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The role of PINK1 in the pathoethiology of Parkinson's disease

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Für meine Eltern...

*Wenn unsere Liebe einen Weg zum Himmel fände
und Erinnerungen Stufen bilden könnten,
wären wir schon längst unterwegs um Dich, Mutti,
zu uns zurückholen.*

Hinterm Horizont geht's weiter – das Ende ist nur der Anfang!

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1 Abstract / Zusammenfassung

1.1 Abstract

Parkinson's disease, the most common neurodegenerative movement disorder, is considered to be a multifactorial disease. Thus, environmental factors, living conditions and aging play a role in this etiology. However, the molecular basis of PD etiology is still largely unknown. Therefore animal models are indispensable tools to decipher the causes of this disease. The aim of this study was the systemic analysis of *PINK1*, a recessive Parkinson linked gene, in a transgenic mouse model to get a closer insight to the physiological roles of *PINK1*. Like for all genetic Parkinson mouse models *Pink1*-deficient mice do not reproduce the major hallmark of PD, i.e. the degeneration of dopaminergic neurons in the substantia nigra indicating the presence of possible compensatory mechanisms. I wanted to elucidate these mechanisms by focusing on mitochondrial function, since it is believed that mitochondrial dysfunction may be a major cellular process underlying PD etiology. The main focus was placed on the integrity and morphology of mitochondria *in vivo* and *in vitro* at different time points after acute knockdown of *Pink1* and in constitutive *Pink1*-deficient animals. An important new finding from our mitochondrial integrity study is the slightly compensation of mitochondrial fragmentation – a disease related cellular dysfunction – with time and *in vivo*. This fact indicates that disease causing molecular mechanisms may be compensated efficiently *in vivo*, which we partly could show in our study. In addition we could show an obvious impairment in mitochondrial transport, especially alternations of the mobility and direction changes in young and matured neurons, as well as changes in the speed, mobility and stop phases of mitochondria in aged neurons of acute *Pink1*-deficient primary cortex neurons. Furthermore, mRNA expression levels of mitochondrial transport factors in acute *Pink1*-deficient neurons show a decrease in *Dnhd1*, a retrograde transport cargo of the dynein complex. Taken together, *PINK1* influences mitochondrial transport in neurons and this in turn may be another factor influencing the onset of PD. Neurons are very sensitive to changes in calcium metabolism, especially dopaminergic neurons with their pacemaking activity. This sensitivity is strongly correlated with age and also triggers oxidative stress. Furthermore, calcium homeostasis is important for a variety of transport mechanisms in neurons and changes in calcium homeostasis may be a high stressor for neurons. Therefore mitochondrial transport impairment due to elevated calcium concentration was examined. Stressed *Pink1*-deficient neurons show an increase of mitochondrial trafficking deficits which is getting worse with time. This means that in addition to a genetic defect, a stressor reinforces the phenotype. Therefore, it fits very well to the hypothesis of a multifactorial disease. Indeed, despite not showing overt PD symptoms, the *Pink1*-deficient animals in our systemic analysis showed slight behavioral impairments also indicative for a system slightly

out of balance. In particular, we found a gait phenotype in our aged constitutive Pink1 knock-out mice, resembling the altered gait pattern in human Parkinson's disease patients in early phases of the disease. Furthermore, in a phenotype screen in the German Mouse Clinic we found evidences for a systemic inflammation response syndrome and impaired glucose tolerance. Taken together, our previous findings in cognitive and olfactory deficits and now gait disturbances validate our PINK1^{-/-} mice as a model for pre-symptomatic PD and in addition to a preliminary phase of type 2 diabetes. Compensatory mechanisms may take place, which are assumed to be present, and thus may hinder the onset of the full blown motor symptom phase of PD. Therefore, PINK1^{-/-} mice with early onset symptoms of PD are optimal models in the research field to find PD biomarkers and compensatory mechanisms and also to reveal the role of mitochondria within PD. Thus, they contribute to the understanding of the disease and this may lead to new therapeutic treatments.

1.2 Zusammenfassung

Morbus Parkinson zählt zu den häufigsten neurodegenerativen Bewegungsstörungen. Sie wird als sogenannte multifaktorielle Erkrankung betrachtet, bei der Gene, Umweltfaktoren, Lebensumstände und Altern eine Rolle spielen. Die Ätiologie der Parkinsonschen Erkrankung ist jedoch nach wie vor weitgehend unbekannt. Tiermodelle sind daher unverzichtbare Werkzeuge, um die Ursache der Erkrankung aufzudecken. Ziel meiner Doktorarbeit war die systematische Analyse von *PINK1*, einem Gen, das frühe rezessive Formen der Parkinson-Erkrankung auslöst. Die physiologische Rolle von PINK1 wurde mit Hilfe eines transgenen Mausmodells erforscht. Wie bei allen anderen transgenen Parkinson Mausmodellen weisen Pink1 defiziente Mäuse keine Hauptmerkmale der Parkinsonschen Erkrankung auf, wie z.B. die Degeneration von dopaminergen Neuronen in der Substantia Nigra. Dies könnte ein Hinweis darauf sein, dass möglicherweise Kompensationsmechanismen vorhanden sind. Diese Mechanismen wollte ich mit Hilfe mitochondrialer Funktionen aufklären, da allgemein angenommen wird, dass die mitochondriale Dysfunktion eine große Rolle in der Ätiologie von Parkinson spielt. Insbesondere lag hier mein Fokus auf die Integrität und Morphologie von Mitochondrien *in vivo* und *in vitro* zu verschiedenen Zeitpunkten nach Knockdown von Pink1 und in konstitutiven Pink1-defizienten Tieren. Eine wichtige neue Erkenntnis aus dieser Integritätsstudie ist die zeitliche Kompensation der mitochondrialen Fragmentierung – eine mit der Krankheit verbundene Funktionsstörung – sowie die Kompensation *in vivo*. Was verdeutlicht, dass dysfunktionale molekulare Mechanismen *in vivo* effektiv kompensiert werden können. Das konnten wir auch teilweise in unseren Studien zeigen. Darüber hinaus waren wir in der Lage eine deutliche Beeinträchtigung des mitochondrialen Transportes, insbesondere in der Mobilität und Richtungsänderungsverhalten der Mitochondrien bei jungen und ausgereiften Neuronen zu

entdecken. Bei Mitochondrien von gealterten akut Pink1 defizienten primären Neuronen des Cortex wurde eine Änderung der Geschwindigkeit, Mobilität und Stopp-Phasen deutlich. Eine weiterführende Analyse über die Genregulation von mitochondrialen Transportfaktoren in gesunden und Pink1 defizienten Neuronen zeigt eine deutliche Abnahme von Dnhd1, ein Bestandteil des Dynein-Komplexes der hauptsächlich für den retrograden Transport zuständig ist. PINK1 beeinflusst also stark den mitochondrialen Transport in Neurone und kann dadurch wiederum den Beginn der Parkinson-Erkrankung beeinflussen. Neurone sind überdies sehr empfindlich gegenüber Veränderungen der Kalziumhomöostase, insbesondere dopaminerge Neurone, die ständig aktiv sind. Diese Sensitivität ist zudem stark mit dem Alter korreliert und löst zusätzlich oxidativen Stress aus. Des Weiteren ist die Kalziumhomöostase für eine Vielzahl von Transportmechanismen in Neurone wichtig und Veränderungen im Kalziumhaushalt können ein hoher Stressfaktor für die Zelle sein. Deshalb haben wir auch mögliche Störungen im Mitochondrientransport aufgrund erhöhter Kalziumkonzentrationen untersucht. Gestresste Pink1 defiziente Mitochondrien zeigen schon früher Störungen im mitochondrialem Transport als deren ungestresste Konterparts. Diese Funktionsstörungen verstärken sich zunehmend mit der Zeit. Zusätzlich zum genetischen Defekt verstärkt ein Stressor also diesen Phänotyp. Der vorherrschenden Hypothese einer multifaktoriellen Erkrankung bei Parkinson wird hiermit Nachdruck verliehen. Obwohl sie keine offensichtlichen Parkinson Symptome zeigen, weisen Pink1-defiziente Tiere in unserer systemischen Analyse leichte Verhaltensänderungen auf, was für ein etwas aus dem Gleichgewicht geratenes System spricht. Insbesondere fanden wir eine Veränderung des Gangmusters in gealterten konstitutiven Pink1 Mäusen, ähnlich dem Gangbild der bei Parkinson Patienten in der frühen Phase der Erkrankung auftritt. Außerdem wurde eine groß angelegte Untersuchung des Pink1-Mausphänotypes in der German Mouse Klinik durchgeführt. Dabei fanden wir Hinweise auf eine systemische Entzündungsreaktion des Organismus (systemisches inflammatorisches Response-Syndrom), sowie eine veränderte Glucose-Toleranz. Zusammenfassend kann gesagt werden, dass mit unseren bisherigen Erkenntnissen der kognitiven und olfaktorischen Defizite, die in einer früheren Arbeit erzielt wurden, sowie mit den Gangstörungen, unsere PINK1^{-/-} Mäuse hervorragend als Modell für die prä-symptomatische Phase der Parkinsonschen Erkrankung in Frage kommen, wie auch als Vorläufer des Typ-2-Diabetes. Kompensatorische Mechanismen, von denen allgemein vermutet wird, dass sie vorhanden sind, scheinen auch hier statt zu finden und den vollen Ausbruch der motorischen Phase von Parkinson zu verhindern. Unsere PINK1^{-/-} Mäuse, die nur frühe Symptome der Erkrankung zeigen, sind optimale Forschungsmodelle, um Biomarker für Parkinson zu finden, sowie kompensatorische Mechanismen und die Rolle von Mitochondrien in Morbus Parkinson zu erforschen. Dies trägt wiederum zum Verständnis der Krankheit bei, was zu neuen therapeutischen Ansätzen führen kann.

2 Introduction

2.1 Parkinson's disease

Parkinson's disease is the most common movement disorder and the second most common neurodegenerative disorder after Alzheimer's disease. The average age of onset is 55 with markedly increased prevalence and incidence with advancing age. PD affects 1% of the population over 55 years of age [Less et al., 2009; Blesa et al., 2012] and among the population over 80 years old up to 4 % are already affected by the disease [De Lau and Breteler, 2006; Yamaguchi and Shen, 2007] with rising tendency due to demographic changes. It was first described as "paralysis agitans" (shaking palsy) nearly 200 years ago, 1817, from James Parkinson in his publication of "An Essay on the Shaking Palsy", a treatise about 6 individuals with distinct syndromes. 60 years later, Jean Martin Charcot renamed the disease "Parkinson's disease" and found some remarkable discoveries in this field between 1868 and 1881. Among other things, he distinguished between rigidity, weakness and bradykinesia [Herzberg, 1987], which are some of the yet known cardinal symptoms of PD. Nowadays clinical symptoms of PD can be distinguished in an early occurring premotor phase with the so-called "non-motor symptoms" like for example dysfunction of the autonomic nervous system, olfactory impairments, depression, cognitive impairment and sleep disturbances. With ongoing progression of the disease the symptomatic phase rises with "motor symptoms" like pronounced resting tremor (tremor occurring in a relaxed and supported limb or other body part), bradykinesia (inability to perform purposeful movements), akinesia (absence or loss of control of voluntary muscle movements), rigidity (inflexibility or stiffness of muscles), postural instability (loss of balance), which are referred to as the above mentioned cardinal symptoms of the Parkinson's disease [Fahn, 2003; Shulman, 2010]. Other also frequently occurring motor symptoms include gait disturbances with rapid shuffling steps and posture problems with a forward-flexed posture when walking [Jankovic; 2008]. Furthermore, patients in advanced stages show mostly psychiatric impairments, such as psychosis and pronounced depression, as well as speech and swallowing problems [Russell et al., 2010]. Interestingly, men seem to have a 1.5 times higher prevalence to the disease than women [Fahn and Sulzer 2004; Wooten et al. 2004; De Lau and Breteler, 2006; Haaxma et al. 2007; Rodríguez-Navarro et al., 2008; Misiak et al., 2010;]. But other studies could not find a difference in PD prevalence between men and woman [reviewed by De Lau and Breteler, 2006]. In women, the age of onset correlates with the duration of the fertile life [Rodríguez-Navarro et al., 2008]. There are also gender differences in the clinical manifestations, the course of disease and the response to drug treatment [Haaxma et al. 2007; Tao et al., 2011].

The major symptoms of PD are associated with the neuropathological finding in PD, characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta, a brain structure located in the midbrain (mesencephalon). The substantia nigra plays an important role in the control of the execution of movement via the neuronal circuitries of the basal ganglia (Fig. 1). However, the cardinal symptoms only appear when about 50 - 80 % of these neurons are already lost forever [Pham et al., 2010; Kwon et al., 2011]. Interestingly, and highly related to the non-motor symptoms of PD, also other brain areas are affected although not quite as strong as the substantia nigra pars compacta (SNpc). Among them are the locus coeruleus with noradrenergic neurons, the raphe nuclei with serotonergic neurons, the nucleus basalis and dorsal nucleus of vagus nerve (posterior motor nucleus of vagus) with cholinergic neurons, as well as the olfactory bulb, cerebral cortex and the autonomic nervous system.

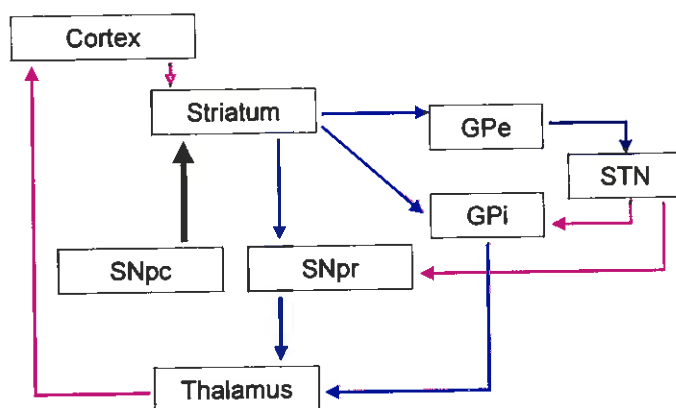


Fig. 1: Schematic drawing of the basal ganglia circuits which are mainly affected in Parkinson's disease.

Dopaminergic connections are represented with a black arrow, whereas GABAergic pathways are shown in blue and glutaminergic in magenta. **Abbreviations:** GPe: external part of the globus pallidus; GPi: internal part of the globus pallidus; SNpc: substantia nigra pars compacta; SNpr: substantia nigra pars reticulata; STN: subthalamic nucleus.

In most cases, the loss of dopaminergic neurons is associated with the appearance of specific cell inclusions, the Lewy bodies [Forno, 1996; Samii et al., 2004]. Lewy bodies are abnormal protein aggregates which can be found in nerve cells of most Parkinson patients, as well as Lewy Body Dementia and some other disorders. They consist of different proteins like α -synuclein, ubiquitin, neurofilament protein, α -B crystalline and also tau proteins can be included. Several studies suggested that Lewy bodies represent an aggresome response in the cells [Tanaka et al., 2004]. Interestingly, in autosomal recessive early-onset Parkinson's disease like *PINK1*, parkin and DJ-1 mediated PD, they normally do not occur [Hayashi et al., 2000; Rizzu et al., 2004], and a causal function in PD etiology is still debated.

2.2 Treatment of the disease

At the moment there is no cure existing for Parkinson's disease. But good health care, medications, advancing knowledge in brain surgery and interacting medical fields and management can improve the symptoms of the disease, delay the progressive course and improve the living conditions of the affected patients and their family members.

Drug treatment in PD starts when the patient is functionally impaired. Depending on the patient's age and severity of symptoms the first drugs which are used are either levodopa or a dopamine agonist [Fuente-Fernández et al., 2010]. Levodopa has been the most widely used treatment for over 30 years [Collaborating Centre for Chronic Conditions, 2006]. It converts to dopamine in the dopaminergic neurons via dopa decarboxylase. But only 5-10 % of L-DOPA crosses the blood-brain barrier and the remaining agent is converted to dopamine somewhere else, which causes some side effects [reviewed in Fuente-Fernández et al., 2010]. In addition, after 4 – 6 years of treatment, about 40 % of patients develop motor fluctuations and dyskinesia [Fuente-Fernández et al., 2010], which has to be treated with additional drugs. Several dopamine agonists existing like pramipexole (Mirapex), ropinirole (Requip) and bromocriptine (Parlodel). All of them binds to dopaminergic post-synaptic receptors in the brain and have similar effects. Dopamine agonist usually is used in mild, early forms of Parkinson's disease or as an additional drug. Its side effects are usually mild. Another well tolerated but unfortunately less effective agent is monoamine oxidase B (MAO-B) inhibitors like selegiline and rasagiline. Dopamine is broken down by MAO-B. Inhibiting this metabolite increases the level of dopamine in the basal ganglia. MAO-B is used as monotherapy to improve motor symptoms and delay the need for levodopa in early disease [reviewed in Fuente-Fernández et al., 2010].

Furthermore neuroprotective agents are also used for treatment of Parkinson's disease. Some of them are promising, others were found not to be effective. Among the promising agents are creatine, inosine and calcium channel blockers. While creatine shows a possible positive affect on complex I of the respiratory chain, Inosine acts via elevating uric acid [Fuente-Fernández et al., 2012]. Coenzyme Q10, Pramipexole and Rasagiline belong to the yet non-effective adjuvant.

The last option in the treatment of PD is the deep brain stimulation (DBS) with previous surgical treatment. For this purpose a so-called brain pacemaker, a medical device, is placed into the brain and sends electrical impulses to specific parts of the brain. DBS is typically applied in severe PD cases or at a point of the disease where no further medical therapy is useful.

Annual healthcare costs for Parkinson's patients amount to several billions worldwide. Solely in the U.S. the annual costs are estimated at up to 23 billion dollars. Not only to reduce the costs but most of all to improve the quality of life of PD patients and their family members is a

goal which has to be achieved in the coming years. Further intensive studies of this disease are urgently needed to improve medical care with the great goal to understand the reasons for getting Parkinson's disease and possibly even cure this scourge of mankind. Although the disease was first described by James Parkinson, already in the ancient Greece, about 2000 years ago, anatomists like Ersistratos have reported about movement disorders characterized by rigidity and akinesia. Nevertheless, the cause of PD remains unknown. Meanwhile, multifactorial causes are assumed. Furthermore, in the Parkinson's research exist a widely accepted so-called "Multiple-Hit-Theory". A summation of several (multiple) factors like environmental influences, aging and genetic mutations is needed to trigger (hit) Parkinson. One change alone will normally not be enough to induce PD, except for rare cases like repeated MPTP recordings. This would allow the development of new therapeutic and protective strategies for the treatment of PD [Gasser, 2007].

2.3 Parkinson's disease and environment

Numerous epidemiological studies have been done to identify non-genetic risk factors of PD but often with limited resources and methods. The most frequently replicated relationships are an increasing risk to develop PD in people exposed to pesticides and oddly enough a reduced risk in smokers [De Lau and Breteler, 2006]. The risk of smokers is up to 1/3 lower than that of non-smokers [De Lau and Breteler, 2006]. Nicotine may stimulate dopamine release and act as an antioxidant or it might alter the activity of monoamine oxidase B [Priyadarshi et al., 2000; Quik M., 2004; reviewed by De Lau and Breteler, 2006; Dutta et al., 2008]. But a detailed justification for this phenomenon is not yet known.

