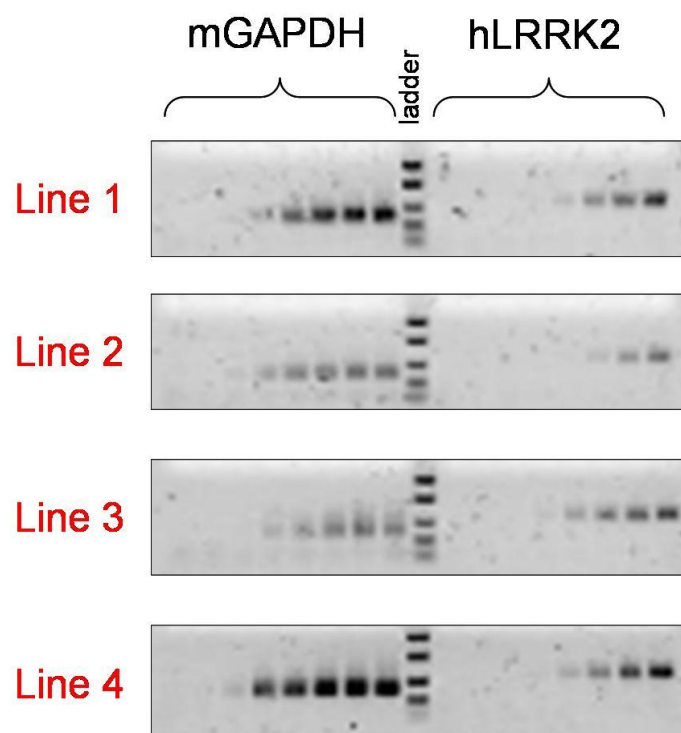


Fig.26 sqPCR with founder DNA. Determination of LRRK2 integration number with sqPCR using house-keeping gene primer and LRRK2 primer. Samples were stopped after different reaction cycles (16, 18, 21, 24, 27, 30, 33 and 36 cycles) and loaded on a gel from left to right. The number of LRRK2 gene integrations in the genome starting with the highest is line 3 > 1 > 4 > 2.

3.2.1.3 Quantitative PCR (qPCR)

Results from semi-quantitative PCR were confirmed by qPCR. The signal obtained from the amplification of the IL2 gene with two copies in the genome was compared to the transgene signal (hLRRK2; LRRK2 primers 1). The amount of LRRK2 integrations in the genome was determined subsequently. In this analysis the tg founder gDNA was included. According to Fig.27.A, LRRK2 reaches threshold earlier than IL2. Therefore more LRRK2 insertions than IL2 are present in the genome. Results from melting peak determination confirmed specificity of both primers (Fig.27.B).

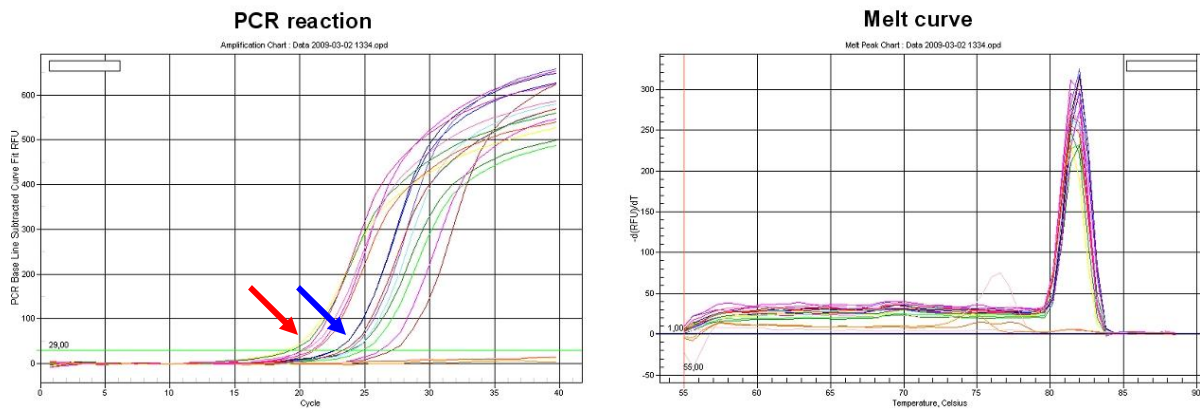


Fig.27 qPCR with founder genomic DNA. Determination of transgene integration number in the genome of founder animals by RT-PCR using IL2 primer and LRRK2 primer. An illustration of RT-PCR cycles is shown on the left side (LRRK2 signal red arrow, IL2 signal blue arrow). Melt curve of PCR samples after PCR reaction is shown on the right side, indicating the purity of the amplicon.

After data analysis we calculated the approximate integration number of LRRK2 in the genome of founder animals compared to the IL2 gene (two copies per genome). Results showed the highest LRRK2 integration number in the genome of the animal in line 3, followed by line 1 and 4. The lowest number was detected in line 1 (Fig.28). These results were consistent with the results obtained by semi-quantitative PCR.

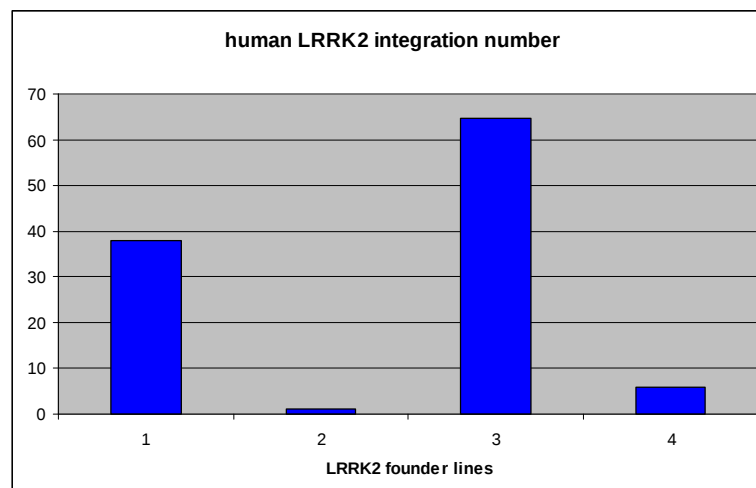


Fig.28 qPCR with LRRK2 founder DNA. LRRK2 integrations in the genome show the highest number in line 3 followed by line 1 and 4. The lowest copy is present in line 2.

3.3 Initial characterization of the 4 tg lines

Founder animals and the offspring from four different lines were analysed by PCR to check their genotype for further breeding (Fig.25, 29, 30). Progenies of line 3 and 4 were analysed starting with two months of age.