Among the pesticides some are in reasonable suspicion or are even proved to trigger Parkinson's disease like rotenone and N,N'-dimethyl-4,4'-bipyridinium dichloride (paraquat). Paraquat is one of the most widely used herbicides against broadleaf plants and grasses in the world. It can be absorbed very quickly through the plant surface where it acts non-selective and kills green plants due to the radical formation in the chloroplasts. His large-scale application is particularly present in coffee, tea, oil palm and banana plantations, as well as in orchards and vineyards. Already in 2006, a study on paraquat-induced toxicity in rats exhibited a neurological degenerative mechanism like Parkinson [Ossowska et al., 2006]. In 2011, a study of the National Institutes of Health in US showed a link between paraquat and Parkinson's disease in farm workers [Tanner et al., 2011]. Whereas rotenone occurs naturally in the roots and stems of several plants and is used in many insecticides and pesticides. It is lipophilic and can cross the blood-brain barrier. In primary neuronal and microglia cultures of rats a low dosage of rotenone induced oxidative damage and acted lethal of dopaminergic neurons [Gao et al., 2003]. In addition, rotenone injection into rats

causes Parkinson's disease-like symptoms within these [Caboni et al., 2004]. Rotenone has normally short lifetime of six days in the environment but in water it may last up to six months. Also herbicides like Agent Orange, which came to the cruel use to defoliate the jungle during the Vietnam War, appear to play a role in Parkinson's disease (added on the list of conditions associated with Agent Orange exposure of the U.S. Department of Veterans Affairs, 2010). Furthermore, the fungicide Manganethylen-1,2-bis-dithiocarbamat (Maneb) is also suspected to trigger PD via disturbing mitochondrial function [Ciccetti et al., 2009]. Maneb appears as residuum in fruits and vegetables. Solely in the year 2007 100-250 t of Maneb were used only in Germany. Thus, living in rural environment, has been proposed to be a risk factor in PD.

Interestingly, most of these toxins inhibit complex I of the electron transport chain of mitochondria and are also influencing the mitochondrial transport. Molecular mechanisms which are also implicated in the molecular function of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridin (MPTP), MPTP is known as a trigger of PD since its sad origin as a meperidine analogue designer drug in Northern California. Repeated use of this drug led to akinetic redig syndrome with or without resting tremor within one to two weeks [Langston and Ballard, 1983; reviewed in Blandini and Armentero, 2012]. MPTP is like rotenone lipophilic and can cross the blood-brain barrier. It is now known to cause dopaminergic cell loss in the nigrostriatum and induces Parkinson in humans, generally in primates and in rodents [reviewed in Blandini and Armentero, 2012].

All of these toxins have been used to generate reliable animal models for PD which exhibit degeneration of the dopaminergic neurons in the SNpc. However, except for MPTP, which is selectively taken up by the dopaminergic neurons, via the dopamine transporter (DAT), the selective vulnerability of these neurons for these toxins remains a mystery. Currently the hypothesis, that these toxins increase - via inflicting mitochondrial dysfunction - oxidative stress in neurons, and that dopaminergic neurons are specifically susceptible to this increase, is the most accepted one (Fig. 2)

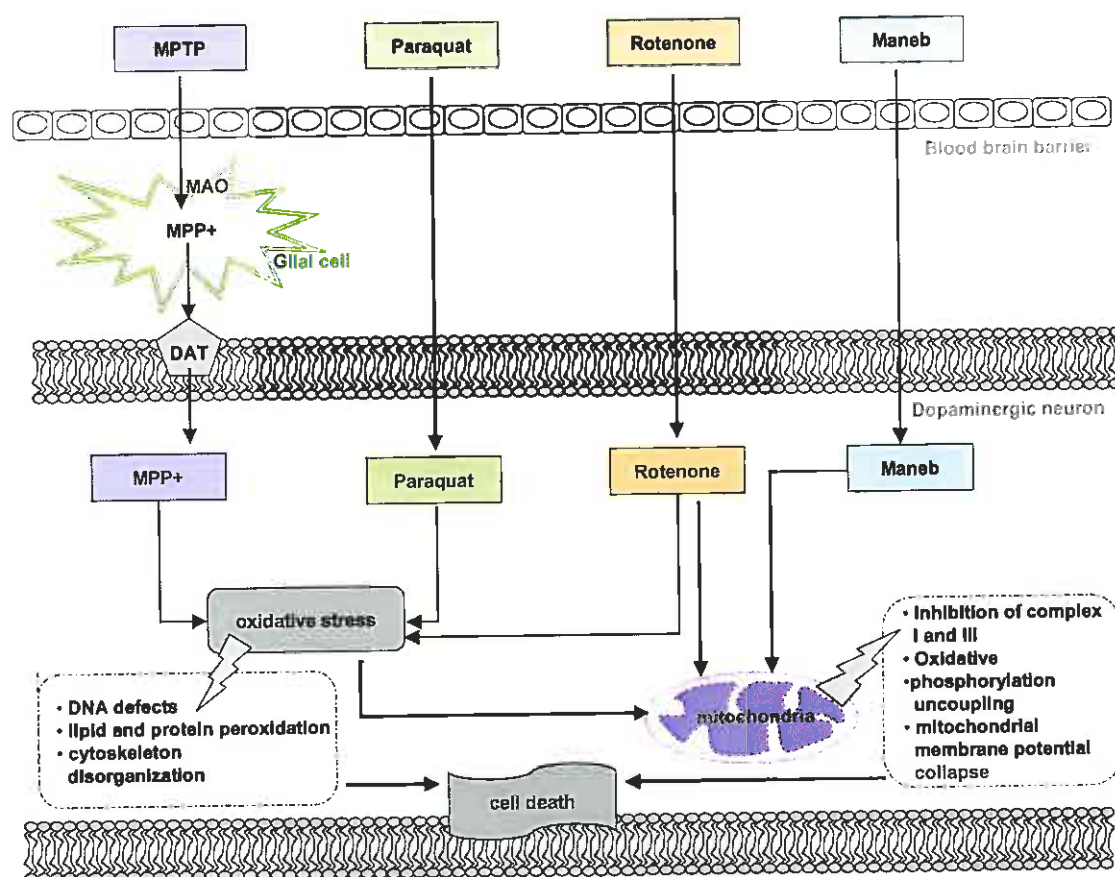


Fig. 2: Molecular pathway of some environmental factors playing a role in PD, like MPTP, rotenone and paraquat. Adapted from Ciccetti et al., 2009. Most of the environmental factors lead to increased oxidative stress and damage of mitochondria.

Additionally, chronic heavy metal exposure is reported to increase the risk of PD: Especially chronic Manganese (Mn^{2+}) exposure is cytotoxic and alters dopaminergic and GABAergic neurons within the basal ganglia [Stanwood et al., 2009]. Intraperitoneal $MnCl_2$ injections in mice for one month reduced 20% TH-positive neurons in the substantia nigra pars compacta (SNpc). Acute manganese exposure induces cytoskeletal dysfunction prior to degeneration [Stanwood et al., 2009]. In humans, a typically chronic manganese exposure over 6 months to 16 years is leading to the onset of manganism [Schapira, 2007]. The motor symptoms of this disorder are, however, strikingly similar to those observed in Parkinson's disease [Stanwood et al., 2009]. Manganese is normally occurring in nature but is applied in higher concentrations in the industries (welding, mining and steel production).

Another risk factor is apparently a low serum selenium concentration, which was found in PD patients, while urinary excretion was normal [Jiménez-Jiménez et al., 2011]. These concentrations were not influenced by drugs against PD and did not correlate with age [Jiménez-Jiménez et al., 2011]. But interestingly, in elderly people additionally a disturbance of resorption occurs (difficulty in absorbing nutrients), organic or caused by drugs. And in fact, selenium is a trace element found in small amounts in food and function normally as an

essential component of the antioxidant enzyme glutathione peroxidase, whose activity is reduced in the substantia nigra of Parkinson's disease patients [Jiménez-Jiménez et al., 2011].

A widespread drink is often consumed in the Caribbean, sour sop drink. Among other things it is composed of Annonacin, which is a complex I inhibitor. Studies with daily Annonacin consumption in rats caused brain lesions consisted with Parkinson's disease. [Lannuzel et al., 2003; Champy et al., 2004].

In addition to environmental factors, also inflammation, changes in lifestyle like stress and diet, as well as aging are under suspicion or have been proven to trigger sporadic Parkinson's disease. Most of the PD cases are sporadic with environmental or yet unknown reasons [De Lau and Breteler, 2006]. Just a rare amount, up to 10%, are familial, inherited cases [Dauer and Przedborski, 2003; Blesa et al., 2012].

2.4 Parkinson's disease and genes

During the last decade, analyses and gene association studies from different geographical areas confirm up to now 18 different genetic loci, the PARK family, which are associated to inheritent (familial) PD in an autosomal dominant (PARK 1, 3-5, 8, 11) or recessive way (PARK 2, 6, 7, 9, 14, 15) [Thomas and Beal, 2007; Belin and Westerlund, 2008; Paisán-Ruiz et al., 2010; Tan et al., 2010; Xiromerisiou et al., 2010; Corti et al., 2011; Martins et al., 2011 and Lesage and Brice, 2012]. However, most of the PD cases are idiopathic and as above mentioned approximately 10 % of patients are known to have monogenetic, familial forms [Gasser, 2007; Lesage and Brice, 2009; Seibler et al., 2011]. Among them the mitochondrial PTEN (phosphatase and tensin homologue-) induced kinase 1 (*PINK1*), which is described even closer in the next passage (chapter 2.6). The incidence of autosomal recessive PD forms like parkin, *PINK1* and DJ-1 is very rare, less than 5% of all PD cases [Grünblatt, 2012]. A brief overview of the Parkinson associated genes with their full names and inheritance form is given in Tab. 1. Interestingly it is now known, that many Parkinson associated genes functionally interact with each other. (Fig. 3). Thus, modulation of similar or even the same molecular pathways by dysfunction PD-associated genes is now regarded as being highly likely. This is supported by the fact, that in many post-mortem studies even a link between familial to sporadic PD was found [Grünblatt, 2012], e.g. alternations in the transcription of SNCA, as well as UCHL-1 and DJ-1 mRNA were found to be modified in sporadic PD [Grünblatt, 2012]. Therefore, it is expected that the same pathogenic mechanisms which are responsible for mendelian PD cases could play a relevant role also in

the more frequent sporadic PD cases [Valente et al., 2012]. Although a common pathway was not yet discovered, the search for it is running at full speed.

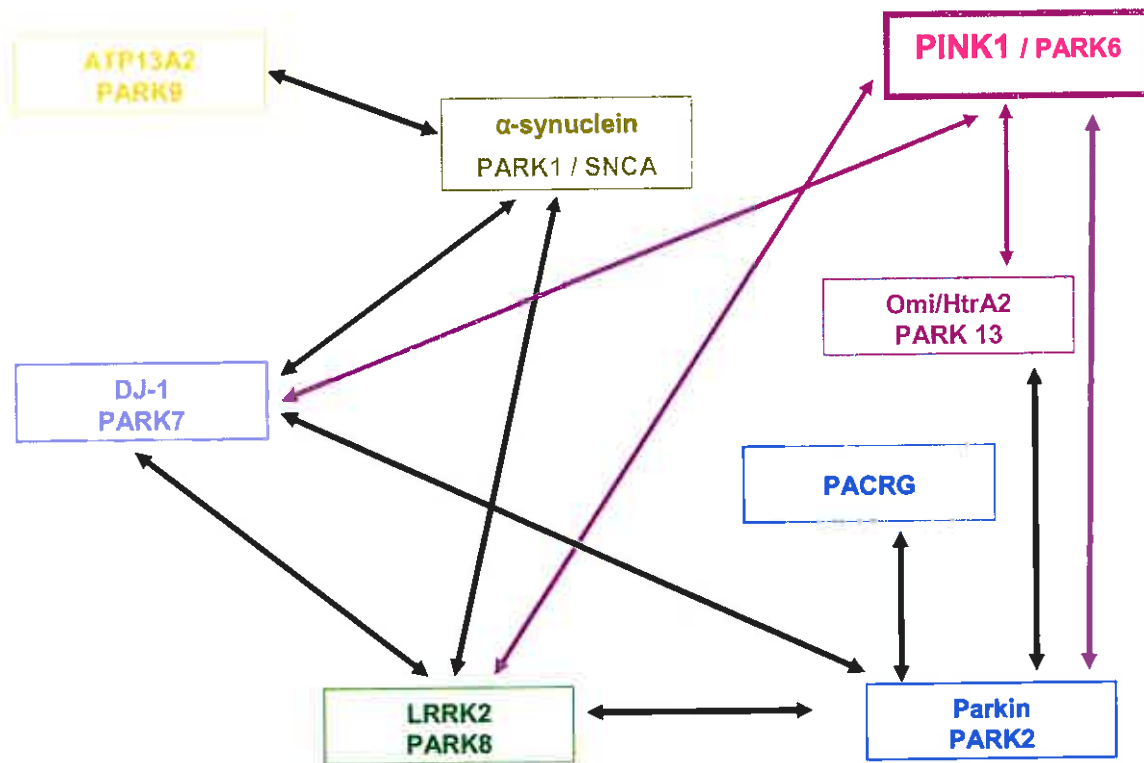


Fig. 3: Interaction partner of some Parkinson associated genes. PINK1 was shown to interact with parkin [Deng et al., 2008; Poole et al., 2008; Yang et al., 2008; Dagda, 2009; Lutz et al., 2009; Weihofen et al., 2009; Whitworth and Pallanck, 2009], LRRK2 [Venderova, 2009], Omi/HtrA2 [Dagda, 2009], DJ-1 [Thomas, 2011] and the Parkin co-regulator PACAG [West et al. 2003; Dawe, 2005; Wilson, 2010]. A publication of Giller et al., 2009 revealed the connection of ATP13A2 with α -synuclein. In addition α -synuclein interacts with LRRK2 [Lin, 2009; Tong and Shen, 2009; Carballo-Carbajal et al., 2010] and DJ-1 [Meulener, 2005; Zhou and Freed, 2005; Batelli, 2008; Liu, 2008]. LRRK2 interacts in addition with parkin [Deas and Dunn, 2010], which also interacts with PACRG [West et al. 2003]. Magenta arrows exhibit PINK1 interaction, whereas black arrows exhibit remaining gene interactions.

locus	gene and function	synonymous	inheritance
PARK1 = PARK 4	synuclein, alpha (non A4 component of amyloid precursor) ; function as lipid membrane association protein	SNCA	AD
PARK2	parkin; ubiquitin E3 ligase and play a role in mitochondrial quality control	PRKN	AR
PARK3	sepiapterin reductase (7,8-dihydrobiopterin: NADP+ oxidoreductase)? – but still not entirely clear	SPR?	AD
PARK4 = PARK 1	synuclein, alpha (non A4 component of amyloid precursor)	SNCA	AD
PARK5	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase); ubiquitin C-terminal hydrolase	UCHL1	AD and idiopathic
PARK6	PTEN induced putative kinase 1; mitochondrial kinase and plays major role in mitochondrial quality control and trafficking	PINK1	AR
PARK7	DJ-1; chaperone, antioxidant	DJ-1	AR
PARK8	leucine-rich repeat kinase 2; cytosolic kinase	LRRK2	AD and idiopathic
PARK9	ATPase type 13A2; P-type ATPase	ATP13A2	AR
PARK10	unknown	unknown	idiopathic
PARK11	GRB10 interacting GYF protein 2; unknown	GIGYF2?	AD and idiopathic
PARK12	unknown	unknown	?
PARK13	HtrA serine peptidase 2; mitochondrial serine protease	Omi/HTRA2	idiopathic
PARK14	PLA2G6; phospholipase	PLA2G6	AR
PARK15	F-box protein 7; ubiquitin E3 ligase	FBXO7	AR
PARK16	unknown	unknown	AR
PARK17	vacuolar protein sorting 35 homolog (S.cerevisiae)	VPS35	AD
PARK18	eukaryotic translation initiation factor 4 gamma,1	EIF4G1	AD

Tab. 1: Associated genes of Parkinson's disease. This list gives an overview of all known PARK loci with link to PD, their synonymous name (generally used name in science), mode of inheritance and function. The data are adapted from Thomas and Beal, 2007; Belin and Westerlund, 2008; Paisán-Ruiz et al., 2010; Tan et al., 2010; Xiromerisiou et al., 2010; Corti et al., 2011; Martins et al., 2011 and Lesage and Brice, 2012. AD means autosomal dominant, whereas AR means the autosomal recessive form.

2.5 Mitochondrial integrity in Parkinson's disease

As stated above one common theme emerging for the pathoetiology of PD is mitochondrial dysfunction. Most of the Parkinson-associated genes encode proteins which are at least partly localized and correlated to mitochondria and with certain aspects of mitochondrial function. Among them are α -synuclein (localized to mitochondria under certain conditions and can decrease complex I function of the respiratory chain), LRRK2 (serine-threonine kinase which interacts with parkin), DJ-1 (relocates to mitochondria when oxidative stress increases), HTRA2 (serine protease), parkin (E3 ligase in ubiquitin-proteasomal system, partially localized to mitochondria, involved in mitochondrial biogenesis and quality – together with PINK1) and PINK1 (serine-threonine kinase, protects against oxidative stress and involved in mitochondrial biogenesis as well as trafficking, close connection to parkin) [Henchcliffe and Beal, 2008]. Mitochondrial activity can also be affected by environmental factors, e.g. like rotenone, MPTP, Annonacin, acute CO poisoning, high manganese exposure and of course with aging. In recent years, more and more evidence occurs, that mitochondrial dysfunction may play a role in Parkinson's disease.

2.5.1 Mitochondrial dynamics

Mitochondria are highly dynamic organelles whose shape, size, number, internal structure and function can be controlled by the opposing processes, the so-called mitofission and mitofusion [Chan, 2006]. The quality of mitochondria (morphology and function) depends on the balance between fission and fusion (Fig. 4). Mitofusion is necessary for normal respiratory function in cells and is needed for information exchange between two mitochondria. It is also suggested that mitofusion is used to repair small defects in DNA [Chen and Chan, 2004; Chan, 2006].

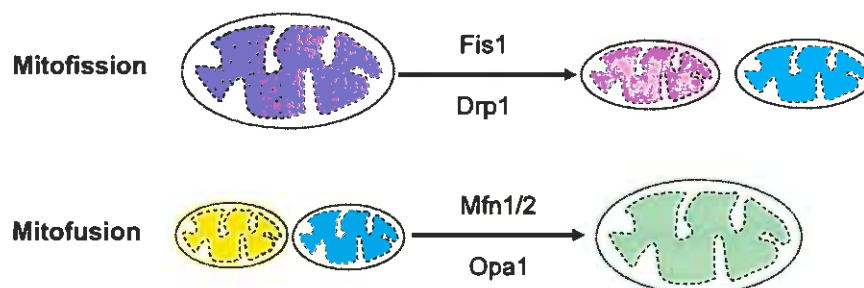


Fig. 4: Mitochondrial fission and fusion process. During the mitofission process a "mother" mitochondria is separated in two similar "daughter" mitochondria, whereas in mitofusion two mitochondria are fused together to a bigger, enlarged one. **Abbreviations:** Drp1: Dynamin-related protein 1; Fis1: Anti-Mitochondrial fission 1 protein; Mfn1 / 2: Mitofusion 1 and 2; Opa1: optic atrophy.

Furthermore, mitochondria are critical for neuronal function and are involved in respiratory chain efficiency, primary energy production, regulating cytoplasmic calcium homeostasis and in apoptosis processes, as well as in autophagy [Chan, 2006; De Vos et al., 2008; Henchcliffe and Beal, 2008]. The energy production occurs via the electron transport chain (ETC), which is localized in the inner membrane of mitochondria. The ETC is needed for oxidative phosphorylation of ADP to ATP and consists of five multiprotein complexes (complex I – V). As already mentioned, complex I was found to be disturbed in PINK1-deficient cells [Gu et al., 1998, Shults, 2004; Morais et al., 2009]. As a consequence, depletion of ATP-dependent cellular processes occurs and free radicals are generated that cause oxidative stress [Shults, 2004].

Recently, some PD-associated genes have been found to influence the balance of mitochondrial fission and fusion: *PINK1* and *Parkin*. *PINK1* has a N-terminal mitochondrial targeting sequence [Nakajima et al., 2003] and *Parkin* can associate with mitochondria [Plun-Favreau et al., 2007]. With *Pink1* and *parkin* deficiency the following mitochondrial functions have been shown to be affected: respiratory chain efficiency, energy production, membrane potential, morphological changes (fission/fusion) [reviewed by Büeler, 2010; Chu, 2010; Xi et al., Zhu and Chu 2010; Exner et al., 2012; Pils and Winklhofer, 2012]. Most of these studies were done in *Drosophila melanogaster* showing that *PINK1/Parkin* pathway promotes mitofission [Yang et al. 2008; Deng et al. 2008; Poole et al., 2008; Park et al. 2009]. *PINK1* deficiency causes mitochondrial pathology and can be rescued by *parkin*. But in addition, there could exist differences between arthropods and mammals in the regulation of mitochondrial dynamics and dysfunctions [Lutz et al. 2009]. In the year 2007, Exner and group for example could show that in human immortalized neuroblastoma cells (SH-SY5Y) a *PINK1*-deficiency also impairs mitochondrial morphology and the *PINK1* knockdown phenotype can be rescued by *parkin*. But interestingly, *parkin*-deficiency induces fragmentation of mitochondrial network. Furthermore, they could show that *parkin* or *PINK1*-deficiency is rescued by an increase in mitofusion (via *Mfn2* or *Opa1*) and a decrease in mitofission (via *Drp1*) [Exner et al., 2007]. *In vivo* studies of mitochondrial morphology in mammals revealed contradictory results [Gautier et al., 2008; Gispert et al., 2009; Glasl et al., 2012]. Further *in vivo* analyzes are needed to confirm the *in vitro* effect of *PINK1* deficiency on mammalian mitochondrial function [Glasl et al., 2012].

2.5.2 Mitochondrial trafficking

A recent study from Weihofen et al. (2009) could show that *PINK1* forms a multiprotein complex with *Miro* and *Milton* linking *PINK1* function to mitochondrial trafficking. If two mitochondria cannot travel across the cell to dock with one another, they cannot fuse. More fragmented mitochondria should appear. In contrast mitofission is independent of

mitochondrial movement [Baloh, 2007]. Furthermore parkin was shown to bind to and stabilize microtubules [Yang et al., 2005].

Mitochondria are transported within neurons along microtubules via kinesin (anterograde direction, meaning towards the synapses) or via dynein (retrograde direction, meaning towards the soma) and for a short distance along actin filaments via myosin motors [De Vos et al., 2008], see Fig. 5. But actin filament transport seems to emerge primarily in the area of the synapses [De Vos, 2010]. Mitochondrial transport, which is commonly referred as mitochondrial trafficking, undergoes saltatory and bidirectional movement with individual mitochondria frequently stopping and changing direction [Baloh, 2008; De Vos et al., 2008; Cai and Sheng, 2009]. At a given time the majority of mitochondria (75 - 90%) are immobile [Baloh, 2008; Cai and Sheng, 2009; Mispeld, 2006].

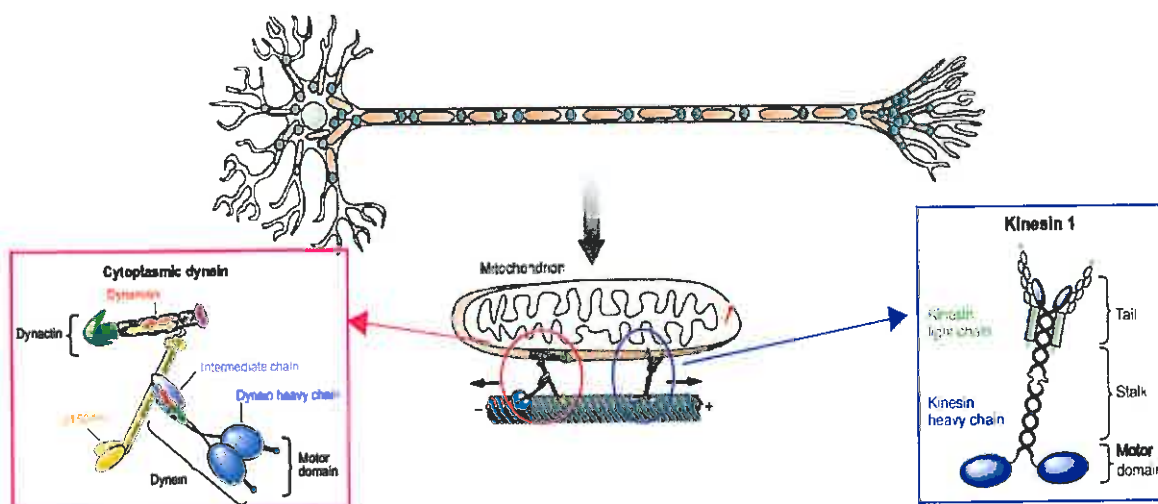


Fig. 5: Mitochondrial trafficking in neurons. Mitochondria can travel from the soma to synapses mostly via kinesin binding. This kind of transport is called anterograde transport. On the other hand, transport from the synapses towards the soma is guaranteed by binding to the dynein complex, a transport which is so-called retrograde. The Figure is adapted from De Vos et al., 2008.

2.6 Phosphatase and tensin homolog (PTEN)-induced novel kinase 1 (PINK1)

In my thesis I have concentrated on the PD associated gene *Phosphatase and tensin homolog (PTEN)-induced novel kinase 1 (PINK1)*, *PARK6*. *PINK1* linked parkinsonism is characterized by an early onset of recessively inherited disease and mutations are thought to lead to a loss of function [Valente et al., 2004; Beilina et al., 2005; Yanwei, 2010]. *PINK1* mutations are the second most common autosomal recessive mutations after parkin and cause up to 7 % of all early onset forms of Parkinson's disease [Gasser, 2009]. 2001 was the

first time *PINK1*, originally known as EST, was mentioned and described as transcriptionally transactivated in an expression profile of cancer cells after the introduction of exogenous PTEN, a tumor suppressor [Matsushima-Nishiu et al, 2001; Unoki and Nakamura, 2001]. Therefore, *PINK1* was shown to have anti-apoptotic properties in cancer cells. Shortly thereafter Unoki and Nakamura cloned a full-length *PINK1* cDNA and found out, that *PINK1* encodes a 581 amino acid protein with a molecular mass of 62.8 kD [Unoki and Nakamura, 2001]. Additional three years later Valente et al. identified *PINK1* as responsible gene for *PARK6*-linked parkinsonism [Valente et al., 2004] and contain 8 exons spanning 1.8 kb in humans. Its location is on chromosome 1 at position 36 (1p36). The *PINK1* protein exhibit high homology to other species, e.g. it shares a 43 % sequence with *Drosophila* and a 31 % sequence with *C. elegans*. Today several different mutations in the *PINK1* gene, homozygous and heterozygous, have been found to cause PD. Homozygous *PINK1* mutations are associated with significant dopaminergic neuron loss in the substantia nigra and with motor behaviour impairment [Yanwei et al., 2010], whereas heterozygous mutations are a high risk factor for PD, supposed to produce subclinical dopaminergic dysfunctions [Valente et al., 2004; Abou-Sleiman et al., 2006]. Two different *PINK1* isoforms were found in human brain extracts and via *PINK1* overexpression *in vitro* [Beilina et al., 2005]. Both isoforms (with molecular mass of 66 kD and 55 kD) localize to the inner and outer mitochondrial membrane [Silvestri et al., 2005; Zhou et al., 2008]. The ratio of these isoforms seems to be important in a yet unknown manner [Weihofen et al., 2008]. Furthermore, *PINK1* is ubiquitously expressed with high demand in mitochondrial rich tissue, like heart, skeletal muscle and testis [Scheele et al., 2007] and low-level expression in cytosolic and microsomal-rich fractions [Weihofen et al., 2008].

Structurally, *PINK1* contains a conserved serine/threonine kinase domain with an N-terminal mitochondrial targeting motif and C-terminal kinase domain facing the cytoplasm [Nakajima et al., 2003; Dawson et al., 2010; Shulman et al., 2011]. *PINK1* was the first gene directly implicating mitochondrial function in the pathogenesis of PD. Therefore, *PINK1* seem to have a direct impact on mitochondrial function. Indeed, loss of *PINK1* leading to a malfunction in mitochondria and hence to oxidative stress [Plun-Favreau et al., 2007; Pridgeon et al., 2007; Yanwei, 2010; Xiromerisiou et al., 2010]. Changes in the efficiency of the respiratory chain, mainly of complex I, production of reactive oxygen species (ROS production), changes in the membrane potential and in the mitochondrial morphology are mentioned as a malfunction of mitochondria due to *PINK1*-deficiency [Valente et al., 2004; Silvestri et al., 2005; Abou-Sleiman et al., 2006; Deng et al., 2008; Yang et al., 2008; Lutz et al., 2009; Morais et al., 2009; Poole et al., 2010; reviewed by Winklhofer and Haass, 2010; Liu et al., 2011; Yu et al., 2011]. Most interestingly, however, it has been reported that *PINK1* act in the same pathway than parkin, another PD-associated gene. Mitochondrial morphology and dynamic changes

in PINK1 null mutant *Drosophila*, was shown to be rescued and compensated by an overexpression of parkin [Poole et al., 2008; Gispert et al., 2009] but not vice versa. Implying that PINK1 and parkin function in a common genetic pathway with parkin acting downstream of PINK1 [Poole et al., 2008; Yang et al., 2008; Deng et al., 2008; Narendra et al., 2010; Winklhofer and Haass, 2010]. Furthermore, cellular localization studies of recombinant and endogenous PINK1 protein in mammalian cells showed that PINK1 exists also in the cytosol [Gandhi et al., 2006; Plun-Favreau et al., 2007; Zhou et al., 2008] and may also regulate mitochondrial calcium dynamics [Gandhi et al., 2009]. With depolarization of the mitochondria membrane, due to dysfunctional mitochondria, PINK1 accumulates from the cytosol to the mitochondrial membrane, from where it recruits parkin to depolarize mitochondria and triggers autophagosome formation, in particular mitophagy. Thereupon damaged mitochondria will be phagocytosed [Deng et al., 2008; Poole et al., 2008; Yang et al., 2008; Geisler et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010; Winklhofer and Haass, 2010; Narendra and Youle, 2011; Kanamaru et al., 2012]. Mitophagy is accompanied by an increase in mitochondrial fission and ROS production [Deng et al., 2008; Poole et al., 2008; Yang et al., 2008; Vives-Bauza et al., 2010; Narendra et al., 2010]. Taken together, PINK1 may phosphorylates mitochondrial proteins in response to cellular stress and thus, protecting mitochondrial dysfunctions [Valente et al., 2004; Sleiman et al., 2006; Petit et al., 2005]. In addition dysfunctional mitochondria are sorted out by mitophagy. With PINK1-deficiency, these processes are out of balance.

Then, finally three years ago, two studies from Marongiu et al. and S  mann et al. also suggested a link between PINK1 and axonal outgrowth with an important role in mitochondrial function. Mutant PINK1 displayed a defect in axonal outgrowth and showed abnormal mitochondrial structure in addition to reduced ATP levels [Marongiu et al., 2009; Samann et al., 2009; Yanwei et al., 2010]. The cause of these defects in PINK1 deficient axons could be impaired mitochondrial quality control (mitofission and –fusion effects) and / or defects in mitochondrial transport to the growth cones [Yanwei et al., 2010]. It is now known that PINK1 and parkin play an important role in mitochondrial dynamics [Deng et al., 2008; Poole et al., 2008; Yang et al., 2008; Gispert et al., 2009]. Furthermore, a study from Weihofen et al., 2009 could show that PINK1 forms a multiprotein complex with Miro and Milton linking PINK1 function for the first time to mitochondrial trafficking.

Most of the PINK1 studies for mitochondrial dynamics were done *in vitro* or in a *Drosophila* model and showed partially controversial results. Not much is known about the *in vivo* situation or the influence of possible impaired mitochondrial trafficking in PD due to PINK1-deficiency. Thus, evaluating the function of PINK1 *in vivo* becomes more and more relevant. To approach the *in vivo* function of PINK1 in PD several animal models were generated. Among them are the most studied animal models like *Drosophila melanogaster*, zebra- and

medaka fish and mouse models. Our transgenic PINK1 knockout mouse model shows promising approaches for an early form of PD.

2.6.1 Animal models of PINK1

2.6.1.1 *Drosophila melanogaster*

PINK1-deficient *Drosophila melanogaster* shows very striking phenotypes characterized by reduced life span, male sterility (defective spermatogenesis), locomotor defects due to apoptotic flight muscle degeneration, muscle and neuronal degeneration, hypersensitivity to oxidative stress, reduced ATP production, swollen mitochondria and fragmented mitochondrial cristae [Clark et al., 2006; Park et al., 2006; Wang et al., 2006; Yang et al., 2006; Gispert et al., 2009]. In addition, the lack of PINK1 in the eye of fruit flies leads to the degeneration of the ommatidia in the compound eye [Wang et al., 2006]. In accordance with the fact that Pink1 and Parkin act in the same molecular pathway *Drosophila* Pink1 null mutants share phenotypic similarities with parkin null mutants. Although *Drosophila* PINK1 and parkin loss-of-function models have dramatic phenotypes, which can be suppressed by an increase in mitochondrial fission or a decrease in fusion [Poole et al., 2008; Yang et al., 2008; Deng et al., 2008], the major abnormalities reside outside the nervous system. Moreover, the DA system is only mildly affected [Clark et al., Park et al., 2006; Wang et al., 2006; Yang et al., 2006; Yanwei et al., 2010].

2.6.1.2 Zebrafish and medaka fish

Among the animal models for Parkinson's disease, also zebrafish and medaka fish models (*Oryzias latipes*) exist. Xi et al. found out that morpholino-mediated loss of Pink1 function in zebrafish larvae results in impaired response to tactile stimulation and a decreased locomotor activity (reduced swimming behaviour). All of these defects can be rescued by expression of an exogenous Pink1 that is not a target of the morpholinos used. In addition, weak or absent response of PINK1 morphants to tactile stimuli can be rescued by treatment with the dopamine D1 receptor agonist SKF-38393 [Xi et al., 2010]. The ability to respond to dopaminergic stimulation remained intact. Furthermore, they found an abnormal DA neuron expression patterning in PINK1-deficient zebrafish, but the total number of DA neurons is seemingly not affected, which indicates the involvement of PINK1 in DA neuron development, in positioning and for the establishment of neuronal circuits in which dopaminergic neurons are implicated, at least in zebrafish [Yanwei et al., 2010] and supported by the fact that Pink1 is strongly expressed during development.

Within the Pink1-gene-disrupted medaka fish (*Oryzias latipes*) loss of Pink1 causes late-onset decrease in spontaneous swimming movement [Matsui et al., 2010]. Furthermore,

to be normal, but moderate mitochondrial defects were displayed under intrinsic or environmental stress [Kitada et al., 2007; Gautier et al., 2008; Xi et al., 2010].

2.6.1.4 Our PINK1 knockout mouse model

Our PINK1 loss-of-function mouse model is based on a deletion of exon 2 and 3 and the mice are viable and fertile without external signs of abnormal body weight, posture or mortality. Morphologically, we could not find any degeneration of dopaminergic neurons in our 6 and 19 month old PINK1^{-/-} mice. Therefore, no gross motor dysfunction was observed. Nonetheless, symptoms which develop mainly before the cardinal motor symptoms were clearly evident in our PINK1-deficient mice [Glasl et al., 2012]. We found a change in the sense of smell, a main symptom in the non-motor phase of PD, as well as a reduction in the serotonergic innervation of the olfactory bulb. Specifically, the density of serotonergic fibers in the glomerular layer of the olfactory bulb was significantly reduced. In the odor test, male PINK1-deficient mice showed a clear social recognition deficit, a process which is mainly controlled in mice by the smell, whereas object recognition, which is mainly done by visual processes, were not affected. Male PINK1^{-/-} mutants showed a deficit in the fine discrimination of binary mixtures of odorants and reduced olfactory sensitivity [Glasl et al., 2012].

In addition, gait alternations were present in 24 month old PINK1-deficient male mice, tested by the automated gait analysis system "CatWalk" [Glasl et al., 2012]. In detail, the base of support (the average distance between the mass midpoints of the prints at maximum contact) was significantly decreased for the hind paws of our PINK1^{-/-} mice, as well as the paw print length and stand duration of the hind paws. In addition, the maximal contact was markedly increased in the hind paws.

At the age of 3 and 24 month, our PINK1-deficient mice showed the same locomotor activity and also the total distance travelled was not altered. Furthermore, the mice did not reveal differences in anxiety-related or depression-like behaviour.

The observed subtle phenotypes in this ubiquitous knock-out model indicate that beyond the dopaminergic system other neurotransmitter systems may be involved in the pathoetiology of the non-motor phenotypes. Our PINK1-deficient mouse model exhibits behavioral symptoms of early non-motor phases of PD, in which compensation may still impede the onset of neurodegeneration [Glasl et al., 2012].

Interestingly, neither of the yet genetic generated mouse models of PINK1, nor the models of parkin or DJ-1, reproduce the major hallmarks of PD with degeneration of the dopaminergic neurons in the substantia nigra and the subsequent development of motoric symptoms. However, a systematic and systemic analysis of these mice has not been done. Thus, during

the course of my thesis a knock-out mouse model for PINK1 was comprehensively analysed with age and specifically for symptoms of PD.

2.7 Aim of my thesis

Parkinson's disease is considered as a multifactorial disease in which genes, environmental factors, life science and aging play a role. The etiology of PD is largely unknown. Therefore animal models are indispensable tools to decipher the causes of this neurodegenerative disease. The superordinate aim of this study is to investigate the role of the *PINK1* gene in a transgenic mouse model of Parkinson's disease.

In particular, the focus of my PhD thesis is placed on the integrity and morphology of mitochondria *in vivo* and *in vitro*, an organelle which was found to play a major role in PD, as well as transport mechanisms via live cell imaging at different developmental stages of primary neurons. In addition, mitochondrial trafficking during permanent stress application was measured. Furthermore, several further factors like endosomal transport, protein and mRNA expression of mitofission and -fusion processes, as well as protein and mRNA expression related to transport mechanisms, and the influence of PINK1 in aged neurons were investigated.

Another part of the thesis is focused on the systemic analysis of the PINK1 model including behavioral and peripheral phenotypes in order to grasp PINK1 function not only in the brain but in the whole organism, since it is expressed throughout several organ systems.