3.3.1 Genotyping PCR of transgenic LRRK2 mice

Tail biopsies from offspring of all four lines (animal 12-34), were used for genotyping PCR. LRRK2 specific primers 1 and 4/5 were used for transgene detection, mGAPDH primers as positive controls. Thy1.2-LRRK2 DNA was used as positive control (plus) and H₂O for detection of contamination (minus). After PCR, samples were separated by gel electrophoresis (Fig.30).

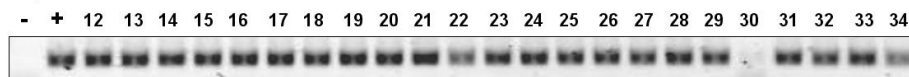
According to Fig.20, GAPDH was detected in all animals except of animal 30. The Transgene specific signal was detected in 5 out of 23 pups (animal 13, 21, 23, 33 and 34) (red arrow). Positive control was detected with all primers and the water control showed no signal. The transgenic animals obtained were used together with wild type littermates for further behavioural and biochemical analysis to detect transgene expression.

LRRK2 animals

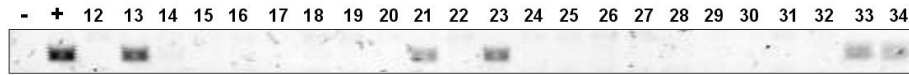
No.	sex	date of birth	genotype	Line	animal status
1	f	28.07.2008	wt	1	founder
2	f	28.07.2008	wt	1	founder
3	m	28.07.2008	tg	1	founder
4	f	28.07.2008	tg	2	founder
5	m	28.07.2008	wt	2	founder
6	m	28.07.2008	tg	3	founder
7	f	28.07.2008	wt	3	founder
8	f	28.07.2008	wt	3	founder
9	f	28.07.2008	wt	4	founder
10	f	28.07.2008	wt	4	founder
11	m	28.07.2008	tg	4	founder
12	m	25.11.2008	wt	3	offspring
13	f	25.11.2008	tg	3	offspring
14	f	25.11.2008	wt	3	offspring
15	f	25.11.2008	wt	3	offspring
16	f	27.11.2008	wt	1	offspring
17	f	27.11.2008	wt	1	offspring
18	m	27.11.2008	wt	1	offspring
19	m	27.11.2008	wt	1	offspring
20	m	27.11.2008	wt	1	offspring
21	m	25.11.2008	tg	4	offspring
22	f	25.11.2008	wt	4	offspring
23	f	05.12.2008	tg	4	offspring
24	f	05.12.2008	wt	4	offspring
25	f	07.12.2008	wt	1	offspring
26	f	07.12.2008	wt	1	offspring
27	f	07.12.2008	wt	1	offspring
28	m	07.12.2008	wt	1	offspring
29	m	07.12.2008	wt	1	offspring
30	m	07.12.2008	wt	1	offspring
31	m	07.12.2008	wt	1	offspring
32	f	19.12.2008	wt	4	offspring
33	f	19.12.2008	tg	4	offspring
34	f	19.12.2008	tg	4	offspring

Fig.29 Table of LRRK2 mice, including founder (1-11) and offspring (12-34). m...male, f...female, wt...wild-type, tg...transgene

mGAPDH primer



hLRRK2 primer 1



hLRRK2 primer 4/5

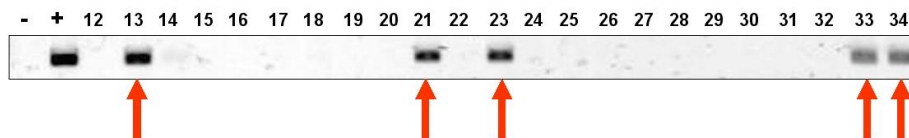


Fig.30 Genotyping PCR with pups DNA. GAPDH specific house-keeping bands were detected in all animals except animal 30 where genomic DNA was absent. Signals with LRRK2 specific primer lead to 5 transgenic animals (13, 21, 23, 33 and 34) out of 23 pups (red arrow).

3.3.2 Genotyping qPCR of transgenic LRRK2 mice

Consequently we were interested in the number of transgene integrations in the genome of the offspring and compared it to founder animals.

Therefore qPCR was performed with gDNA from founder animal (11, line 4) and pups (13, line 3 and animal 23, line 4) with IL2 specific primers and a LRRK2 specific primer pair (primers 1). Results showed that line 3 had again the highest integration number followed by line 4 as obtained before (data not shown).

In order to characterize the phenotype of the lines 3 and 4, 6 animals were chosen (4 from line 4 and 2 from line 3). Two animals (male tg animal 23, wt animal 24 from line 4) with two months of age were chosen for behavioural and biochemical analysis. Two months later four animals were analysed with four months of age (male tg animal 12 and wt animal 21 from line 4, female tg animal 13 and wt animal 14 from line 3).

3.3.3 Analysis of the motor function from transgenic LRRK2 mice

The motor function of six animals was monitored by different tests. At the first day of training, the grooming behaviour of the animals was observed and no difference in behaviour between wt and tg was detected.

Beam, pole and grid test were performed as described in section 2 (Material and Methods).

After data collection and comparison (Fig.31) we did not detect any difference in behaviour between wt and tg animal, probably due to the low number of animals tested.

Subsequently animals were sacrificed for biochemical analysis of transgene expression.

animal	line	sex	age (month)	genotype	Test			
					beam (errors/step)	pole (sec.)		grid (sec.)
						U-turn time	run time	
23	4	m	2	tg	n.d.	2,2	7,4	91
24	4	m	2	wt	n.d.	2,7	10,3	106
12	4	m	4	wt	0,4	4,2	5,2	118
21	4	m	4	tg	0,3	2,2	13,3	120
13	3	f	4	tg	0,2	2,9	5,7	120
14	3	f	4	wt	0,2	2,3	4,8	120

Fig.31 Summary of behavioural tests. LRRK2 offspring (12, 21, 13, 14, 23 and 24) tested by beam, pole and grid test.

As shown before the biochemical analysis of transgene expression was started with male pups 23 and 24 (two months of age, line 4). Furthermore, the effects of the high integration number in line 3 on transgene expression was tested and compared to effects obtained from analysis of animals from line 4. Therefore female tg animal 13 and wt animal 14 from line 3 with four months of age were chosen. Transgene expression was also tested in four month old male animals 12 and 21 from line 4.