3 Materials and Methods

3.1 Materials

3.1.1 Instruments

autoclave	Aigner, type 667-1ST
balances	Sartorius, LC6201S, LC220-S, Basic 210-S
hybridization bottles	ThermoHybaid
cassettes for autoradiography	Amersham, Hypercassette
centrifuges	Sorvall, Evolution RC; Eppendorf, 5415D, 5417R; Heraeus, Varifuge 3.0R, Multifuge 3L-R
chambers for electrophoresis (DNA)	MWG Biotech; Peqlab
cryostat	Mikrom, HM560
developing machine	Agfa, Curix 60
digital camera	Zeiss, AxioCam MRc, HRC
DNA sequencer	Applied Biotech, DNA Analyzer 3730
electro homogenizer	Ika, Ultra Turrax T25 basic
freezer (-20°C)	Liebherr
freezer (-80°C)	Heraeus HFU 686 Basic
fridges (4°C)	Liebherr
gel-/blottingsystem "Criterion"	BioRad
gel-/blottingsystem "Xcell"	
SureLock™ "Mini-Cell"	"Mini-Cell" Invitrogen
gel documentation system	Herolab, E.A.S.Y.
glass pipettes	Hirschmann
glassware	Schott
hybridization oven	Memmert, UM 400; MWG-Biotech, Mini 10; Thermo Electron, Shake'n'Stack
ice machine	Scotsman, AF 30
incubators (for bacteria)	New Brunswick Scientific, innova 4230
incubators (for cells)	Heraeus
laminar flow	Nunc Microflow 2
light source for microscopy	Leica KL 1500
liquid scintillation counter	Hidex, Triathler
luminometer	Berthold, Orion I

magnetic stirrer / heater	Heidolph, MR3001
microscope	Cell Observer, Carl Zeiss Jena confocal microscope (LSM510), Carl Zeiss Jena fluorescence microscope (Axioplan 2); Carl Zeiss Jena stereo microscope (Stemi SV6), Carl Zeiss Jena STEMI Leica (Zellkultur)
microwave oven	Sharp R-937 IN
Namedrop ND-1000	PeqLab
Neubauer counting chamber	Brand
PCR machine	Eppendorf, MasterCycler Gradient
perfusion pump	Watson-Marlow Bredel, 401U/D1
pH-meter	InoLab, pH Level 1 WTW, pH 538
photometer	Eppendorf, Biophotometer 6131
pipette boy	Eppendorf, Easypet; Hirschmann, Pipettus akku
pipettes (1 µl - 1000 µl)	Gilson; Eppendorf
power supplies for electrophoresis	Consort, E443; Pharmacia Biotech, EPS200; Thermo, EC250-90, EC3000-90
radiation monitor	Berthold, LB122
Real-Time PCR system	Applied Biosystems, 7900HT
shaker	Heidolph, Promax 2020, Polymax 1040, Brand, Nutator
slide warmer	Adamas instrument, BV SW 85
slicing microtom	Leica, SM2000R
thermomixer	Eppendorf, comfort
ultramicrotom	Microm, HM 355S
UV-DNA/RNA-crosslinker	Scotlab, Crosslinker SL-8042; Stratagene, UV-Stratalinker 1800
UV-lamp	Benda, N-36
vortex	Scientific Industries, Vortex Genie 2
water bath	Lauda, ecoline RE 112; Leica, HI1210; Mettler, WB7
water conditioning system	Millipore, Milli-Q biocel

3.1.2 Chemicals

a- ³² P-dCTP	Amersham
b-Mercaptoethanol	Sigma, Gibco
3,3'-diaminobenzidine (DAB)	Sigma
acetic acid	Merck
acetic anhydride	Sigma
agarose (for gel electrophoresis)	Gibco Life Technologies, Biozym
ammonium acetate	Merck
ampicillin	Sigma
Ampuwa	Fresenius
antifade solution, Aqua Poly/Mount	Polysciences Inc.
ascorbic acid	Sigma
B-27 supplement	Gibco
bacto agar	Difco
bacto peptone	BD Biosciences
bicine	Fluka
boric acid	Merck
bovine serum albumin (BSA, 20 mg/ml)	NEB, Sigma
bromphenol blue	Sigma
calcium chloride	Sigma
Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	Sigma
carrier DNA	Sigma
caesium chloride (CsCl)	Sigma
chlorobutanol	Sigma
chloroform	Sigma
cresyl violet acetate	Sigma
Criterion TM XT Bis-Tris-gels, 10% (protein)	BioRad
DAPI (4',6-Diamidino-2-phenylindole)	Sigma
DMEM	Gibco
DMSO	Sigma
dNTP (100 mM dATP, dTTP, dCTP, dGTP)	MBI
Doxycycline hyclate S	Sigma
ECL detection reagent	Amersham
EDTA	Sigma
Eosin Y	Sigma

ethanol absolute	Merck
ethidiumbromide	Fluka
fetal calf serum (FCS)	PAN, Hybond
Ficoll 400	Sigma
formamide	Sigma
freezing medium	Tissue Tek, OCT compound
gelatine	Sigma
GeneRuler 1 kb Ladder	Fermentas
glucose	Sigma
glycerol	Sigma
HEPES	Gibco
hydrochloric acid (HCl)	Merck
hydrogen peroxide, 30%	Sigma
isopropanol	Merck
kanamycin	Sigma
L-Glutamin	Sigma
L-Glutamine 200 mM (100x)	Gibco
Lipofectamin™ 2000	Invitrogen
magnesium chloride ($\text{MgCl}_2 \cdot 4\text{H}_2\text{O}$)	Merck
magnesium sulfate (MgSO_4)	Sigma
Mannitol	Sigma
methanol	Merck
mineral oil	Sigma
MOPS	Sigma
N2-supplement (100x)	Gibco
Neurobasal medium	Gibco
Neurofect™, transfection agent	Invitrogen
Neurofect	Gelantis
Nonidet P40 (NP-40)	Fluka
NuPAGE Novex Bis-Tris gels, 10% (protein)	Invitrogen
Opti-MEM® I Reduced-Serum medium (1x)	Invitrogen
orange G	Sigma
Paraformaldehyde (PFA)	Sigma
PBS (for cell culture)	Gibco
Penicillin - Streptomycin 100x Solution	Gibco
perchloric acid	Merck

PIPES	Sigma
Poly D-lysine (1 mg/ml)	Millipore
potassium chloride (KCl)	Merck, Sigma
potassium ferricyanide ($K_3Fe(CN)_6$)	Sigma
potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$)	Sigma
potassium hydroxide (KOH)	Sigma
potassium phosphate ($KH_2PO_4 \cdot H_2O$, K_2HPO_4)	Roth
protease inhibitor	Roche
Quick Start Bradford Protein Assay	Bio-Rad
RapidHyb buffer	Amersham
Retinoic acid	Sigma
RNaseZAP	Sigma
Roti-HistoKit II	Roth
Roti-Histol	Roth
saccharose	Sigma
skim milk powder	BD Biosciences
SmartLadder DNA marker	Eurogentec
sodium acetate (NaOAc)	Merck, Sigma
sodium chloride (NaCl)	Merck
sodium citrate	Sigma
sodium desoxycholate	Sigma
sodium dodecylsulfate (SDS)	Merck
sodium hydroxide (NaOH)	Roth
sodium nitrite (NNaO ₂)	Sigma
sodium phosphate ($NaH_2PO_4 \cdot H_2O$, Na_2HPO_4)	Sigma
Sodium pyruvate	Gibco
spermidin	Sigma
sucrose	Sigma
TriReagent	Sigma
Tris (Trizma-Base)	Sigma
Triton-X 100	Biorad
Trizol	Invitrogen
tRNA	Roche
trypsin	Gibco
Tween 20	Sigma
xylol	Fluka

yeast extract

Difco

3.1.3 Common and stock solutions

General stock solutions

LB (lysogeny broth, (Bertani, 2004)) agar	98,5% LB medium 1,5% Bacto agar
LB (lysogeny broth, (Bertani, 2004)) medium	10 g Bacto peptone 5 g yeast extract 5 g NaCl ad 1 l H ₂ O
loading buffer for agarose gels	15% Ficoll 400 200 mM EDTA 1 - 2% orange G
Kanamycin selection agar	LB agar with 50 µg/ml kanamycin
Kanamycin selection medium	LB medium with 50 µg/ml kanamycin
Para formaldehyde solution (PFA, 4%)	4% PFA w/v in PBS
PBS (1x)	171 mM NaCl 3.4 mM KCl 10 mM Na ₂ HPO ₄ 1.8 mM KH ₂ PO ₄ pH 7.4
SSC (saline sodium citrate, 20x)	3 M NaCl 0.3 M sodium citrate pH 7.0
sucrose solution (20%)	20% sucrose w/v in PBS
TAE (10x)	0.4 M Tris base 0.1 M acetate 0.01 M EDTA
TBE (10x)	0.89 M Tris base 0.89 M boric acid 0.02 M EDTA
TBS (10x)	0.25 M Tris-HCl pH 7.6 1.37 M NaCl
TBS-T (1x)	1 x TBS 0.05% Tween 20

TE (Tris-EDTA)	10 mM Tris-HCl pH 7.4 1 mM EDTA
Tris-HCl	1 M Tris base, pH 7.5

Cell culture solutions

dissection medium	48.8 ml HBSS 500 μ l HEPES (1M) 600 μ l MgSO_4 (1 M) 500 μ l Penicillin/Streptomycin (100x)
culture medium:	48 ml Neurobasal 1 ml B-27 500 μ l L-Glutamine 500 μ l Penicilline/Streptomycin (100x)

Solutions for southern blot analysis

Church buffer	0.5 M Na_2HPO_4 0.5 M NaH_2PO_4 1% BSA 7% SDS 1 mM EDTA pH 8.0 0.1 mg/ml salmon sperm DNA
Denaturation solution	0.5 M NaOH 1.5 M NaCl
Hybridization Buffer	Rapid Hyb Buffer, Amersham/GE; Church Buffer
Lysis buffer for genomic DNA extraction	0.1 M Tris pH 8.5 5 mM EDTA 0.2% SDS 0.2 M NaCl 0.1 mg/ml Proteinase K
Neutralization solution	0.1 M Tris - HCl pH 7.5 0.5 M NaCl
Stripping solution	0.4 M NaOH
Wash solution I	2 x SSC 0.1% SDS

Wash solution II	0.5 x SSC
	0.5% SDS
Wash solution III	0.5 x SSC
	1% SDS

Western blot solutions

Blocking solution	4% skim milk powder in TBS-T
Laemmli buffer (5x)	313 mM Tris-HCl pH 6.8
	50% glycerol
	10% SDS
	0,05% bromphenolblue
	25% β -mercaptoethanol
MES running buffer (10x, for NuPAGE gels)	500 mM MES
	500 mM Tris
	1% SDS
	10 mM EDTA
	pH 7.2
10X MOPS:	0.2 M 3-Morpholinopropan-sulfonsäure
	50 mM Natriumacetat
	10 mM EDTA
	500 mM MOPS
	500 mM Tris
	1% SDS
	10 mM EDTA
	pH 7.7
NuPAGE transfer buffer (10x, for NuPAGE gels)	250 mM bicine
	250 mM bis-tris
	10 mM EDTA
	0.05 mM chlorobutanol
NuPAGE transfer buffer (1x, for NuPAGE gels)	10% 10x transfer buffer
	10% Methanol
RIPA buffer	50 mM Tris-HCl pH 7.4
	1% NP-40
	0,25% sodium desoxycholate
	150 mM NaCl

	1 mM EDTA
	1 tablet protease inhibitor
	in 50 ml H ₂ O
Tris glycine blotting buffer (10x)	0.25 M Tris
	1.92 M glycine
Tris glycine blotting buffer (1x)	10% 10x blotting buffer
	10% methanol

3.1.4 Consumables and others

100 bp DNA ladder	Fermentas
1kb+ DNA Ladder	Invitrogen
cell culture dishes (Ø 60 mm, 100 mm, 150 mm)	Nunc
cell strainer (40 µm)	BD Biosciences
cell culture dishes	Nunc
centrifuge tubes 15 ml, 50 ml	Falcon
cleaning columns	Amersham, MicroSpin S-300 Roche
coverslips for sections on slides	Menzel Gläser
Coverslip (round, 14 mm)	Hecht Assistant
Criterion™ XT Bis-Tris-gels, 10 % (protein)	BioRad
embedding pots	Polysciences, Peel-A-Way
films for autoradiography	Kodak, Biomax MS, Biomax XAR, Biomax MR
films for chemiluminescence detection	Amersham, Hyperfilm
filter paper	Whatman 3MM (Kat.-Nr.:3030 917)
filter tips 10 µl, 20 µl, 200 µl, 1 ml	Art, Starlab
gloves	Kimberley-Clark, Safeskin PFE Safeskin, Nitrile
HyperLadder I	Bioline
ibidi slides	ibidi
multiwell plates (6, 12, 24, 96 wells)	Nunc
MicroSpin S-300	Amersham
NuPAGE® Novex Bis-Tris gels, 10 % (protein)	Invitrogen
Novex® Sharp pre-stained protein standard	Invitrogen
nylon membrane for DNA transfers	Amersham, Hybond N Plus

Pasteur pipettes	Brand
parafilm	American National Can, USA
PCR reaction tubes (0.2 ml) Biozym	Biozym
PCR sealing film (78 x 120 mm) Kisker	Kisker
Phase Lock Gel™, heavy	Eppendorf
pipette tips	Gilson
pipette tips 10 µl, 20 µl, 200 µl, 1000 µl Art	Gilson, Starlab
plastic pipettes (1, 5, 10, 25 ml) Greiner	Greiner
PVDF membrane for Western blotting	Pall Biosciences
reaction tubes (0.5 ml, 1.5 ml, 2 ml)	Eppendorf
SeeBlue® Plus2 pre-stained protein standard	Invitrogen
slides	Menzel Gläser, Superfrost Plus
SmartLadder DNA marker Eurogentec	Eurogentec
Superfrost Plus slides Menzel Gläser	Menzel Gläser
tissue cassettes	Merck
tissue embedding molds	Polysciences, Inc.

Stereotactic injection equipment

Glass capillaries	World Precision Instruments
Glass capillary puller (PC-10)	Narishige
Lab Standard Stereotaxic Instrument	Stoelting
Nanoliter 2000 injector	World Precision Instruments
one-way needles (20G, 27G)	Terumo, Neolus
one-way syringes (1 mL, 10 mL, 20 mL)	Terumo
Suture (Marlin violett HR 17)	Catgut

3.1.5 Enzymes

Alkaline Phosphatase, Calf Intestinal (CIP)	NEB
Cre recombinase	NEB
DNA Polymerase (Taq)	Qiagen
DNase I (for cell culture) Roche	Roche
Klenow Fragment der DNA Polymerase I:	NEB
PCR-Mastermix 5x 5'Prime	Eppendorf, 5 Rediprime
Proteinase K (20 mg/ml)	Roche
restriction enzymes	Roche, MBI, NEB, Promega

RNA Polymerasen (T7, Sp6; 20 U/ μ l):	Roche
RNase A	Serva
RNase Inhibitor:	Roche
Taq DNA Polymerase	Qiagen, Invitrogen
Taqman Universal PCR Mastermix:	Applied Biosystem

3.1.6 Commercial Kits

Bradford Assay	Sigma
DNA Maxi Prep Kit:	Qiagen
DNA Mini Prep Kit	Qiagen, Sigma
Organelle Lights TM Intracellular Targeted Fluorescent Proteins, Bac Mam 1.0	Molecular Probes
PCR Purification Kit	Qiagen
Qiagen Plasmid Maxi Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
RediPrime TM II DNA Labelling Kit	Amersham/GE
RNeasy Mini Kit	Qiagen
RNeasy Midi Kit	Qiagen
SuperScript TM II First-Strand Synthesis System for RT-PCR	Invitrogen
TOPO TA Cloning Kit:	Invitrogen
Vilo Kit	Invitrogen
Wizard Genomic DNA Purification Kit	Promega

3.1.7 Mouse lines

C57Bl/6J	Wild type inbred mouse line Helmholtz Zentrum München original arrived from Jackson Laboratories
<i>Cre deleter</i>	<i>Cre</i> deleter mouse line, <i>Cre</i> expression is X-linked and occurs during early embryogenesis before implantation
<i>Pink1 cko</i>	<i>Pink1</i> conditional knockout, mouse line with floxed <i>Pink1</i> exons 2 and 3, and neo resistance cassette flanked by FRT sites lying between the first loxP site and exon 2 of the <i>Pink1</i> locus (5.2.1 The conditional <i>Pink1</i> knockout construct)
<i>Pink1 ko</i>	<i>Pink1</i> knockout mouse line, <i>Pink1 cko</i> after breeding with <i>Cre</i> deleter line, exons 2 and 3 of the <i>Pink1</i> locus are missing throughout the whole body

3.1.8 Cell lines

E. Coli; DH5 α	Gibco Life Technologies
HEK 293T	
primary mouse cortex neurons	all primary neurons arrived from mouse
primary mouse striatal neurons	strains C57/Bl6-J, Pink1 ^{-/-} and PINK1 ^{+/+} of
primary mouse hippocampal neurons	our own internal mousehouse

3.1.9 Antibodies

Primary antibodies

Anti-Active Caspase-3pAb (G7481) rabbit	Promega
anti-glial fibrillary acidic protein (GFAP), clone C-A-S, mouse (monoclonal)	Sigma (G3893)
β -III-Tubulin rabbit (polyclonal)	abcam (ab18207)
β -actin mouse (monoclonal)	GeneTex (GTX26276)
Cleaved Caspase (D175) (SA1E) anti rabbit	Cell Signaling
Darpp-32 (19A3) anti rabbit	Cell signalling
Dctn 1 (Dynactin)	Abcam(75034) rabbit
Dnhd1	Abnova rabbit
Dnm1L (=Drp 1)	Abcam ab(54038) Rabbit
Fis1	Abnova Rabbit Santa Cruz Sc-48865
Mfn1	Millipore ABC41
Mfn2	Abnova Rabbit Millipore rarabbit
Milton (Trak2)	
Opa 1	Abcam Rabbit
Pink	Santa Cruz (sc-33796) Acris
RhoT1 (Miro 1, Trak1, OIP106)	Genway rabbit
RhoT2 (Miro2)	Santa Cruz(sc165360)

Secondary antibodies

anti-rabbit Alexa Fluor® 488 donkey ICC	Invitrogen A21206 1:500
CyTM3-conjugated affinity pure donkey anti-rabbit	
IgG (H-L) anti rabbit	Jackson Immuno Research
Cy2 anti mouse	Jackson Immuno Research

3.1.10 Oligonucleotides

name	sequence	conditions	size of product
neocass-for	GACAGTACTTGCCTAGCGTAGGTAG	94°C 5 min	688 bp
		94°C 35 sec	34x integrated flox/flox
neocass-rev	TTGCTCAGCGGTGCTGTCCATCT	63°C 30 sec	
		72°C 50 sec	
		72°C 5 min	
neocass-for	GACAGTACTTGCCTAGCGTAGGTAG	94°C 5 min	34x 781bp wildtype band
		94°C 35 sec	
ex2-rev	CAGACACGCGCTTGGTTTTCTGTGT	63°C 30 sec	
		72°C 50 sec	
		72°C 5 min	
neocass-for	GACAGTACTTGCCTAGCGTAGGTAG	94°C 5 min	34x 736 bp knockout band
		94°C 35 sec	
ko-rev	CTTGCCCTGGGTGAATGGTGACA	63°C 30 sec	
		72°C 50 sec	
		72°C 5 min	
SBxho-for	GCTTTCTTAGGAACTCCAGGAGCC	XhoI probe for SB	wt 6,1 kb
SBxho-rev	ACTGCCTTGGCCATAGAAGGGATTG		integrated 8,0 kb ko 4,2 kb
SBnew-for	CCAGCAGCCATCAGGATCTCAG	ScaI probe for SB	wt 6,8 kb
SBnew-rev	AGATGGGGCCAGCATCAGAGACA		integrated 8,6 kb ko 4,8 kb
SBbam-for	CAAGGGCGGAGTTAGTTGGATAG	BamHI probe for SB	wt 9 kb
SBbam-rev	CCAACCAAAGAGTACACATGGATGG		integrated 4,5 kb ko 4,5 kb
PINK1 shRNA	5'- GCG GTA ATT GAC TAC AGC AAA - 3'		

All Primers which were used for RT-PCR are listed at page 52 and 53.

3.1.11 Plasmids

name of plasmid	construct from	description
pEGFP-D1-rLC3	Prof. Dr. Köster R.	Autophagic marker taking part in the formation of autophagosomal membranes,
hRab5a	Prof. Dr. Zerial M.	The G protein Rab5a belongs to the GTPase superfamily and is required for both fusion of plasma membranes and building of early endosomes.
P2-Pink1 SB	Barbara di Benedetto	Contains Pink1 3'-Southern blot probe which is 680 bp long, starting 97 bp upstream of the end of exon 7, ending 583 bp downstream the end of exon 7 in intron 7 (digested with EcoRI)

3.2 Methods

3.2.1 Generation of PINK1 knockout mice

The generation of our PINK1 knockout mice was made for a previous project at the Institute of Developmental Genetics (IDG) few years ago [Glasl et al.; 2012]. For my recent work I was using this line and thus, the generation of it will only shortly be summarized for a better understanding of the genetic background. For a detailed description I refer to the Thesis of Anne Röthig, chapter 5.2 and 5.3 and Glasl et al.; 2012.

The conditional PINK1 knockout targeting construct was built to remove exons 2 and 3 out of the Pink1 locus.

A neomycin resistance cassette flanked by FRT sites (dark blue triangles Fig.6) was inserted in front of exon 2. In addition, the FRT-flanked neo cassette and exon 2 and 3 were flanked with LoxP sites (light blue triangles; Fig.6). For Southern blot verification a 3'-probe (blue horizontal line Fig.6) using *ScaI* digestion was located at the beginning of intron 7 outside the homologous arms. A neo-probe for the correct insertion of the floxed homologous arm digestion with *MfeI* and *HindIII* was performed, since insertion of the loxP site introduced a new *HindIII* restriction site. 5' homologous arm, floxed homologous arm, and 3' homologous arm are shown as black arrows (Fig.6).

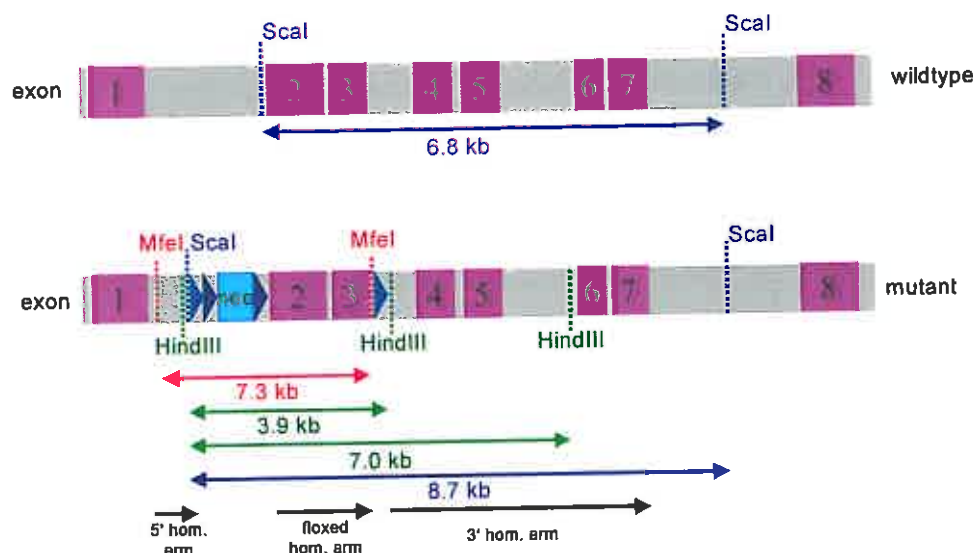


Fig. 6: Construct for conditional Pink1 knockout mice. Adapted from Glasl et al., 2012.

When Pink1 conditional knockout mice were mated with Cre deleter mice, Cre recombination occurs (Fig.7) while the FRT sites, the neomycin resistance cassette, exon 2 and 3 are removed. Just one loxP site between exon 1 and 4 keeps active, which generates a frame shift in the Pink1 coding sequence on RNA level and consequently creates a stop codon briefly behind exon 1. On the protein level a truncated form of Pink1 results consisting only of those amino acids encoded by the first of the eight exons.

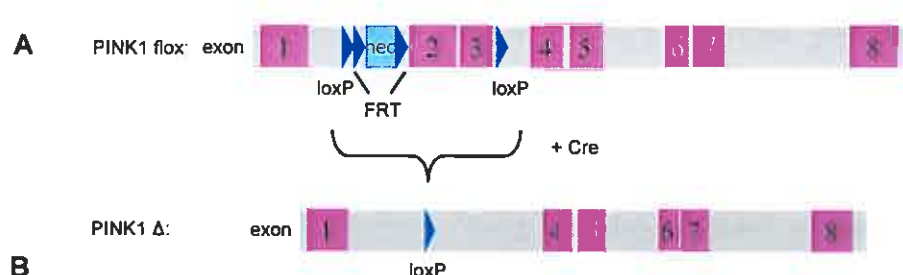


Fig. 7: Schematic drawing of floxed *Pink1* allele before (A) and after (B) Cre recombination. Adapted from Anne Röthig, "Generation and analysis of a *Pink1* deficient mouse model for Parkinson's disease".

3.2.2 Genotyping

To genotype our *PINK1* line DNA isolation from mouse tails were made using Wizard genomic DNA purification kit (Promega) following manufacture's instruction.

3.2.2.1 Genotyping via Polymerase Chain Reaction (PCR)

The genomic DNA was used as a template in a duplex PCR reaction to discriminate between the wild type and the deleted *PINK1* allele: wt-for (5'-gacagtacttgcttagcgtaggtag-3') and 5'-cagacacgcgcttggtttctgtgt-3' amplifying the 736bp knockout band and wt-form and 5'-cttgccctgggtgaatggtgaca-3' amplifying 781bp wild type band. For genotyping our *PINK1* conditional knockout mice a triplex PCR was performed with additional Primers for floxed *PINK1* (wt-for and 5'-ttgctcagcgggtgctgtccatct-3' amplifying 688bp lox-P side).

The reaction mix consists of 1 µl DNA template, 10 µl 5' Prime PCR Master Mix, 1.25 µl of each primer and 11.5 µl H₂O to get a total reaction volume of 25 µl. An additional water control was added to detect possible contamination.

Initial denaturation of the DNA into single strands was done at 94°C for 5 minutes followed by a 34 fold cycle of a denature phase at 94°C for 35 seconds, an annealing phase at 63°C for 30 seconds and an elongation phase at 72°C for 50 seconds. A final elongation step after the cycle was done at 72°C for 5 minutes and the samples were stored at 4°C until further use. PCR products and a 1kb Ladder (Invitrogen) were filled up on a 1% agarose gel in TAE buffer containing ethidium bromide (EtBr). Each sample was mixed with a Loading Dye (orange G) and the gel was run for 30 minutes at 120 mA before visualizing the DNA bands under UV light and taking a picture for documentation.

3.2.2.2 Genotyping via Southern blotting (SB)

20 µg of genomic DNA were digested at 37°C over night with restrictions enzyme *ScaI* (10U/l form Fermentas) and appropriate buffer in a total volume of 50 µl. After digestion a 0.7 % agarose gel in 1xTBE including ethidium bromide was prepared in which DNA was separated and run for up to 20 hours at ~ 40 V and thereafter was photographed to check the status of

digestion. Gels were rinsed briefly in H₂O to wash off removing TBE buffer. DNA was depurinated with 0.2 M HCl for a maximum of 10 minutes at RT under gentle agitation. This step induces a mild DNA fragmentation and subsequently to a better blotting efficiency of large fragments. Two denaturation steps for each 15 minutes in denaturation solution followed according with gently shaking. Finally the DNA fragment solution was transferred into a neutralization solution for up to 45 minutes with frequently solution changes during this time to make the gel pH neutral again. The single stranded DNA was than blotted overnight via capillary transfer onto a nylon membrane by using 10x SSC. The next day the membrane was briefly rinsed with 2x SSC and crosslinked via UV light to fix the bands onto the surface. The membrane now could be stored dry at 4°C until further use.

Before hybridization the membrane was prehybridized at 63°C for at least two hours in a hybridisation tube containing 10 ml Rapid-Hyb™ Buffer (Amersham) or Church Buffer to reduce unspecific binding of the probe. A 680bp long 3' external probe (amplified with the primers 5'-cagctaccaggaagctcagc-3' and 5'-ggaggctgcaggagttggct-3') on Scal digested genomic DNA was used to distinguish between endogenous, floxed and deleted alleles (6.8 kb, 4.8 kb and 8.7 kb, respectively). 50-100 ng of the 5'-flanked probe was labelled with α -32P-dCTP using the Rediprime II labeling kit following manufacturer's instructions. The radioactive labeled probe was centrifuged in a Microspin S-300 column to purify and get rid of left over nucleotides and radioactively labelled DNA fragments. To appoint the labelling efficiency, 1 μ l purified probe was measured in a liquid scintillation counter which yielded around 200,000 cpm/ μ l. The radioactive labelled DNA probe was denatured at 95°C for 10 minutes, chilled on ice and the hybridization buffer was added to the membrane. The whole probe stayed at 65°C in a hybridization oven overnight. The membrane was than washed with 2x SSC + 0.1% SDS for 30 minutes to one hour at 60°C, followed by 0.4x SSC + 0.1% SDS washing step at 60°C for about 30 minutes and a last washing step by 0.4x SSC + 0.1% SDS for about 30 minutes to clean the membrane from unspecific bound probe and to determine the amount of radiation on the membrane. In between the radioactivity was measured using a Geiger-Müller counter. Subsequently the hybridized membrane was transferred in a transparent film to keep it humid and exposed to an autoradiography film (Biomax MS for to detect also weak signals) for one week at -80°C. After one week the film could be developed in the darkroom using a developing machine with associated developing, washing and fixation solution. The hybridized signals revealed distinct bands for wild type allele (6.8 kb), cre-deleted knockout allele (4.8 kb) and it there was also PINK1 cko mice floxed allele (8.7 kb).

3.2.3 Mouse husbandry

All of our mice were kept and bred in the Helmholtz Zentrum Munich animal facilities in accordance with national and institutional guidelines. The mice were group-housed in individually ventilated cages with up to five animals per cage as maximum and separated by gender. Mice were kept in a 22 ± 2 °C and 55 ± 5 % relative humidity environment at 12 hour light/dark cycle with food and water given *ad libitum*.

For stereotactical investigations as well as for behavioural analysis, some mice were transferred to the German Mouse Clinic under the same housing conditions but smaller cages. For gait analysis and tracing as well as electrophysiology experiments mice were transferred into another mouse house but with same housing conditions.

For breeding, single or sometimes double mating was set up. Pups were weaned at an age of three to four weeks and earmarks were made for identification. In parallel, tail clips were taken for genotyping. The mice were then again separated according to their gender. Mouse husbandry was maintained in a special database.

3.2.4 Lentivirus production

HEK 293T cells were used for producing lentiviruses as described in Consiglio et al., 2001. Producing a lentivirus with siRNA for PINK1 (previously described in Lutz et al., 2009), the nucleotides 1353 to 1373 corresponding PINK1 shRNA sequence 5'- GCG GTA ATT GAC TAC AGC AAA - 3' were cloned into pLL3.7 vector by Hpa I and Xho I cloning sites and shRNA was driven by a U6 promotor. The original indicating GFP part of pLL3.7 vector was exchanged via CMV-driven mito-EYFP cloning (pEYFP-mito, Clontech, Mountainview, CA, USA) into Nhe I and Eco RI site. shRNA was designed using the pSico- Oligomaker 1.5 (developed by A. Ventura). On the internet the construct is freely available at <http://web.mit.edu/ccr/labs/jacks/protocols/pSico.html>. For control the same virus was used without the PINK1 shRNA sequence.

To determine the virus titer, coverslips (12 mm diameter) were baked at 180°C for four hours and transferred to 24 well plates. 5×10^4 HEK 293 cells were plated on these coverslips. Four hours later a concentration series was done according to the following volumes of virus-medium mixture: 400 µl medium and 2 µl virus, which had to be examined. This mixture was added into each well to infect the cells at different concentrations: 200 µl (containing 1 µl virus), 100 µl (containing 0.5 µl virus), 20 µl (containing 0.1 µl virus), 10 µl (containing 0.05 µl virus), 1 µl (containing 0.005 µl virus) and 0.1 µl (containing 0.0005 µl virus). After two days of incubation, cells were washed with PBS, fixed with fresh PFA 4% for 5 minutes and washed again with PBS. In addition, a DAPI staining was carried out with an additional PBS washing step. Finally the adherent cells on the coverslips were mounted with antifade on standard slides. All infected cells of the different virus concentrations were counted by

epifluorescence microscopy. To calculate the titer, e.g. 50 cells counted on the coverslip with 1 μ l virus-medium mixture had to be multiplied with 10^4 and divided through the used virus volume 0.005 μ l, which equals a titer of 1×10^8 colony forming units (cfu).

3.2.5 Mitochondrial morphology study

3.2.5.1 Mitochondrial morphology study *in vivo*

Stereotactic surgery and quantification of mitochondria morphology *in vivo* of PINK1 mice

To study mitochondrial morphology *in vivo*, two *Pink1*^{-/-} mice and two *PINK1*^{+/+} mice were anesthetized via i.p. injection with 0.1ml per 10g body weight of an anaesthetic containing 1ml Ketamin (10%), 0.25ml Xylazin (2%) and 6ml NaCl (physiologic 0.5%) and placed into their home cage until complete sedation. Mice were then placed on a 37°C warm heat-pillow and fixed in the stereotactic apparatus (Stoelting) using ear bars and a mouse nose clamp. To protect the eyes from corneal drying during surgery they were treated with eye ointment. The fur on the skull was cleaned with 70% ethanol and the skin was cut with a sterile scalpel along the midline. To keep the area open and have a free surgery field, small surgical clamps were used. The skull was repeatedly moistened with PBS and coordinates of the Bregma and Lambda cranial suture were measured. Appropriate coordinates for the striatum were calculated and marked using the stereotactic "Mouse Brain Atlas" from Paxinos and Franklin (2001). The following coordinates for striatal injection were used from Bregma as origin point: 0.0 mm in anterior – posterior, $\pm$ 2.2 mm in medial – lateral and 3.4 mm in dorsal – ventral direction (see Fig. 8). With a small dental drill a little hole was drilled into the skull at the injection position. Afterwards a micropipette, including a lentivirus (CMVgfpIRESmitosred) for mitochondrial detection, was positioned at the correct coordinates and 1.8 μ l of the viral vector solution was injected via a motorized Nanoliter injector (World Precision Instruments) with a speed of 1 μ l per 4 minutes. Mice were rested with the micropipette in the brain for 4 to 5 minutes. The skull was cleaned and moistened again before the skin was sutured with an absorbable thread. The mouse was left on the heat-pillow until recovery from anesthesia and then kept in single-housed cages.



Fig.8: Injection region of the lentivirus CMVgfpIRESmitodsred for mitochondrial detection in Striatum of PINK1 mice. (Adapted from 'The mouse brain in stereotaxic coordinates' from George Paxinos and Keith Franklin.)

Two weeks after surgical operation, the mice were killed with CO₂, immediately fixed with their paws onto a Styrofoam plate and thoracic cavity was opened for access to the heart. Afterwards, a blunt needle was inserted carefully through the left ventricle into the ascending aorta, fixed with a clamp and the right atrium was cut open to let the blood flow out of the body. Via a perfusion pump which was connected to the blunt needle, blood vessels were rinsed with ice-cold PBS until the liver became pale. The perfusion was then carried out with 4% paraformaldehyde (PFA) in PBS for at least 5 minutes until the mouse became stiff. Thereafter, the mouse was decapitated and the brain was dissected by removing bones and meninges. To post-fix the brain, it was kept in 4% PFA for 2 – 4 hours. After fixation, the brain was transferred into a sucrose solution (20% Sucrose + 0.01% sodium azide in PBS) until further use.

In order to produce 16 µm thick slices, brains were embedded with Neg-50 tissue solution, cut via cryostat (HM560 Kryostat), directly attached to glass slides and dried. The slides were rinsed in PBS to remove resting Neg-50 tissue solution and finally they were mounted with Aqua Poly/Mount on standard coverslips (24 x 60 mm). Three dimensional pictures of striatal morphologic distinct neurons with eYFP-stained mitochondria were acquired using confocal microscopy (LSM 510). Mitochondrial length was quantified using the Axiovision 4.7 software and classified into three different categories: fragmented mitochondria (< 0.5 µm), intermediate mitochondria (0.5 – 5 µm) and tubular mitochondria (> 5 µm). In total, two mice per genotype with 20 neurons per mouse and 50-250 mitochondria per neuron were analyzed. Statistical analysis of the fragmented and intermediate mitochondria was carried out using ANOVA. Data were expressed as means ± S.E.M. The p-values of tubular mitochondria were calculated via Fisher's Exact Test. *p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001. All experiments were approved by the animal welfare and use committee of the local governmental body.

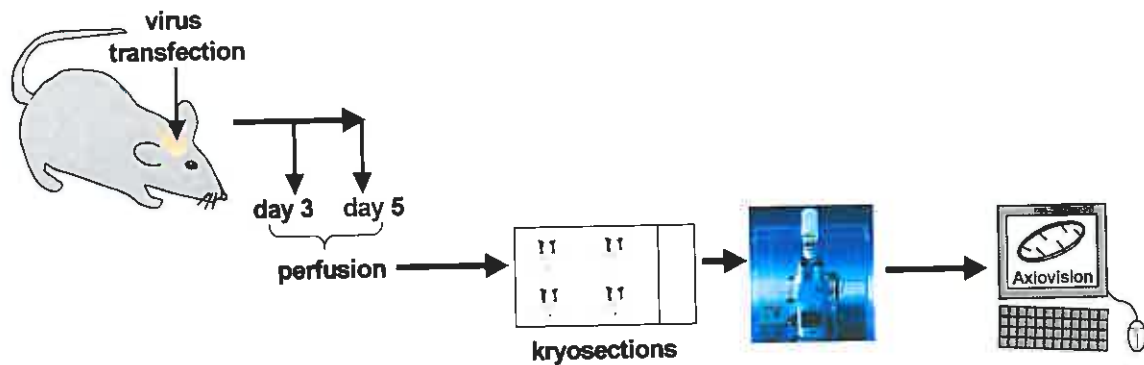


Fig. 9: Experimental setup for mitochondrial morphology experiments *in vivo*.