LRRK2 RNA was detected by PCR and qPCR, LRRK2 protein by Western Blotting and IHC.

3.3.4 Tissue RNA extraction and PCR/qPCR of transgenic mice

Mice were sacrificed and RNA was extracted from different tissues (brain, gut, heart liver, lung, stomach, spleen, muscle, kidney and spinal cord) to test LRRK2 RNA and protein expression.

The extracted RNA not treated with DNase I was leading to specific LRRK2 bands in nearly all tissues of tg animal (see Fig.32). LRRK2 signals were specific because the wt animal displayed no LRRK2 signal in any tissue. Instead, PCR using mGAPDH primers lead to bands in all tissues. As positive control Thy1.2-LRRK2 plasmid DNA was used and water as negative control.

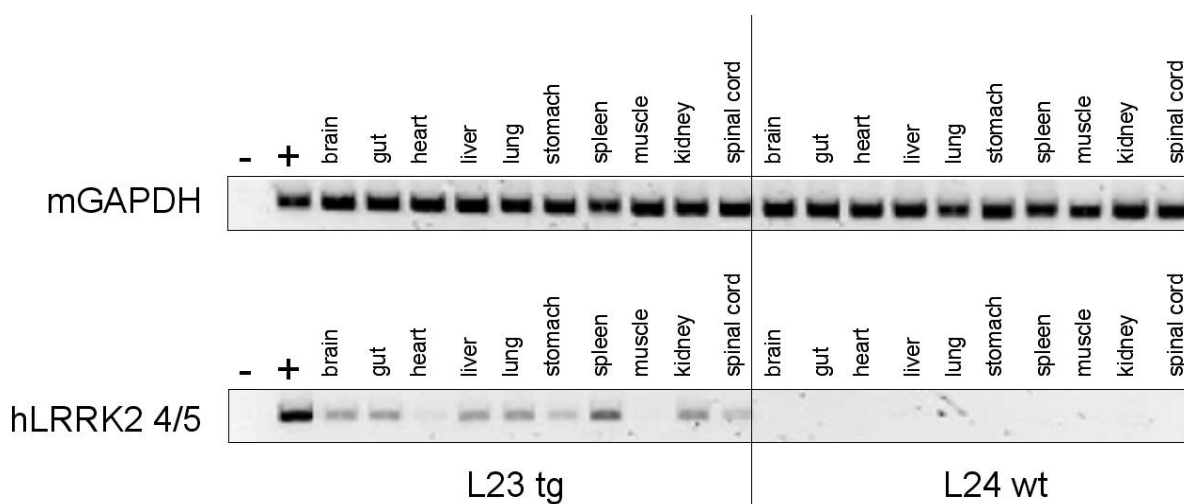
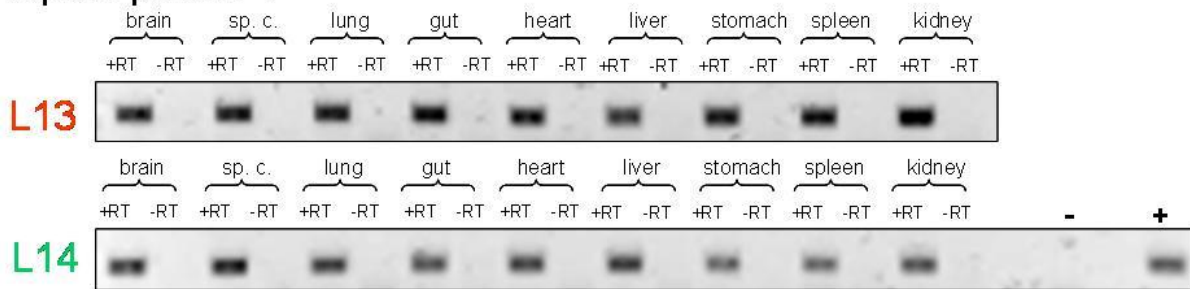


Fig.32 PCR with animal 23 and animal 24 tissue cDNA (not treated with DNase I). mGAPDH primer for house keeping gene gave positive bands in every tissue of both animals. LRRK2 specific primers gave signals only in transgenic animal 23 in all tissues except of heart and muscle.

Female animals wt 13 and tg 14 from line 3 (four months old) were also tested for LRRK2 RNA expression by PCR and qPCR. Results in Fig.33 show Rpl32 RNA expression in all tissues analysed. LRRK2 RNA could only be detected in the tg animal and was restricted to the brain and spinal cord. gDNA contamination was not detectable as indicated by the absence of a signal in samples without reverse transcriptase (-RT).

Rpl32 primer 1



LRRK2 primer 1

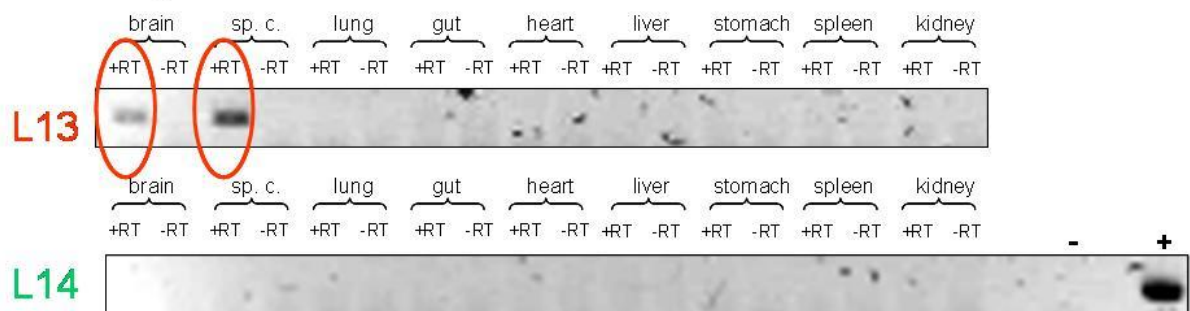


Fig.33 PCR with cDNA from 13 (tg) and 14 (wt) tissue RNA. Rpl32 primer shows similar results in tissues of both animals. LRRK2 cDNA is only present in L13 brain and spinal cord, it could not be detected in other tissues of this animal or in the wild type. red circle ð LRRK2 RNA band, sp.c. ..spinal cord, RT ð reverse transcriptase

Results from qPCR (Fig.34) with RNA from brain, spinal cord and lung (negative control) show expected results. Rpl32 RNA was detected in all tissues, LRRK2 RNA was only detected in brain and spinal cord. Signal from lung indicated presence of unspecific amplification or primer dimerisation. According to Fig.34.A, Rpl32 signal reaches threshold earlier than LRRK2. Results from melting peak determination confirmed specificity of both amplicons (Fig.34.B).