Depending on the experiments, different lentiviruses were used (for stereotactic injection into both Striatum and CortexCMVgfpIRESmitodsred, pLL3.7mito-eYFP, pLL3.7shPINK1mito-eYFP, respectively)

Stereotactic surgery and quantification of mitochondria morphology *in vivo* of C57/Bl6-J mice with acute knockdown of PINK1

For the *in vivo*-analysis of cortex neurons six C57/Bl6-J mice were anesthetized as previously described (Stereotactic injection and quantification of striatal neuronal mitochondria *in vivo*) and stereotactically injected with 1.8µl of lentivirus pLL3.7mito-eYFP into the left Cortex, whereas 1.8µl of pLL3.7shPINK1mito-eYFP was injected into the right Cortex (mm from Bregma: 0.0 mm anterior-posterior; ±2.5 mm medial-lateral; 1.7 mm dorsal-ventral; coordinates from Mouse Brain Atlas, Paxinos & Franklin). One half of the mice were perfused and treated as previously described three days after injection, while the second group was treated five days after injection. In addition, these samples were cutted into 16 µm thick slices to detect apoptotic cells. The mounting with Aqua Poly/Mount (PolySciences, Inc., Warrington, PA, USA) on coverslips (24 x 60 mm; Roth) was performed identically. Morphologic distinct neurons with eYFP-stained mitochondria of control and *Pink1* knock down cortex hemisphere were visualized by using confocal microscopy with z-stack application tool (LSM 510, Carl Zeiss). Mitochondrial length was quantified using the Axiovision 4.7 software (Carl Zeiss) and classified in groups of fragmented, intermediate and tubular length (see above). Three mice per time point with 5 -10 neurons per virus group and mouse (with 50-250 mitochondria per neuron) were analyzed. Statistical analysis of the mitochondrial lengths was carried out using Students t-Test with * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and data were expressed as means $\pm$ S.E.M. All experiments were approved by the animal welfare and use committee of the local governmental body.



Fig. 10: Injection region within the Cortex of C57/Bl6-J mice. Lentivirus for acute knockdown of *PINK1* (pLL3.7+*PINK1*sh mitoYFP) and control virus (pLL3.7 + mitoYFP) was used to detect impairments of mitochondria morphology within *PINK1* deficient neurons. Adapted from 'The mouse brain in stereotaxic coordinates' from George Paxinos and Keith Franklin.

3.2.5.2 Mitochondrial morphology *in vitro* in Bl6 primary hippocampal neurons

Primary hippocampal neuronal cultures (Lutz et al., 2009 and Glasl et al., 2012) of C57/Bl6-J mice from two different litters were prepared at embryonic stage E15.5 and transferred to dissection medium: 48.8 ml HBSS, 500 μ l HEPES (1M), 600 μ l $MgSO_4$ (1 M), 500 μ l Penicillin/Streptomycin (100x). After washing with prewarmed trypsin 0.05% with EDTA two times, the tissue was trypsinated for 15 min at 37°C and thereafter washed three times in culture medium: 48 ml Neurobasal, 1 ml B-27, 500 μ l L-Glutamine, 500 μ l Penicilline/Streptomycin (100x). Cells were dispersed with the fire polished tip of a Pasteur pipette. 4×10^4 cells per 300 μ l Medium (corresponds to 1.33×10^5 cells per 1ml Medium) were cultivated on Poly-D-Lysin-coated (1mg/ml in H_2O) coverslips ($\varnothing$ 12 mm) in 24-well plates. After 4 days of growth, neurons were infected with a lentiviral shRNA vector for acute knock-down of *Pink1* (pLL3.7sh*PINK1*mito-eYFP) and a control lentivirus (pLL3.7mito-eYFP). Three, four and five days after infection, cells were fixed in 4% fresh PFA for ten minutes and washed two times with PBS. Neurons were visualized by immunocytochemistry using antineuron specific beta III tubulin rabbit pAB (Abcam; Cambridge, UK) as primary antibody and CyTM3-conjugated affinity pure donkey anti-rabbit IgG (H+L) (Jackson Immuno Research; West Grove, PA, USA) as secondary antibody (Jackson Immuno Research; West Grove, PA, USA). Neurons were blocked with PBS containing 0.1% Triton X-100 and 10% horse serum for 2 hours at RT and incubated within this blocking solution containing additionally the primary antibody over night at 4°C. The next day, neurons were washed three times with a solution of PBS and 0.1% Triton X-100, followed by an incubation in the blocking solution containing the secondary antibody for 2 hours at RT. Then, the neurons were washed again two times with PBS and finally were fixed with Aqua Poly/Mount (Polyscience Inc.) while transferring to slides (Superfrost[®] Plus; 25 x 75 x 1.0 mm; Menzel-Gläser; Thermo Scientific). Three-dimensional images were acquired using motorized focus of a confocal microscope (LSM 510, Carl Zeiss). Mitochondrial length was quantified with specialized analyzing software Axiovision 4.7 (Axiovision 4.7, Carl Zeiss). Two cultures per condition, with 20 neurons/culture and 50-250 mitochondria/neuron, were analyzed.

Classification of mitochondria was done as follows: fragmented mitochondria ($< 0.5 \mu\text{m}$), intermediate mitochondria ($0.5 - 5 \mu\text{m}$) and tubular mitochondria ($> 5 \mu\text{m}$). Data are expressed as means $\pm$ S.E.M.. Statistical analyses of the mitochondrial lengths was carried out using ANOVA. The statistical analyses of the fragmented and intermediate mitochondria were analyzed via ANOVA whereas the p-values of tubular mitochondria were calculated via Fisher's Exact Test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (Lutz et al. 2009; Glasl et al. 2012).

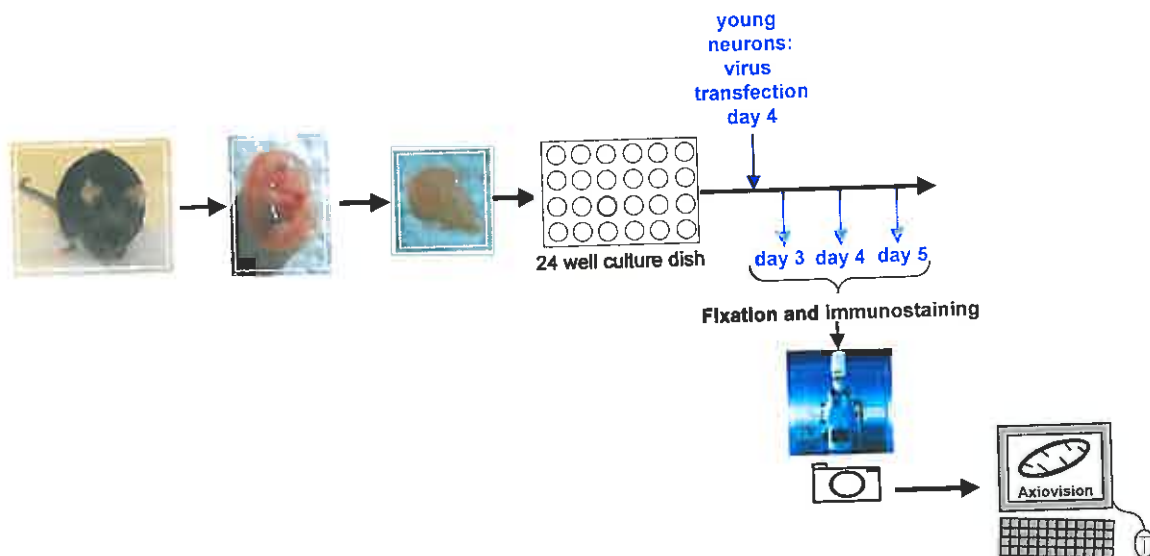


Fig. 11: Experimental setup for mitochondrial morphology experiments *in vitro*.

A lentiviral shRNA vector for acute knock-down of *Pink1* (pLL3.7shPINK1mito-eYFP) and a control lentivirus (pLL3.7mito-eYFP) were added to the primary hippocampal cell culture of E15.5 embryos at day four of growing. Neurons were incubated for additional three and five days before fixing them and making an additional immunohistochemistry staining for β III-tubulin.

3.2.6 Mitochondrial trafficking

3.2.6.1 Mitochondrial trafficking of acute PINK1 knockdown neurons at different developmental stages

Cortices of C57/Bl6-J mice from at least three different litters were prepared at embryonic stage E15.5 and treated as previously described for cell culture analysis. Dispersed cells were cultivated, with a density of 2.66×10^5 cells in 2 ml Culture Medium (corresponds to 1.33×10^5 cells per 1ml Medium), on Poly-D-Lysin-coated (1mg/ml in H_2O) μ -Dish (35 mm) with Grid-500. We investigated three different developmental stages of neurons: young, matured and aged. The cultures were infected during half medium change with a lentiviral shRNA vector for acute knock-down of *Pink1* (pLL3.7shPINK1mito-eYFP) and a control lentivirus (pLL3.7mito-eYFP) at growing day four, eleven and eighteen. Two time points after infection were analysed: Three and five days after infection at each developmental stage

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investigated five neurons per group and litter were recorded with a microscope (Cell Observer) using epifluorescence live cell imaging for mitochondrial trafficking studies. Mitochondrial transport was analyzed using Kymograph software from ImagJ. Detailed data from mitochondrial speed, mobility, direction changes, stop phases and covered distance were collected (the variety of measurements is shown in table 2) and transferred to Excel for further analysis with a script, edited by Daniela Vogt Weisenhorn. Data are expressed as means $\pm$ S.E.M.. Statistical analysis of all data points were carried out using Students t-Test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

speed	average speed of mitochondria (overall speed) average anterograde speed of mitochondria in motion average retrograde speed of mitochondria in motion
mobility	average percentage of stationary mitochondria phase during motion average percentage of anterograde mitochondria phase during motion average percentage of retrograde mitochondria phase during motion percentage of immobile fractions percentage of mobile fractions
direction changes	percentage of mitochondria with direction changes independent of holding phases average number of direction changes during motion average number of direction changes during motion per 10 frames number of speed changes during mitochondrial motion number of speed changes during anterograde movement number of speed changes during retrograde movement
stop phases	percentage of mitochondria with holding stops average duration of stop time during mitochondrial motion number of mitochondrial stop during motion number of stops after anterograde motion number of stops after retrograde motion
covered distance	traveled distance from origin point (net movement) whole travel distance (gross movement) traveled distance into anterograde direction traveled distance into retrograde direction

Tab. 2: Detailed list of measurement points of mitochondrial transport.

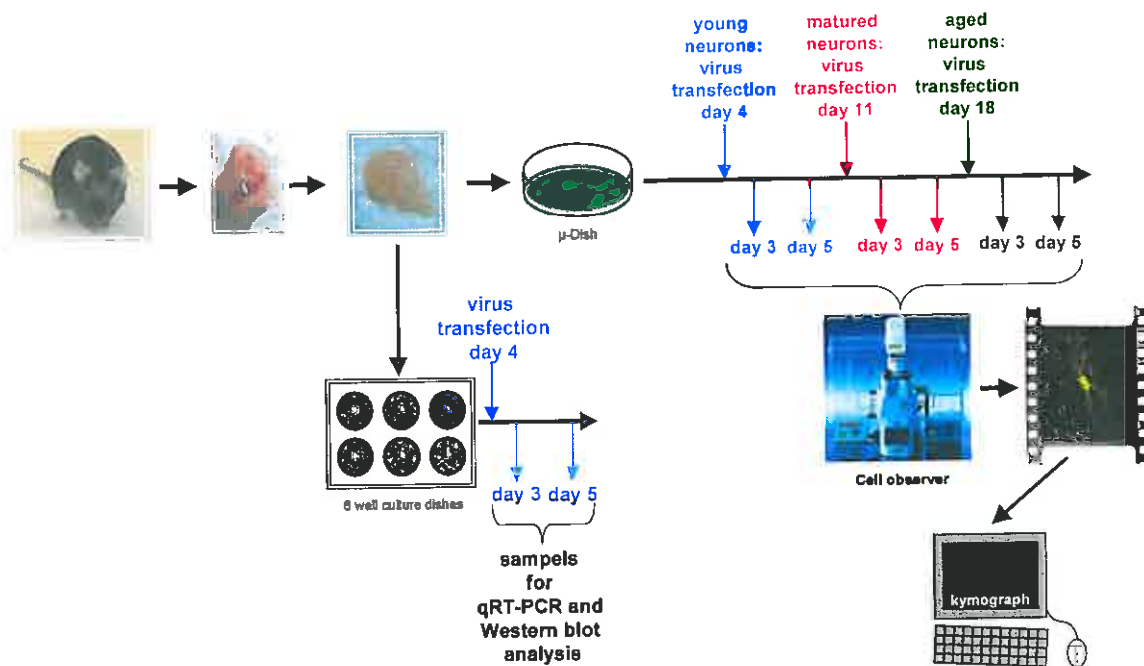


Fig. 12: Experimental setup for mitochondrial trafficking experiments.

Depending on the experiments, the according lentivirus constructs (pLL3.7mito-eYFP, pLL3.7shPINK1mito-eYFP, respectively) were used for transfection of neurons at different developmental stages (day 4, 11 and 18 of growth) and different time points after infection (3 days and 5 days).

3.2.6.2 Mitochondrial trafficking of acute PINK1 knockdown neurons at different developmental stages during stressor application

Primary Cortex culture of E15.5 old C57/Bl6-J mice from at least four different mice of two different litters were prepared and treated as previously described. Dispersed cells were transferred to culture medium with one group containing an additional 1% Calcium overload and the second group containing an additional 10% Calcium overload as stressors with respect to manufacturer's instructions of $1.8 \text{ mM CaCl}_2 \cdot 2\text{H}_2\text{O}$. Plating, cultivation and live cell imaging followed the same protocol as described before. After 4 days of growth and half medium change, neurons from each stressor group were infected with a lentiviral shRNA vector for acute knock-down of *Pink1* (pLL3.7shPINK1mito-eYFP) and a control lentivirus (pLL3.7mito-eYFP). The experiments of unstressed neurons were used as a control. Thus, three and five days after infection five neurons per stressor group, genotype and litter were analyzed in live cell experiments. Mitochondrial transport was analyzed using kymograph software from ImagJ and Excel scripting as described before. Data are expressed as means $\pm$ S.E.M.. Statistical analysis were carried out using Students t-Test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

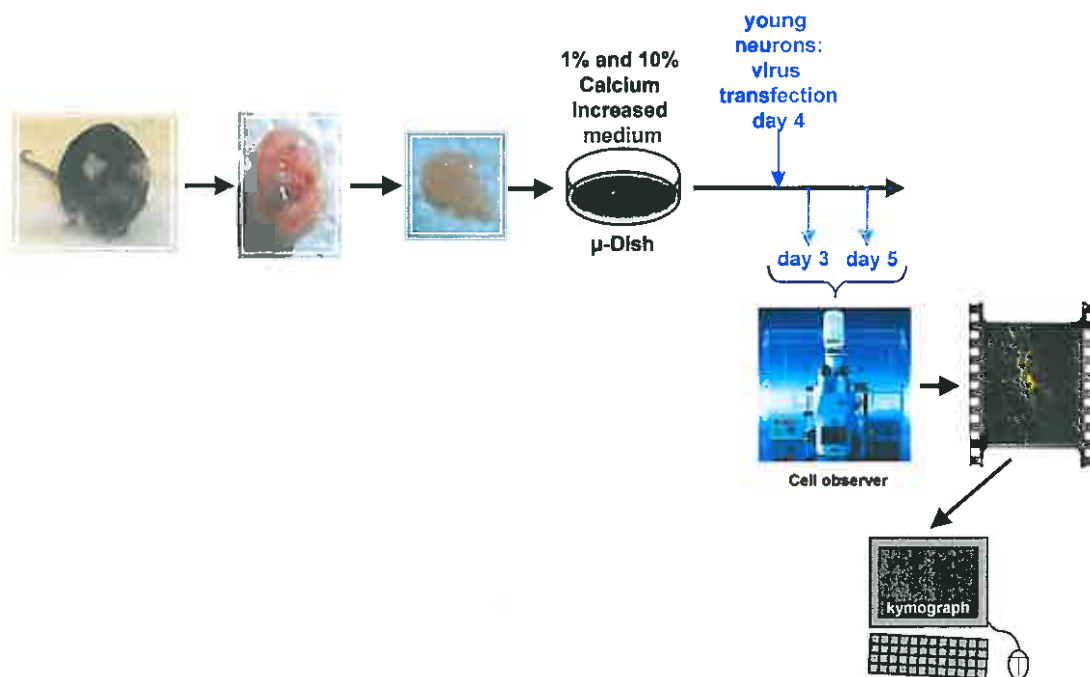


Fig. 13: Experimental setup for stressor application.

A lentiviral shRNA vector for acute knock-down of *Pink1* (pLL3.7sh*PINK1*mito-eYFP) and a control lentivirus (pLL3.7mito-eYFP) were added to the primary cell culture at day four of growing and cells were incubated for additional three and five days before recording mitochondrial transport via live cell imaging.

3.2.6.3 Mitochondrial trafficking of endogenous PINK1-deficient primary neurons at different developmental stages

Primary cortical neuron culture of *PINK1*^{-/-} and *PINK1*^{+/+} mice at an age of E15.5 from three different litters were cultivated as previously described (3.2.5.2). Young, matured and aged cortex neurons were infected with a lentivirus for detection of mitochondria (pLL3.7mito-eYFP) at day four, eleven and eighteen of growth to analyze mitochondrial transport deficits at different developmental stages. Five living neurons of each genotype group and litter were recorded by live cell imaging with epifluorescent microscope (Cell Observer) and analyzed with kymograph software from ImagJ and our analyzing Excel program (from Daniela Vogt Weisenhorn). The main data settings of mitochondrial speed, mobility, direction changes, stop phases, covered distance and number of measured mitochondria with corresponding subdata (see mito trafficking of BL6 neurons) were measured and are expressed as means $\pm$ S.E.M.. Statistical analysis were carried out using Students t-Test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.2.6.4 Analyzing organelle trafficking via Kymograph from ImajJ

Beneath the installation of Kymograph from ImajJ some important additional plug-ins and macros have to be added: Analyze_Reader, Analyze_Writer, HandleExtraFileTypes, Image_Inverter, ImageJ_Updater, Line_Analyzer, MultipleKymograph, MultipleOverlay, reslice_projection_method, StackDifference, Straighten Curved Objects (Straighten), Time_Stamper, tsp0507060, WalkingAverage, ZVI_Reader). These plugins and macros have to be compiled before first use. All macros excluding "tsp0507060" run automatically after starting Kymograph. For "tsp0507060" one has to open the macro in the plugin folder and open the subfolder 'install' to choose "tsp0507060" for installation. Then one can use this macro until closing the program.

The function "walking average" (walkAv) in the plugin folder is used to trace the shape of each individual process in combination with the "segmented button" as "segmented line" (right mouse button). Finally, one has to open the "plug-ins" folder again to activate "kymograph". The kymograph of the previously traced process will be created. The kymograph window tells something about the shape of each single line (which belongs to individual mitochondria). For each line the function "read velocities from tps" from "plug-ins" folder has to be used to create a table including acute time, overall time, actual traveled distance and overall distance as well as the actual frame numbers of the film. "Left to right direction" within the kymograph window means anterograde direction whereas "right to left direction" equals retrograde direction of mitochondria analyzed while a vertical line means no movement at all. This table can be transferred to Excel for statistical analysis. Figure 14 shows typical kymograph pictures.