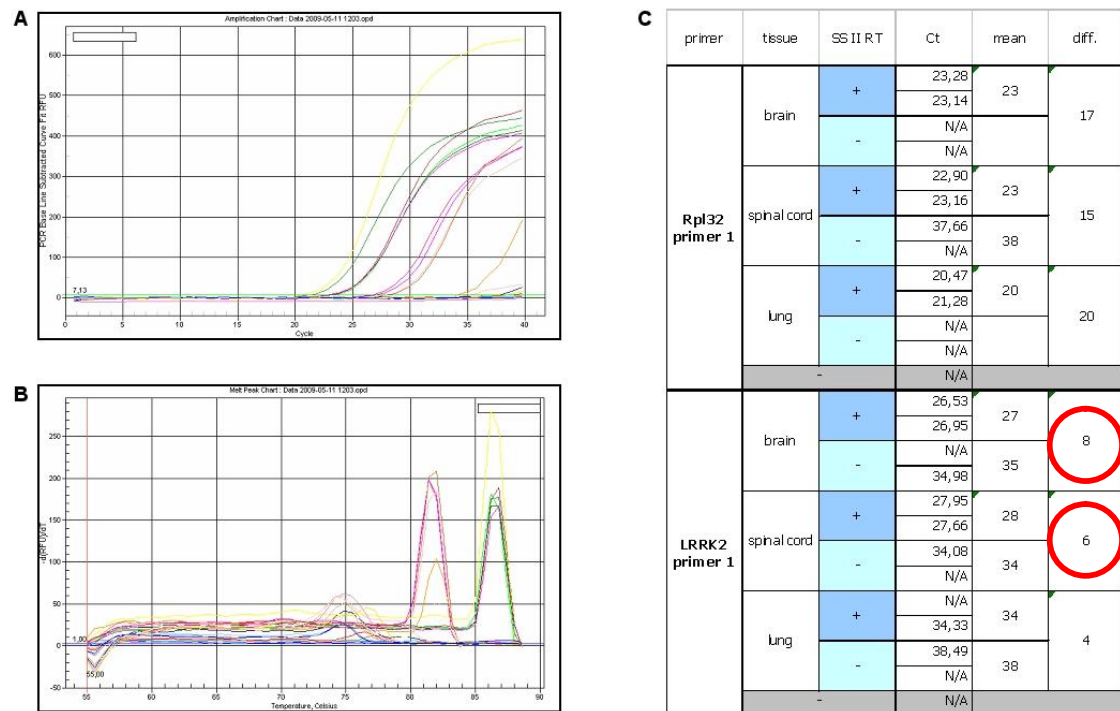


Fig.34 qPCR with cDNA from 13 (tg) brain, spinal cord and lung. (A) Illustration of PCR. (B) Melt curve peaks for amplicon purity determination. (C) Results of qPCR show LRRK2 RNA expression in brain and spinal cord (red circles), results from lung were not counted as LRRK2 RNA expression due to high cycle numbers (Ct value) indicating background signal.

Samples from qPCR were also separated by electrophoresis (Fig.35). As expected, qPCR showed absence of DNA contamination (no bands in ñRT lanes) and LRRK2 RNA presence in brain and spinal cord of the tg animal 21.

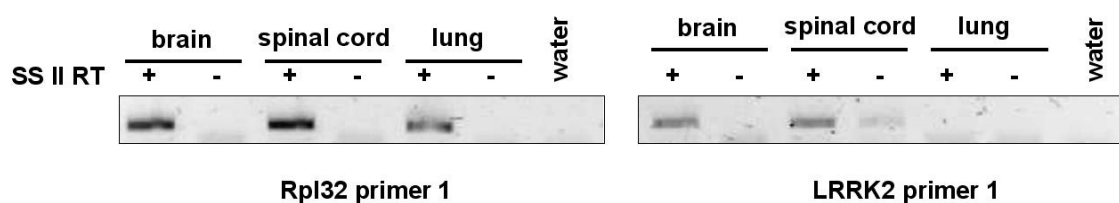


Fig.35 Gel electrophoresis of qPCR samples. PCR with Rpl32 primer showed bands in every tissue. LRRK2 specific primers show RNA expression in brain and spinal cord, but not in the lung. Signals from cDNA and not genomic DNA contamination because samples without reverse transcriptase (SS II RT) do not show any signal.

LRRK2 RNA expression was also tested in four month old male animals from line 4 and compared to two month old animals 23 and 24 (above). Transgenic animal 21 and wt animal 12 were used for PCR and qPCR analysis. Fig.36 showed Rpl32 expression in all tissues, LRRK2 RNA was also present in all tissues except for the spleen thus reproducing the expression profile obtained before.

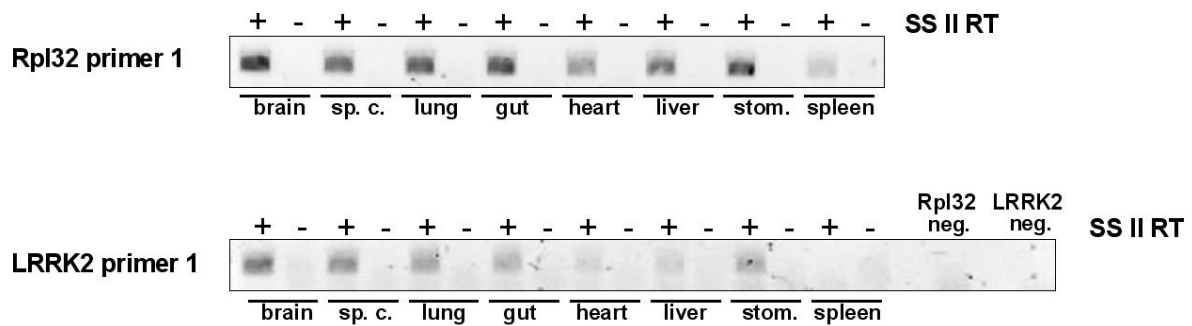


Fig.36 Gel electrophoresis of qPCR samples from animal 21 (tg) cDNA. Rpl32 signal is present in every tissue as positive control for qPCR reaction. LRRK2 cDNA is present in every tissue except of spleen. Samples without reverse transcription show no signal indicating the absence of genomic DNA contamination.