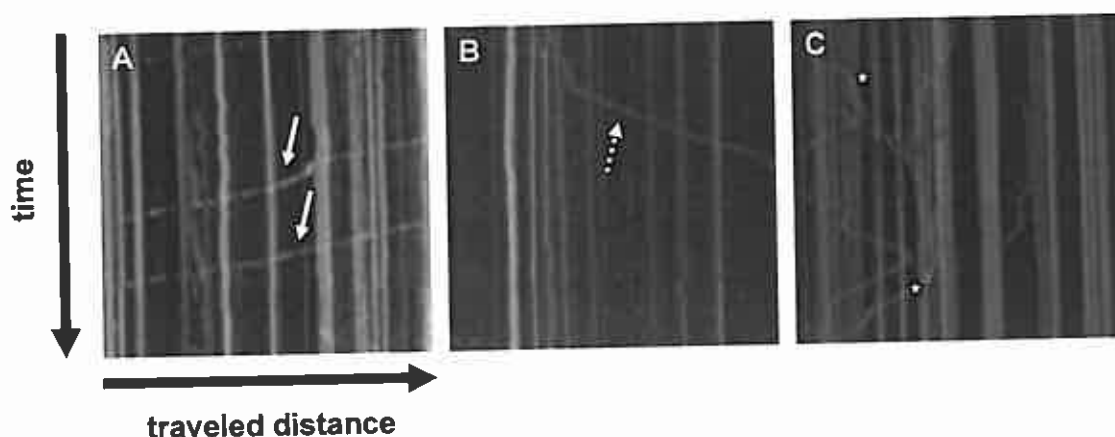


Fig. 14: Three examples of kymograph. Each line or curve represents single mitochondria. The x-axis shows the traveled distance, while the y-axis represents the observation time. (A) The arrowheads on the white arrows show two mitochondria which are traveling in retrograde direction, whereas most of the other mitochondria in this kymograph image are immobile (visible as bright vertical line). (B) The dashed arrow represents mitochondria during anterograde movement. The two asterisk in (C) represent mitochondria which make direction changes during the observation period and do not move uniformly in one direction.

3.2.7 organelle trafficking

3.2.7.1 Endosome-RFP labelling with Organelle Lights™ Intracellular Targeted Fluorescent Proteins

Neurons from Cortex of C57/Bl6-J E15.5 embryos were prepared and cultivated as described before (see mitochondrial trafficking, chapter 3.2.5.2). After an initial growing phase, neurons were transfected with Organelle Lights from Molecular Probes (Organelle Lights™ Intracellular Targeted Fluorescent Proteins, BacMam 1.0) to detect RFP-labelled endosomes (protocol see manufacture's instructions). Furthermore, several additional time and temperature settings were carried out following the advice of the manufacturer:

15 min Organelle Lights + 1:30 h Enhancer; 15 min Organelle Lights + 1:45 h Enhancer; 15 min Organelle Lights at 25°C + 2 h Enhancer; 15 min Organelle Lights at 37°C + 2 h Enhancer; 20 min Organelle Lights at 37°C + 2 h Enhancer; 30 min Organelle Lights + 1:30 h Enhancer; 30 min Organelle Lights at 25°C + 2 h Enhancer and 30 min Organelle Lights at 37°C + 2 h Enhancer.

To detect RFP-labelled endosomes epifluorescent microscope was used.

3.2.7.2 LC3 plasmid transfection

After an initial growing phase, cortical neurons of C57/Bl6-J E15.5 embryos were transfected with microtubule-associated protein 1 light chain 3 (LC3) plasmids (Fig. 15). This is an autophagic marker taking part in the formation of autophagosomal membranes. We used NeuroFect™ from Genlantis according to the manufacture's instruction in a ratio of 1:6 (DNA to NeuroFect). In the following two days, neurons positively stained with LC3 were checked with the aim to investigate late endosome trafficking in an acute knockdown of PINK1. The plasmid was a granted one by Prof. Dr. Köster (TU Braunschweig, formally member of the IDG).

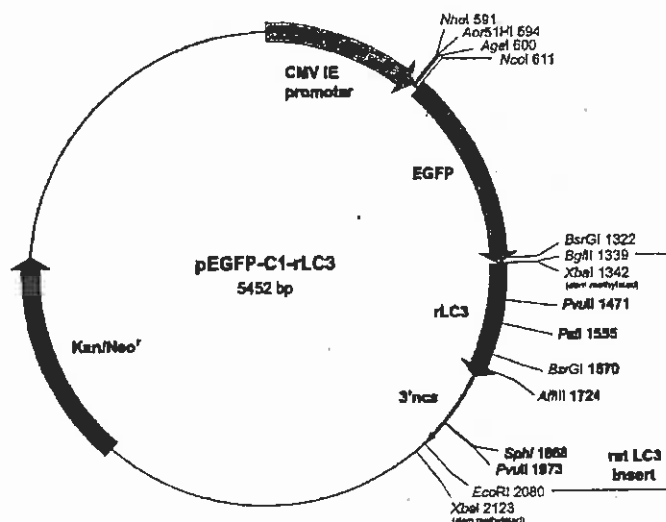


Fig. 15: pEGFP-C1-rLC3 plasmid construct. This vector was a kind gift of Prof. Dr. Köster, TU Braunschweig.

3.2.7.3 LC3 plasmid transfection in highly stressed neurons

LC3 plasmid transfected neurons (see previous section) were treated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP; from Sigma) inhibiting complex I in mitochondria, which leads to high neuronal stress followed by an increase of autophagy. Therefore, LC3 transfected neurons were treated with different concentrations of CCCP (5 μ l, 10 μ l, 25 μ l, 50 μ l, 100 μ l) to check for an increase of the labelling rate of LC3.

3.2.7.4 Rab5 plasmid transfection

The G protein Rab5a belongs to the GTPase superfamily and is required for both fusion of plasma membranes and building of early endosomes. The plasmid used in this work was a kind gift of Prof. Dr. Zerial, MPI Dresden. The cloning procedure of the plasmid was as follows: Competent bacteria of *E. coli* strain DH5 α together with 1 μ l of the hRab5a plasmid were incubated for 30 minutes on ice and thereafter preheated at 42°C for 30 seconds. The DH5 α -hRab5a mixture was filled-up to 1 ml LB-medium without antibiotics and gently shaken at 37°C for 1 hour. 10 μ l, 50 μ l and 100 μ l of the transformed *E. coli* cells were cultivated on LB-agar plates containing 30 μ g/ml kanamycin and stored overnight at 37°C on a shaker. In a next step, clones were picked from the plates, transferred to 5 ml LB-medium containing kanamycin and incubated at 37°C for several hours on a shaker. 250 μ l of this preculture were inoculated in 200 ml LB-medium including antibiotics, and then centrifuged the next day at 4.000 rpm for 15 – 30 minutes. Supernatant was discarded. The pellets were frozen for plasmid Maxi preparation. Qiagen Plasmid Maxi Kit and the containing solutions were used for the following steps following manufacturer's instructions. The bacterial pellet was resuspended in 10 ml Buffer P1 and RNase A. 10 ml buffer P2 was added, gently mixed by inverting 4-6 times and incubated at RT for 5 minutes. Afterwards, 10 ml chilled Buffer P3 was added and mixed immediately but gently by inverting several times, and incubated on ice for 15 – 20 minutes. A filter was moistened and placed on a QIAGEN-tip. The resuspension was loaded onto the QIAGEN-tip and washed 2 times with 30 ml Buffer QC. Supernatant was discarded and DNA was eluted with 15 ml Buffer QF. Eluted DNA was transferred into a Falcon tube and precipitated by 10.5 ml isopropanol at RT. The resulting precipitate was mixed and centrifuged immediately at 5000 x g at 4°C for 30 minutes. The supernatant was decanted carefully and DNA pellet was washed with 1.5 ml of 70°C EtOH and centrifuged again at 15.000 x g for 10 minutes. The DNA pellet was then air-dried for 10 minutes and redissolved in 100 μ l sterile water.

Neurons were transfected with Rab5a plasmid as described above (see 3.2.7.2).

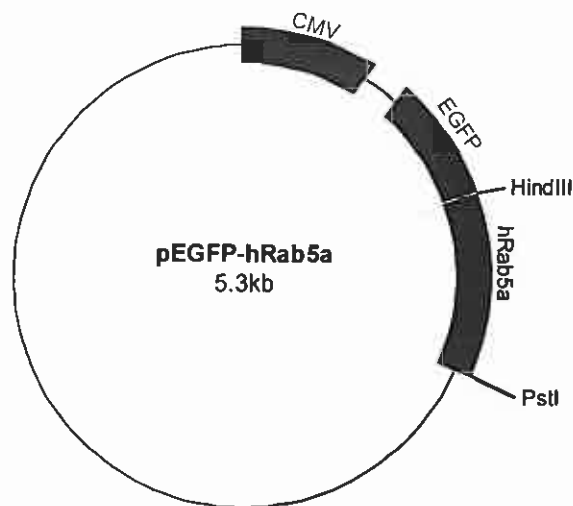


Fig. 16: pEGFP-hRab5a plasmid construct.
This vector was a kind gift of Prof. Dr. Zerial, MPI Dresden.

3.2.8 Gene expression of mitochondrial genes involved in mitofission and –fusion processes as well as mitochondrial trafficking

In order to check for an impaired gene regulation of mitochondrial transport mechanisms in coincidence with mitofission and –fusion processes and in addition to quantify our shRNA knockdown efficiency in primary neuronal culture and the knockdown efficiency in our *PINK1*^{-/-} mice, quantitative Real Time PCR was performed. Primary neuronal cultures from Cortex of E15.5 old C57/Bl6-J, *PINK1*^{-/-} and *PINK1*^{+/+} mice, respectively were prepared and cultivated as described (see 3.2.5.2) but this time at a density of 5.33×10^5 cells per 2 ml culture medium (corresponding to 2.66×10^5 cells per 1ml Medium) in Poly-D-Lysin coated (1mg/ml in H₂O) 6 well plates. After cultivation time of four days neurons were transfected with a lentivirus (pLL3.7sh*PINK1*mito-eYFP, pLL3.7mito-eYFP, respectively). At day 1, 3 and 5 after virus transduction neurons were washed with PBS and directly lysed with 300 µl of a freshly prepared β-Mercapto (10µl) and RTL-buffer (1ml) from RNAeasy Kit (Quiagen) according to manufacturer's instructions. Three wells were pooled and stored immediately at -80°C until further use.

Total cellular RNA was then isolated via RNAeasy kit from Quiagen according to manufacturer's instructions and RNA was checked with Bioanalyzer to control the quality by RIN factor. cDNA was synthesized from 400ng RNA using Vilo-Kit (Invitrogen) according to manufacturer's instructions. qPCR was performed with the TaqMan Gene expression Assay (Quiagen) and triplicates of each primer set for each RNA sample, as well as a triplicate of a water control. A list of the used primers is listed below (Tab. 3). The samples ran for 10 minutes at 95°C, followed by a 40-fold repetition step for 15 seconds at 95° and one minute at 60°C in a 384 well-plate, covered with seal foil (Applied Biosystem).

Quantification was carried out with ABI Prism ® 7900 HT Sequence Detection System and data was acquired using SDS2.3 software (Applied Biosystem). The 5' nuclease activity of the DNA polymerase system was used to cleave a TaqMan® probe during PCR. Cleavage of the Taqman probe during reaction separates the reporter dye at the 5' end and the quencher dye at the 3' end of the probe resulting in an increased fluorescence of the reporter. The relative measurement of transcription expression was calculated by the cycle number at which each PCR reaction reached a significant threshold (Ct). To normalize RNA expression the endogenous genes β -actin and rpl-27 were used as a reference. We had previously identified these two genes as well working for reference by testing overall five different internal controls GAPDH, pgk1, HPRT, rpl27 and β -actin (see table 25, page 121).

Pink1 knockdown efficiency of our virus constructs was tested, also the RNA expression of Miro-1, Miro-2, Milton-1, the Kinesin family member 5A (Kif5a) to detect possible impairments in gene expression of a part in kinesin transport which is normally responsible for anterograde transport. For retrograde transport defects, we tested the gene expression of Dynactin 1 (Dctn1), Dynein heavy chain (Dnhd1) and Dynein cytoplasmic 1 heavy chain 1 (Dync1h1). In addition to the transport genes, we tested the gene regulation of mitofission and -fusion processes, regarding Fission 1 (Fis1), Dynamin-1-like (Drp1), Optic atrophy 1 homolog (Opa1), Mitofusion1 (Mfn-1) and Mitofusion-2 (Mfn-2). To exclude a possible interferon response due to the virus usage in our results, 2'-5' oligoadenylate synthetase 1B (Oas1b) was tested, too. All used primers, alias names, functions and roles in mechanisms are listed in table 3 together with the associated manufacturing company. Relative expression was calculated for each gene and transduction day using the $\Delta\Delta CT$ value. Experiments were repeated at least three times and data are expressed as means $\pm$ S.E.M.. Statistical analysis was carried out using Students t-Test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Involved mechanism	Primer-name + Assay ID	Alias name	Function	Company
Kinesin transport  usually anterograde transport	Miro-1 Mm01304158_m1	Miro1 / Rhot1 2210403N23RIK AA415293 AF244542 AJ834919 Arht1 C430239G08RIK FLJ11040 Miro1 RP23-345E8.2	Ras homolog gene family, member T1	Applied Biosystems; TaqMan® Gene Expression Assay
	Miro-2 Mm00524478_m1	Miro2 / Rhot2 Arht2 Miro2 MGC31218	Ras homolog gene family, member T2 Mus musculus MIRO2 mRNA, complete cds; nuclear gene for mitochondrial product.	Applied Biosystems; TaqMan® Gene Expression Assay
	Milton-1 Mm00549615_m1	Trak2 / GRIF-1 4733401011RIK AJ480838 AW04627 mKIAA0549 Ala2cr3 CALS-C OIP98	Trafficking protein, kinesin binding 2 Grif1 milton??	Applied Biosystems; TaqMan® Gene Expression Assay
	Kif5a Mm00515265_m1	Kinesin family member 5A D108wg0738e KJAA4088 Khc Kif5 Kns mKJAA4088	Members of this kinesin family are part of a multi-subunit complex that functions as a microtubule motor in intracellular organelle transport.	Applied Biosystems; TaqMan® Gene Expression Assay
Cytoplasmic dynein  usually retrograde transport	p150 (glued) Mm00514542_m1 Mm01184845_m1	Dctn1 AL022633, Glued, p150	Dynactin1	Applied Biosystems; TaqMan® Gene Expression Assay
	Dynein heavy chain Mm01162592_m1	Dnhd1 8030491N06RIK	Dynein heavy chain domain1	Applied Biosystems; TaqMan® Gene Expression Assay
	Dynein Cytoplasmic 1 heavy chain 1 Mm00466548_m1	Dync1h1 9930018I23RIK AJ894280 DHC1 DHC1a DNCL Dnchc1 Dnec1 Dnecl Loa MAP1C P22 Swl mKJAA0325 DKFZp686P2245	The cytoplasmic dyneins function in intracellular motility, including retrograde axonal transport, protein sorting, organelle movement, and spindle dynamics.	Applied Biosystems; TaqMan® Gene Expression Assay
Interferon response of virus	Oas1b	Flv, L1, Mmu-L1, Oas1, Oias-2, Oias2, Whv	2'-5' oligoadenylate synthetase 1B	Applied Biosystems; TaqMan® Gene Expression Assay Mm00449297_m1

Involved mechanism	Primer-name + Assay ID	Alias name	function	company
Mitofission	Fls 1 Mm00481580_m1	Fls1 2010003O14Rik, Ttc11	Fission 1(mito outer membrane homolog)	Applied Biosystems; TaqMan®Gene Expression Assay
	Drp1 Mm01342903_m1	Dnm1l 6330417M19Rik, A1450666 , MGC90593	Dynamin-1-like / Drp1	Applied Biosystems; TaqMan®Gene Expression Assay
Mitofusion	Opa-1 Mm00453879_m1	Opa1 1200011N24Rik, AJ225888, AI847218, mKIAA0567	Optic atrophy 1 homolog (human)	Applied Biosystems; TaqMan®Gene Expression Assay
	Mfn-1 Mm00612599_m1	Mfn1 2310002F04Rik, 6330416C07Rik, D3Erd265e, HR2, KIAA4032, mKIAA4032	Mitofusin 1	Applied Biosystems; TaqMan®Gene Expression Assay
	Mfn-2 Mm00500120_m1	Mfn2 D630023P19Rik, Fzo, RP23-406N5.4	Mitofusin2	Applied Biosystems; TaqMan®Gene Expression Assay
Pink1	Pink1 Mm00550827_m1	PARK6	PTEN induced putative kinase 1	Applied Biosystems; TaqMan®Gene Expression Assay
Reference genes	β-actin ACTB(20x): 4352933E	Actb	Actin, cytoplasmic 1	Applied Biosystems; TaqMan®Gene Expression Assay
	rpl27 Mm01245874_g1	MGC103134, RP23-328K2.2	ribosomal protein L27	Applied Biosystems; TaqMan®Gene Expression Assay
	HPRT Mm00446968_m1	C81579, HPGRT, Hprt1, MGC103149, RP23-173F3.3	hypoxanthine guanine phosphoribosyl transferase	Applied Biosystems; TaqMan®Gene Expression Assay
	pgk1 Mm00435617_m1	MGC118097, Pgk-1	phosphoglycerate kinase 1	Applied Biosystems; TaqMan®Gene Expression Assay
	GAPDH Mm99999915_g1	Gapd, MGC102544, MGC102546, MGC103190, MGC103191, MGC105239	glyceraldehyde-3-phosphate dehydrogenase	Applied Biosystems; TaqMan®Gene Expression Assay

Tab. 3: Used primers for RT-PCR with alias names, functions and involved mechanism and ordering company.

3.2.9 protein expression study of mitochondrial proteins involved in mitofission and –fusion processes as well as mitochondrial trafficking

To measure the protein concentration of mitochondrial transport mechanisms as well as mitofission and –fusion processes western blot analyses were performed. Primary neuronal cultures from Cortex of E15.5 old C57/Bl6-J, PINK1^{-/-} and PINK1^{+/+} mice, respectively were prepared and cultivated as described before (chapter 3.2.5.2) at a density of 5×10^5 cells per 2 ml culture medium in Poly-D-Lysin (1mg/ml in H₂O) coated 6 well plates. After a cultivation time of four days neurons from C57/Bl6-J mice were transfected with a lentivirus (pLL3.7shPINK1mito-eYFP, pLL3.7mito-eYFP, respectively), like it was done for the gene expression study and in parallel without any treatment for the PINK1^{-/-} and PINK1^{+/+} neurons. Medium was removed and neurons were washed with prewarmed PBS at day 1, 3 and 5 after virus transduction in C57/Bl6-J neurons and at corresponding time points for PINK1^{-/-} and PINK1^{+/+} neurons. The entire medium of each well had to be totally removed and 150 μ l of trypsin at RT was added for 1-2 minutes with pipetting up and down for a better detachment of the cells. The process of trypsination was stopped with 500 μ l ice-cold PBS to prevent degradation. Neurons were transferred into a 1.5 μ l epi and additional 500 μ l ice-cold PBS was added. Samples were centrifuged at 5.000 rpm at RT, supernatant was removed and neurons were resuspended in 200 μ l ice-cold PBS. The resuspension of three identical samples were collected into one epi and centrifuged again at 3.000 – 5.000 rpm. Supernatant was removed completely and the pellet was frozen immediately at -80°C until further use.