Results from qPCR showed the highest LRRK2 RNA expression in the brain, followed by decreasing amounts of LRRK2 RNA in the spinal cord, lung, heart, stomach, liver and gut (data not shown). In the spleen LRRK2 RNA was not detected, Rpl32 was detected in all tissues.

3.3.5 Western Blotting of tissue homogenates

For Western Blotting, remaining tissue from RNA extraction was used for protein extraction with RIPA buffer. The protein concentration of the homogenates was determined by Bradford measurement (data not shown) and equal amounts were analysed by WB. For the analysis of protein samples from animals 23 and 24, two different amounts of homogenates were loaded due to the low detection limit of the LRRK2 antibody compared to control antibodies (tyrosine hydroxylase TH, β -actin). As additional loading control truncated rec. LRRK2 protein was loaded (205 kD, aa 970-2527, Invitrogen) as well as a cell extract from pCMV6-LRRK2 transfected CHO

cells. After transfer, the membrane was cut into three pieces. For LRRK2 detection a monoclonal LRRK2 specific antibody (16H7-A5) or the polyclonal NB300-268 antibody was used. The anti-TH antibody (60 kD) and anti- β actin antibody (41 kD) were used as loading controls.

Animals 23 and 24 from line 4 were analysed on WB for LRRK2 expression. 16H7-A5 antibody detected recombinant LRRK2 protein and LRRK2 protein in cell extracts as well as in brain homogenates at the expected size (Fig.37). Although the wt animal also showed a faint signal at the expected size LRRK2 expression seemed to be increased in tg brain samples as compared to the wt brain. The detection LRRK2 in the non-transgenic animal indicates, that 16H7-A5 is not only binding to human but also mouse LRRK2 protein in WB. Loading control signal from anti-TH and anti- β actin staining displayed equal loading (1 μ l homogenate was loaded into each lane).

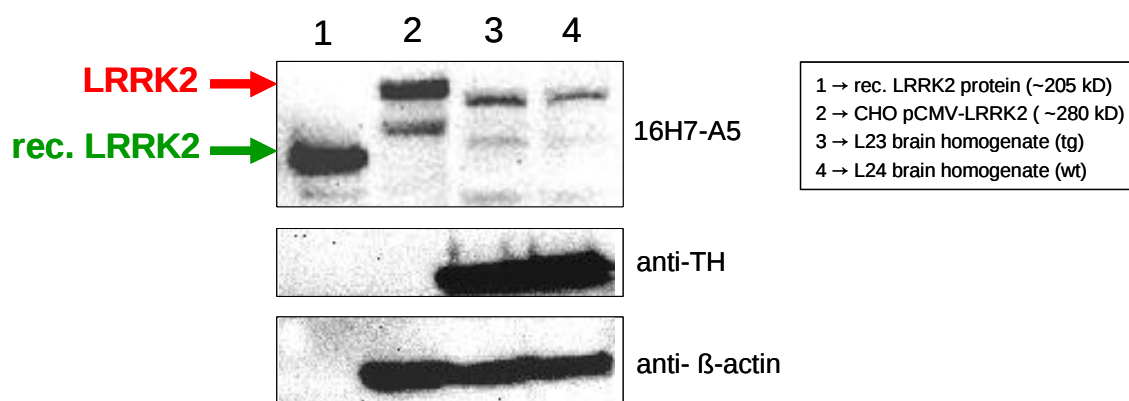


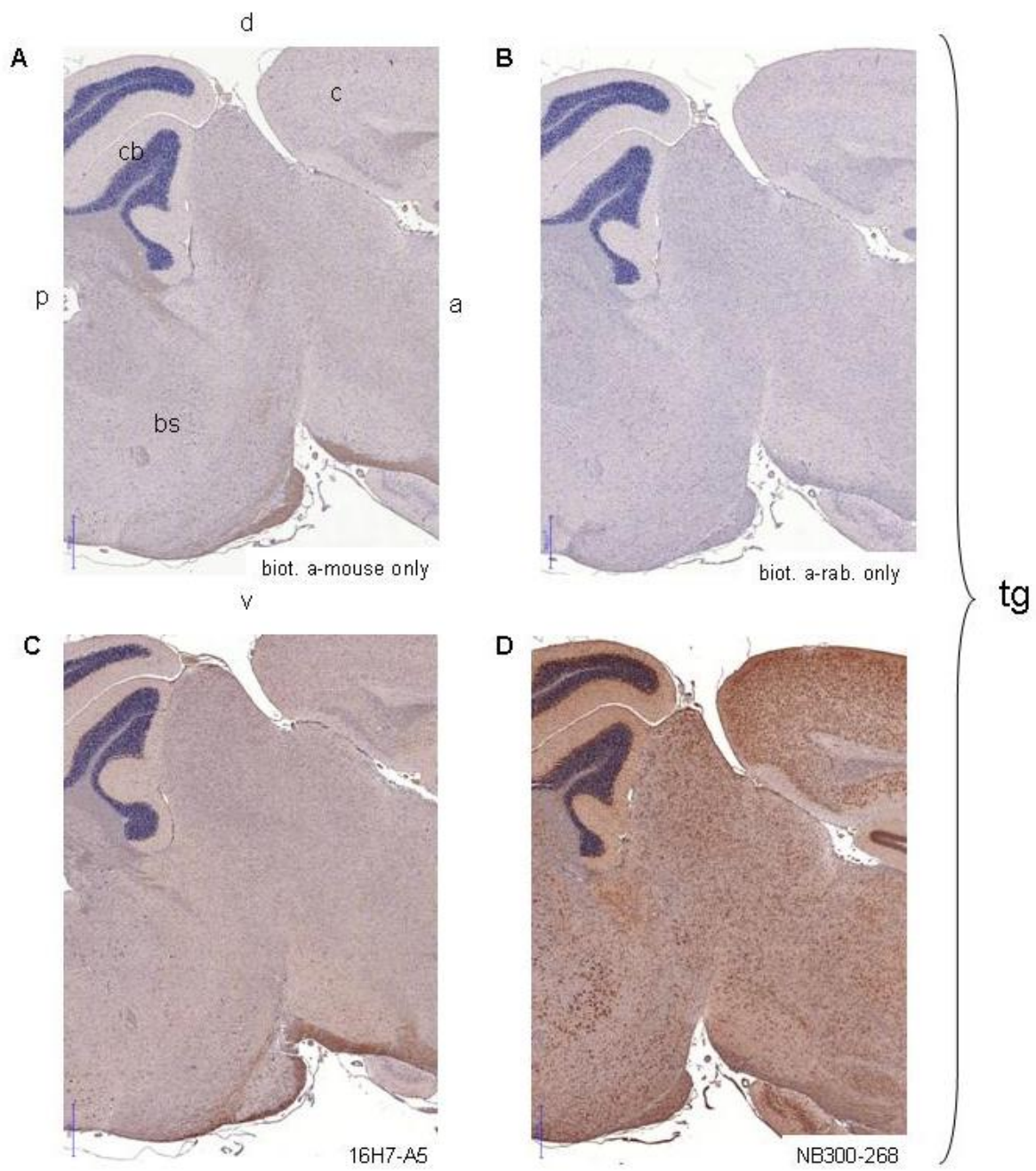
Fig.37 Western Blotting with brain protein homogenates from animal 23 (tg, lane 3) and animal 24 (wt, lane 4) animals. Lane 1 loaded with recombinant LRRK2 protein (~205 kD), lane 2 loaded with CHO pCMV6-LRRK2 cell extracts. Lane 3 was loaded with tg brain (23) and lane 4 with wt homogenates (24). Membranes were incubated with LRRK2 antibody 16H7-A5 and two loading control antibodies, the anti-tyrosine hydroxylase (60 kD) and anti- β actin (41 kD). Both loading control antibodies showed equal loading of brain homogenates. 16H7-A5 antibody showed a signal with expected size of LRRK2 protein (~280 kD, red arrow) in CHO cell extracts and brain samples. Additionally rec. LRRK2 protein was detected (green arrow). The signal of LRRK2 protein was stronger in the transgenic animal than in the wild type.

3.3.6 Immunohistochemistry (IHC)

In order to analyse expression of hLRRK2 in the brain of tg animals, brain hemispheres of animals 23, 24, 12, 21, 13 and 14 were sectioned and subsequently stained with the antibodies NB300-268 and 16H7-A5.

Slides were also incubated with secondary antibody only, to indicate unspecific binding. Staining was documented using a Mirax Scan (Zeiss). Brain regions analysed include brainstem, medulla and midbrain.

Fig.38 shows IHC with brain sections from animal 23 and 24 (line 4). Negative controls using anti-mouse secondary antibody showed weak background only in contrast to anti-rabbit secondary antibody. NB300-268 (rabbit polyclonal antibody) staining showed a high background signal. An overall strong staining in tg as compared to wt littermates was detectable but the background does not allow to define expression on a cellular level. Incubation with 16H7-A5 antibody showed no difference between tg (23) and wt (24).



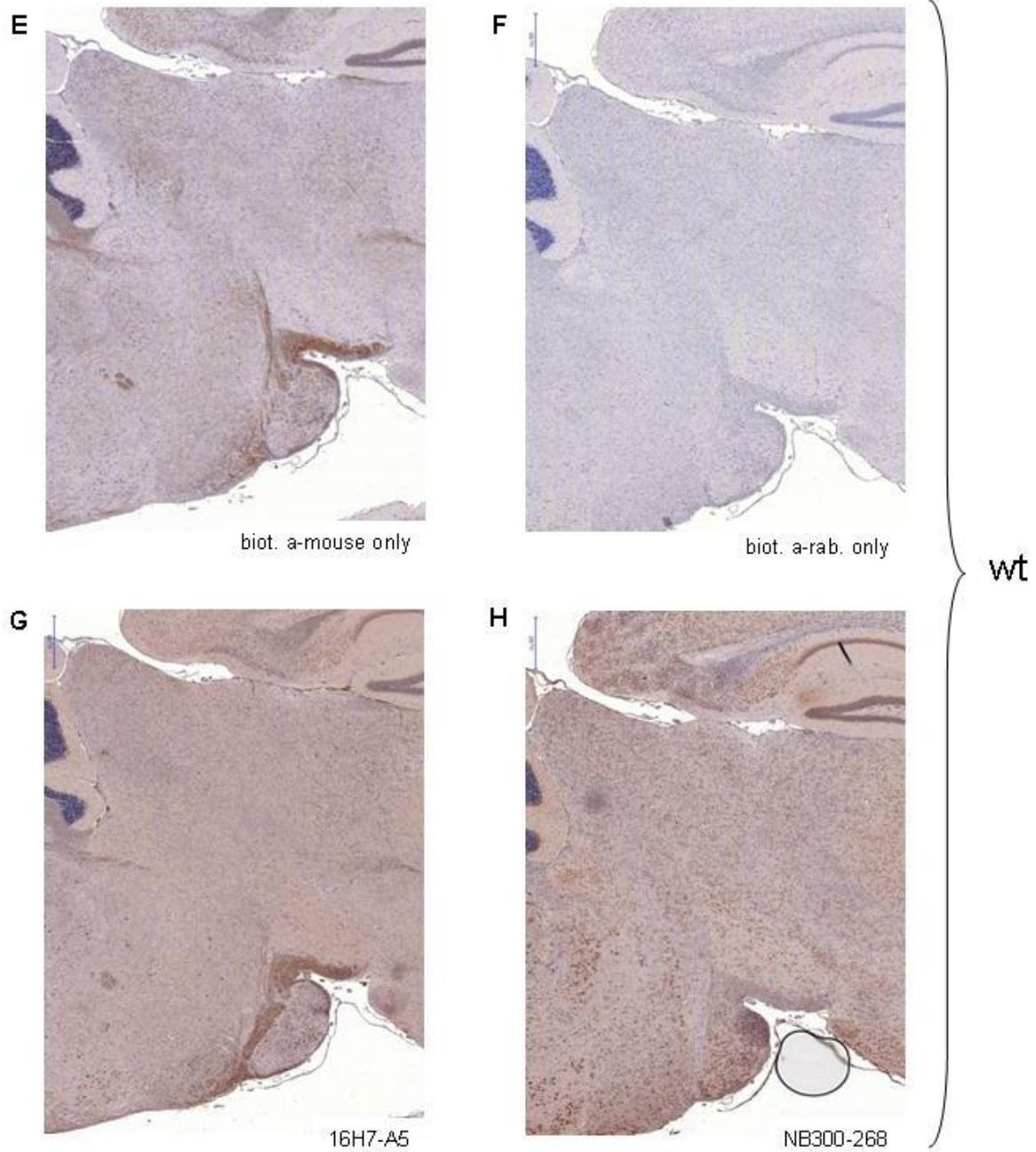


Fig.38 IHC with animal 23 (tg) and 24 (wt) brain sections. Midbrain region from tg and wt animal are displayed in parallel. Background signal with biot. anti-mouse secondary antibody is present (A,E). No detection of background signal with biot. anti-rabbit antibody (B,F). 16H7-A5 incubation (C,G) displayed no difference between wt and tg compared to NB300-268 (D,H). cbē cerebellum, bsē brainstem, cē cortex, aē anterior, pē posterior, dē dorsal, vē ventral

IHC with brain sections from other animals (12, 21, 13 and 14) showed similar results.

Strong signals on NB300-268 stained sections could indicate LRRK2 expression in the brain not only specific for human but also for mouse LRRK2 protein. Thus the antibody tested does not allow determination of hLRRK2 expression due to potential detection of both mouse and human LRRK2 as well because of high background.

Fig.39 shows LRRK2 localization on the cellular level comparing the expression patterns obtained by two different antibodies. The mouse monoclonal antibody 16H7-A5 showed stronger LRRK2 staining intensity in wt than in tg again indicating that the specificity is not limited to human LRRK2 (as shown in WB). Similar staining patterns in wt and tg was achieved by NB300-268. Concluding, this analysis provides evidence that mLRRK2/hLRRK2 protein is localized in the cytoplasm as already indicated by the in-vitro analysis (ICC of LRRK2 over-expressing cells).

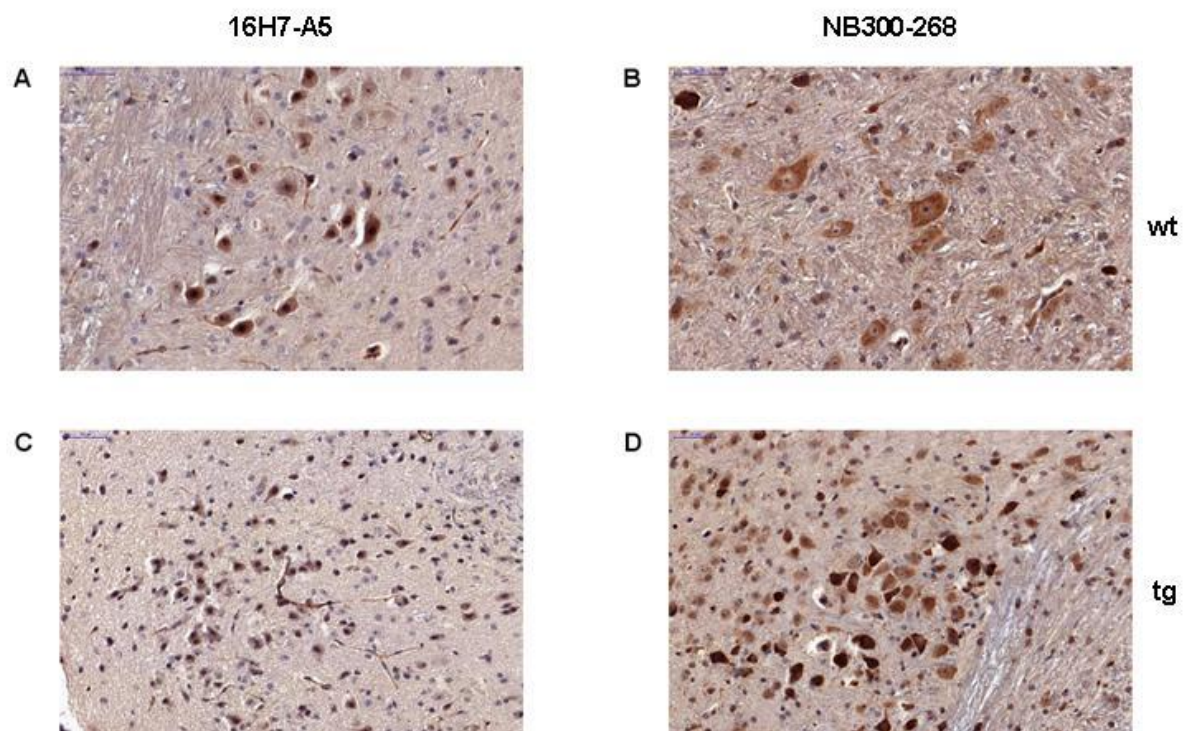


Fig.39 Immunocytochemistry of 12 (wt A, B) and 21 (tg C, D) mice on cellular level (brainstem). Left pictures show staining with 16H7-A5 antibody specific for LRRK2 protein in wt and tg animal. In the wt (A), a stronger cytoplasm staining with 16H7-A5 was detected than in the tg animal (C). NB300-268 staining (B, D) shows similar signals in wt (B) and tg (D) animal. (scale indicates 50 μ m)

Animal	Line	NB300-268	16H7-A5
23	4 (tg)	+ (unspecific)	-
24	4 (wt)	+/- (unspecific)	+
12	4 (wt)	+ (unspecific)	+
21	4 (tg)	+ (unspecific)	-
13	3 (tg)	+ (unspecific)	-
14	3 (wt)	+ (unspecific)	-

Fig.40 IHC results. Summary of IHC results from tested brain sections. Offspring 23, 24, 12, 21, 13 and 14 were analysed. Antibody NB300-268 and 16H7-A5 were used for LRRK2 detection.

4 DISCUSSION

The aim of this diploma thesis was the initial characterization of a novel transgenic mouse line over-expressing hLRRK2 under the control of a neuron specific promoter. This mouse line will be used to elucidate the role of LRRK2 in the nervous system *in vivo* and might thus shed light on the role of LRRK2/Dardarin/PARK8 in the etiology of PD and related disorders. A thorough analysis of LRRK2 function by over-expression of wt LRRK2 could also be instrumental to evaluate whether LRRK2 is a potential target for the development of novel treatment strategies against Parkinson's disease and related neurodegenerative diseases in the future.

As a first step before tg production the vector containing full length hLRRK2 cDNA was tested. *In-vitro* expression analysis of pCMV6-XL5-LRRK2 plasmid was done by PCR and qPCR. Beside full length mRNA, LRRK2 protein was also detected by Western Blotting and FICC/ICC. The hLRRK2 cDNA insert was subsequently cloned into the pUC18-Thy1.2 vector [42], which was used for transgene production. The mouse Thy1.2 expression cassette as used in the design of the transgene described in this thesis is well known to induce expression of the transgene in neurons in peripheral and central nervous system including the cortex, the cerebellum as well as in the midbrain and is also leading to expression in motor neurons as well as in spinal cord nuclei. The onset of transgene expression is reported around postnatal day 6, therefore no interference with early embryonic and nervous system development is expected, as this could preclude any postnatal analysis. Thus hLRRK2 expression under the control of the murine Thy1.2 cassette should be present in areas responsible for movement control affected in PD patients [42, 47] and could thus present a suitable model for studying the role of LRRK2 in normal nervous system as well as in disease.

The plasmid Thy1.2-LRRK2 was used for expression analysis of LRRK2 on RNA and protein level. RNA expression from this plasmid was detected in transfected HEK cells (Fig.22) but not in other cell lines. LRRK2 protein was only weakly detectable by western blot analysis, also FICC/ICC staining demonstrated LRRK2 protein expression albeit only in few transfected cells (Fig.24). Antibody AB9682 used for

western blot analysis was not able to detect transgene and rec. LRRK2 protein in our hands. In contrast antibody NB300-268 detected LRRK2 protein in low concentrations but displayed high unspecific binding in Western Blotting. The antibody 16H7-A5 detected LRRK2 only when high amounts of protein were loaded but showed less unspecific binding in WB.

The difficulties to detect LRRK2 protein in tissue culture using pUC18-Thy1.2 transfected cells could be due to low transfection efficiency leading to a limited amount of LRRK2 expression. For this project, the transfection protocol used has also been changed to increase transfection efficiency by using the CaP precipitation method. Even though this method usually leads to high transfection efficiency in HEK and CHO cells, no increase in efficiency could be observed (data not shown).

Another explanation might be that inappropriate cell lines were used for *in-vitro* analysis. The mThy1.2 promoter might be only active in certain cell lines where glucophosphatidylinositol linked protein (Thy) is normally expressed (nervous system, thymus and connective tissue).

Interestingly, recent experiments performed in the lab also demonstrated that transfection using pCMV6-hLRRK2 as well as Thy1.2-hLRRK2 plasmids lead to increased cell death after 24h (C.Lahsnig, unpublished). Thus, the lack of protein expression and the low number of LRRK2 positive cells detected could well be based on a LRRK2 induced increase in apoptosis following LRRK2 expression. This would also be supported by recent experiments in different labs, which could show that mutant as well as wt LRRK2 is able to increase apoptosis, both stress dependent and independent, applying the extrinsic cell death pathway and Caspase 8 [48]. Thus a different assay to perform an in-depth functional analysis of LRRK2 enzyme activity needs to be established. One possibility would be the isolation of primary neurons derived from hLRRK2 transgenic animals.

To start the *in vivo* characterization of LRRK2 function, the founder animals obtained, carrying the transgene, as well as several animals derived from breeding the different founder animals to C57/Bl6N animals were genotyped and initially characterized. Six littermates (F1 generation), obtained from breeding of tg founders with C57Bl/6N wt animals were tested by behavioural tests, including monitoring of grooming behaviour, as well as the beam, pole and grid test. Due to time constraints, only animals with 2 and 4 months of age were included in this analysis. The animals

tested failed to display a significant difference between wt and tg littermates. However, it has to be mentioned that the number of tested animals was too small for a comprehensive characterization. Especially for behavioural testing, a greater animal number needs to be analysed in parallel to get statistically significant results due to big individual differences. Indeed, even though no phenotypic alterations due to LRRK2 over-expression have been detected in Tg animals at an age of 4 months but might be developing in older animals, which were not available during this analysis the tests have been repeated independently. Importantly, this additional behavioural analysis in a small cohort of animals carrying the transgene with an age of 8 month did show a trend for PD-like motor dysfunction (C. Lahsnig, unpublished information) supporting the idea that a neurodegenerative phenotype might only be detectable later upon aging if the animals. The fact that a PD-like phenotype is expected upon aging is further demonstrated by recent work of Li et al.. These authors could demonstrate that a hLRRK2^{R1441G} BAC tg mouse model developed a strong phenotype. The R1441G mutation used in this study is associated in humans with familial PD [14, 15]. At three month of age, transgenic animals did not show any motor deficits. In contrast at the age of 10-12 months hLRRK2^{R1441G} BAC tg animals showed defects such as immobility and impaired dopamine release. These mice offer, for the first time, an animal model showing cardinal features of PD. Interestingly L-DOPA medication reversed this phenotype as observed in PD patients. Also transgenic mice over-expressing wt hLRRK2 were generated which unexpectedly did not display any disease associated symptoms [41]. Similar effects of wt and mutant LRRK2 were found in *Drosophila*. Especially mutant flies showed reduced lifespan and increased sensitivity to mitochondrial toxins [40].

This observation is in accordance to our wt transgenic model where no phenotype was present in young animals. Nevertheless, six littermates were tested biochemically for LRRK2 RNA and protein expression.

LRRK2 mRNA expression was successfully detected in several tg animals (see below). LRRK2 specific antibodies NB300-268 and 16H7-A5 were used for protein detection. The polyclonal NB300-268 antibody detected LRRK2 protein in lower concentrations than the 16H7-A5, however unspecific binding proved to be higher using NB300-268. Additionally, a band at the expected size of LRRK2 was also detected in wt and tg using both Abs. Therefore, antibodies seem to detect both

human and mouse LRRK2. According to results described [44] NB300-268 should be specific for human LRRK2 only. Interestingly, an increase in LRRK2 signal might be detectable in tg compared to wt animals.

Detection of hLRRK2 expression by IHC on sagittal brain sections of tg and wt animals showed similar results as obtained by WB. Both antibodies were not able to discriminate between murine and human LRRK2. Unspecific binding to other proteins also seems to be more pronounced with NB300-268 antibody, as shown on WB. 16H7-A5 antibody showed weak staining of the tissue slides but a low unspecific binding pattern. The detection of murine and human LRRK2 however does not allow to clearly demonstrate hLRRK2 over-expression so far. Therefore other antibodies should be tested and used for transgene detection, which are able to distinguish between mouse and human LRRK2. Additionally, the staining protocol should be modified to allow hLRRK2 detection in situ.

Currently, still many questions concerning the LRRK2 function in vivo have not been solved and thus there is still much research on this topic needed. Further analysis using this novel mouse model will be necessary to further clarify the biochemical, physiological and pathological function of the LRRK2 protein.

Parkinson's disease has a divergent pathology with many different factors influencing the development of the disease. Understanding and treatment of this neurodegenerative disease is therefore a big topic for current neurobiological research. LRRK2 protein seems to play a crucial role in disease manifestation, and is leading to different pathologies. This property highlights the importance of evaluating the potential of targeting LRRK2 protein by therapeutics which might be used for treatment of patients suffering from Parkinson's disease.

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