A complete proteinase inhibitor tablet (Roche) and a tablet of PhosStop (Roche) was added to RIPA buffer (containing of 150mM NaCl, 50mM Tris (pH7.5), 2mM EDTA (pH8.0), 1 % Triton X-100, 0.25 % Sodium-Deoxy-Cholate and 0.1% SDS) and dissolved. 100 μ l of this RIPA buffer mixture was added to the frozen cells. All further steps were done on ice to avoid degradation. Furthermore, cells were homogenized two times for 5 seconds by electro-homogenisation (Ika, Ultra Turrax T25 basic). After each homogenisation step cells were washed and dried. To recover cells they were left on ice for 10 minutes before they were centrifuged at 10000g for 10 minutes at 4°C . Supernatant was transferred to fresh cooled reaction tubes, sonificated 5 seconds (50% in ice water) and centrifuged again. The supernatant was removed and stored at -80°C until the test for protein concentration was done. Therefore BSA standard dilutions (Bradford Assay from Sigma) with 0, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml were prepared from stock solution and 5 μ l of each of these dilutions were added to 200 μ l BSA. Samples were incubated at 37°C in a water bath for 30 minutes and cooled down to RT to measure 562 nm absorption in a photometer comparing to a BSA standard curve. For correlation of the protein concentration measurement, the slope of linear part of the standard curve was used.

Materials & Methods

10 - 20 μ l of total protein samples, depending on the specificity of the antibody used, were mixed with 5 μ l Laemmli loading buffer (1x; Invitrogen) and denatured at 95 – 100°C for 5 minutes. Samples were chilled on ice, centrifuged at high speed for 1 minute at RT and loaded on Bis-Tris gels (Criterion XT, Biorad) in addition to 5 μ l of a molecular weight marker (SeeBlue® Plus2 or Novex® Sharp prestained protein marker). Electrophoresis was performed at 200V for 1 to 1½ hours, depending on expected protein size, and each gel was afterwards blotted on a PVDF membrane, which had been activated by soaking it with 100% methanol for 1 minute. For the transfer to PVDF membrane, blotting was performed at 30mV for 1.5 – 3 hours at 4°C dependent on the protein of interest. Thereafter, the membrane was washed immediately in TBS for 5 minutes at RT and blocked for 1 hour with blocking solution containing TBS, 0.1 % Tween-20 and 5 % w/v non-fat dry milk powder and then washed again three times with TBS-T at RT. Subsequently, the membrane was incubated with the primary antibody (antibodies used, see in table 4) in blocking solution and a gentle agitation at 4°C overnight. The next day the membrane was washed three times with TBS-T, each for 5 minutes, and incubated with horseradish-peroxidase-conjugated secondary antibody in TBS-T for 1 hour for another gentle agitation. Afterwards each membrane was washed three times with TBS-T for 5 minutes, and incubated for 1 minute with ECL detection reagent (Amersham) to initiate detection. Within a film cassette the membrane was exposed to a chemiluminescent film for 30 seconds up to several minutes, depending on signal intensity. The exposed film was developed with a developing machine and quantified by ImageJ software.

Involved mechanism	antibody + Assay ID	company	conc. 1 st antibody	conc. 2 nd antibody	size
Kinesin transport  usually anterograde transport	RhoT1 (Miro 1, Trak1, OIP106)	112 Genway rabbit	1:500	1:5000	68 kDa
	RhoT2 Miro2	156 Santa Cruz(sc165360) goat	1:500	1:5000	
	Milton Trak2 =Grif1=OIP98				
Cytoplasmic dynein  usually retrograde transport	Dctn 1 (Dynactin)	111 Abcam(75034) rabbit	1:300	1:10000	142 kDa
	Dnhd1	115 Abnova rabbit	1:500	1:5000	
Mitofission	Fis1	113 Abnova Rabbit	1:200	1:10000	143 kDa
	Dnm1L (=Drp 1)	149 Santa Cruz Sc-48865 110 Abcam ab(54038) Rabbit	1:300	1:10000	81 kDa

Mitofusion	Opa 1	109 Abcam Rabbit	1:500	1:10000	92.86 kDa 111kDa predicted
	Mfn1	Santa Cruz 154 Millipore ABC41			
	Mfn2	114 Abnova Rabbit 155 Millipore rabbit	1:200	1:10000	~80 kDa
Pink1		117 Santa Cruz (sc-33796) Rabbit poly 118 Acris	1:500	1:5000	66 kDa

Tab. 4: Used antibodies for protein expression analysis.

3.2.10 GMC-screen

A cohort of our PINK1 knockout mice (mutant male $n = 19$, wild type male $n = 19$, mutant female $n = 20$, wild type female $n = 20$) were sent to the German Mouse Clinic (GMC) for a broad standardised phenotypic screen at a minimum age of eight weeks. This primary screen contains basic screening procedures in fields of dysmorphology, behaviour, neurology, nociception, eye development, energy metabolism, clinical chemistry, immunology, cardiovascular system, allergies, steroid metabolism, lung function, pathology and molecular phenotyping. After a one week adaption phase in the GMC the different screens were done in the ongoing time frame. For a detailed timetable see Fig. 17.



Fig. 17: Overview of GMC screen experiments with according time units.

[<http://www.mouseclinic.de/index.php?id=11119>]

A short and adapted overview of the standard screens done by the GMC is given by the next view pages. For detailed information please look at the homepage of the GMC at <http://www.mouseclinic.de/index.php?id=11096>.

Dysmorphology screen (week 9 and 14):

In week 9 and 14 our mice were tested for anatomical, bone and cartilage abnormalities. Therefore, the GMC followed a 54 parameter containing protocol (like the clickbox-test) to detect and evaluate malformations and malfunctions of different organ systems. In addition, X-ray analysis (with Faxitron MX-20) and bone densitometry (with pDEXA Sabre X-ray Bone Densitometer) were done to detect deficits in skeleton, bone mineralization and homeostasis.

Behavior screen (week 9 and 11):

At the age of 9 weeks mice pass the open field test (OF) to examine locomotion, exploration and anxiety-related behaviour. Whereas at the age of 11 weeks they absolve sensorimotor gating by prepulse inhibition of the acoustic startle reflex.

Neurology (week 9 and 10):

With a modified SHIRPA-Protocol (Smithkline Beecham, MRC Harwell, Imperial College, Royal London hospital, phenotype assessment), a semi-quantitative screening method, the GMC assessed basic neurological and motor functions. In addition, grip strength tests were used to measure muscle strength in the forelimbs and a rotarod test to exhibit motor coordination and balance deficits.

Nociception (week 12):

The hotplate test was done to measure changes in nociceptive thresholds usually occurring after inflammation. Therefore, first-respond time was measured and after a while the test was repeated and the second-respond time was measured.

Eye development (week 13 and 16):

Via Slit Lamp Biomicroscopy the anterior part of the eye, mainly cornea and lens, was measured, and followed by a Funduscopy / Ophthalmoscopy where the posterior part of the eye, mainly retina and optic nerve, was measured. At the end of the eye screen, Laser Interference Biometry (LIB) was done to determine eye size parameters like axial length.

Energy metabolism (week 12 and 13):

To measure body composition, fat and lean content in our PINK1 knockout mice a non-invasive NMR Scan was used. For a closer look on energy metabolism dysfunctions, mice

underwent indirect Calorimetry, a method to determine energy metabolism from gas exchange. With this method different parameters like oxygen consumption, carbon dioxide production, respiratory exchange ratio, heat production, *ad libitum* food consumption are measured. In addition, locomotor activity, body weight and rectal body temperature after the measurements were taken.

Clinical chemistry (week 13 - 15):

For detection of haematological changes, organ system defects, metabolic pathway changes or changes in electrolyte homeostasis, a set of routine clinical chemistry procedures like the Intraperitoneal Glucose Tolerance Test (IpGTT), AU400 and ABC Animal Blood Counter were taken.

In the IpGTT, which is a first hint of subclinical diabetes and impaired glucose tolerance, our mice were removed from food for 16-18 hours before they got a basal glucose level (Glucose i.p. 2g/kg body weight). Basal fasted glucose levels were taken at start point of glucose intake, 15, 30, 60 and 120 minutes later and expressed as "Area under the curve" (AUC) T0-T30, T30-T120.

To determine parameters of clinical chemistry and haematological parameters blood samples were taken from the GMC via retro-orbital puncture under isoflurane anaesthesia. One part of the blood samples were purified to blood plasma to investigate the chemical screen AUC400, where Cholesterol, HDL-cholesterol, triglycerides, non-esterified fatty acids (NEFA), glycerol, glucose and non-HDL cholesterol can be measured under fasted conditions. Also changes in sodium, potassium, chloride, calcium, inorganic phosphorus, iron, total protein, albumin, creatinine, urea, cholesterol, triglycerides, alpha-amylase, aspartat-aminotransferase (ASAT/GOT), alanine-aminotransferase (ALAT/GPT), alkaline phosphatase (AP) and lactate-dehydrogenase (LDH) can be analyzed. The other part of the blood samples were transferred in EDTA-coated tubes and used for animal blood counter (ABC) to measure basic haematological parameters like red blood cell count (RBC), white blood cell count (WBC), platelet count (PLT), haemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), mean platelet volume (MPV).

Immunology (week 14):

Blood cells resting from the blood purified from Clinical Chemistry screen underwent a 10 color polychromatic flow cytometry, where live and dead cells could be discriminated as well as identification of T cells, B cells, granulocytes, NK cells and subsets within a CD45+ gate.

Blood plasma was used for Bioplex and ELISA analyses to detect levels of IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, IgA, IgM (Bioplex), anti-DNA antibodies and rheumatoid factor (indirect ELISA).

Allergy (week 14):

Again a part of the blood plasma taken in Clinical Chemistry screen was used to search for IgE levels (standard allergy parameters) in our PINK1 mice by ELISA technique.

Steroid metabolism (week 14):

Corticosterone, testosterone and androstenedione, the three key steroids, were quantified from blood plasma by high-throughput LC-MS/MS technology (published in Nature Protocols) using HPLC (Shimadzu Prominence) and Triple Quadrupole Mass Spectrometer (4000 Qtrap, AB Sciex).

Cardiovascular (week 15):

Echocardiography (ECG) with Vevo® 2100 High-Resolution Imaging System were used for a non-invasive analysis of left ventricular internal diameter (diastole and systole; LVIDd and LVIDs), left ventricular posterior wall (diastole and systole; LVPWd and LVPWs), interventricular septum (diastole and systole; IVSd and IVSs), heart rate and respiratory rate.

Lung function (week 17):

Lung function screen evaluates changes in spontaneous breathing patterns. Therefore, a Fine Pointe RC System (Buxco Electronics) was used to investigate lung resistance (R), dynamic lung compliance (C_{dyn}) and tidal volume (TV). Pulmonary Function Testing System (Buxco Electronics) was used to detect functional residual capacity maneuver (FRC), fast flow volume maneuver (FV) like forced expiratory volume (FEV₁₀₀), forced vital capacity (FVC), peak expiratory flow (PEF), expiratory reverse volume (ERV) and quasistatic pressure volume (PV) like inspiratory capacity (IC), chord compliance (C_{chord}), vital capacity (VC), indirect residual volume (RV), total lung capacity (TLC).

Pathology (week 17):

One part of our PINK1 mice (12 mutants: 6 females and 6 males; 6 wild types: 3 females and 3 males) were killed with CO₂ and passed a complete macroscopic examination, including body weight and internal organ measurements. The following organs were analyzed: skin, musculoskeletal system, eyes, brain, cerebellum, heart/arteries, trachea, lung, teeth, salivary gland, oesophagus, stomach, small intestine, large intestine, liver, mamma, pancreas, cervical lymph nodes, thymus, spleen, thyroid, parathyroid, adrenal gland, kidneys, urinary bladder, testes, epididymis, funiculus spermaticus, ovaries, uterus, vagina, body weight.

Afterwards, organs were fixed in 4% neutrally buffered formalin, embedded in paraffin, sliced and stained for histological examination using standard haematoxylin-eosin stain (H&E).

Molecular phenotyping (samples from week 17):

By the time of completion my doctoral thesis, this analysis was not performed. However, corresponding samples exist and could be measured by Illumina Bead Array Technology for a whole-genome expression profiling.

3.2.11 Gait analysis of PINK1 mice

A detailed gait study of our PINK1^{-/-} and PINK1^{+/+} mice was performed in cooperation with Lisa Glasl from the group of Dr. Sabine Hölter-Koch (Behavioural Neuroscience, IDG, Helmholtz Centre Munich) using an automated gait analysis system CatWalk® (Noldus). The resulting data were previously published in Glasl et al. (2012) and summarized here in paragraph 2.6.1.4. In addition Lisa Glasl performed a stepping test according to Blume et al. (2009), but we were also interested to detect akinesia and additional gait deficits. Therefore, we were using three PINK1^{+/+} and five PINK1^{-/-} mice at an age of 22 – 28 month. They were pulled backwards by the tail for a fixed distance over the glass plate of the walkway. Adjusting steps of the front paws of three trials per mouse were tracked by the system (Pulnix camera RM-765 and Catwalk® software Version 7.1; Noldus) to get additionally information about spatial and temporal parameters of the adjusting steps. Data are expressed as means ± S.E.M.. Statistical analyse was carried out using Students t-Test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4 Results

4.1 Mitochondrial morphology studies *in vitro* and *in vivo*

The function of PINK1 has been implicated in controlling mitochondrial morphology. Several studies in different animal models and cell lines reveal different results [Deng et al., 2008; Gautier et al., 2008; Poole et al., 2008; Yang et al., 2008; Gispert et al., 2009; Lutz et al., 2009; Park et al., 2009; Sandebring et al., 2009; Weihofen et al., 2009; Winklhofer and Haass, 2010]. Further *in vitro* and *in vivo* analyses were needed to confirm the role of PINK1-deficiency in mammalian cells and to get insight into impairment of mammalian mitochondrial function. Therefore we analyzed mitochondrial morphology in endogenous PINK1^{+/+} mice. Based on the obtained results we had to consider a possible time-dependent and transient compensatory mechanism *in vivo*. Therefore, I investigated mitochondrial morphology at different time points after acute knockdown of PINK1 *in vivo*. If this possible transient compensation is circuit dependent or a general effect we checked *in vitro*. Indeed, also *in vitro* we see a strong compensation over time. All data are expressed as means $\pm$ S.E.M.. Statistical analysis was carried out using ANOVA except for the p-values of tubular mitochondria, which were calculated by Fisher's Exact Test due to the low number. Significances were achieved when $p \leq 0.05$ (*), whereas high significance values include a p-value ≤ 0.01 (**) and highest significance values start from $p \leq 0.001$ (***).

4.1.1 *In vivo* study of mitochondrial morphology

Stereotactic injections into the brain of PINK1^{+/+} and PINK1^{+/+} mice were performed to detect endogenous impact of PINK1. In addition, I performed stereotactic injections into adult C57/BL6-J mice to induce an acute knockdown of PINK1 and to obtain possible time-dependent compensation. Therefore, mitochondrial morphology was investigated at different time points after injection

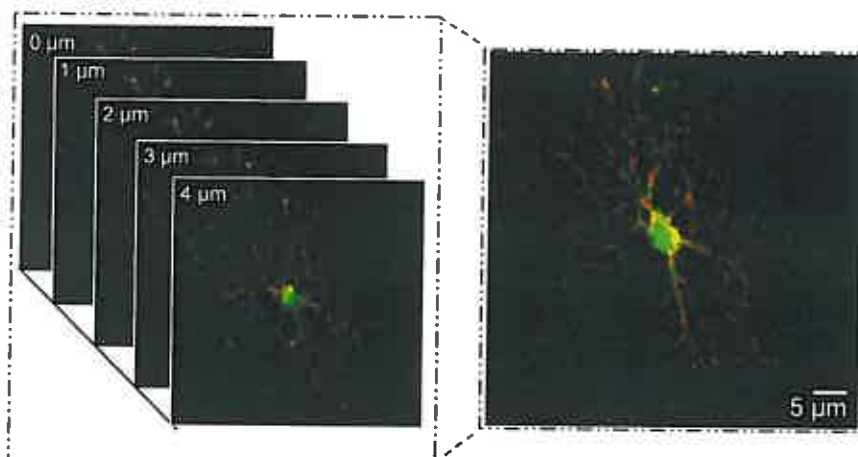


Fig. 18: 3D projection of cortex neuron in a brain section from a PINK1^{+/+} mouse infected with a lentivirus expressing CMVgfpIRESmitodored. The neuron is colored in green, whereas the mitochondria are labeled in red.

4.1.1.1 Mitochondrial morphology in endogenous PINK1-deficient neurons

In vivo analyses of our PINK1^{-/-} and PINK1^{+/+} mice two weeks after lentiviral injection into the striatum (mito-eYFP), revealed that mitochondrial length in entire neurons, as well as divided in soma and processes, was not changed significantly (Tab. 5 and Fig. 19).

mitochondrial length in PINK1 ^{+/+} and PINK1 ^{-/-} mice		
	Pink1 ^{+/+}	Pink1 ^{-/-}
soma	0.81 ± 0.04	0.88 ± 0.03
p-value	0.19	
processes	0.93 ± 0.03	1.00 ± 0.04
p-value	0.10	
whole cell	0.87 ± 0.03	0.94 ± 0.03
p-value	0.11	

Tab. 5: Average mitochondrial length in PINK1^{+/+} and PINK1^{-/-} striatal neurons has not changed. Mitochondria were visualized with a lentivirus expressing mito-eYFP.

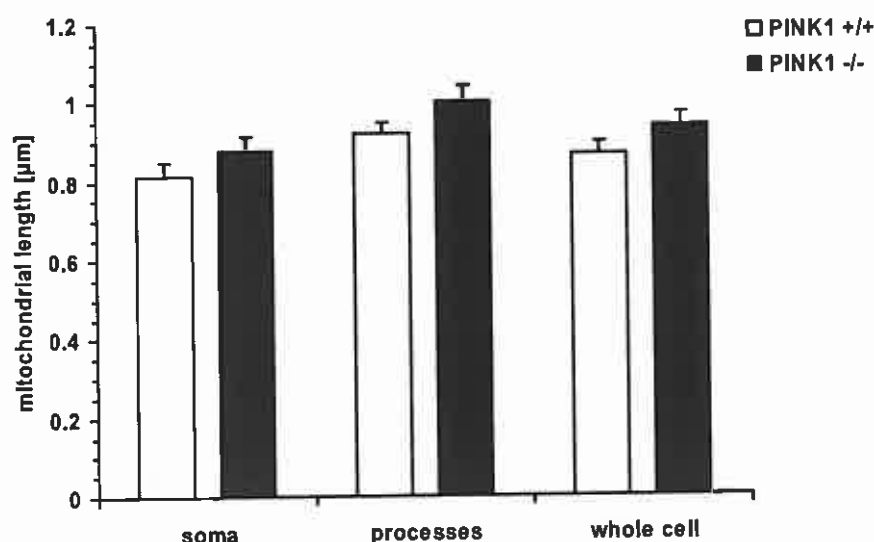


Fig. 19: Quantification of mitochondrial length in PINK1^{+/+} and PINK1^{-/-} striatal neurons shows a slight but not significant increase in PINK1^{-/-} mice.

Considering the percentage distribution of the different groups, our PINK1^{-/-} mice show a significant decrease in mitochondrial fragmentation (PINK1^{+/+}: 22.71% ± 1.96; PINK1^{-/-}: 14.98% ± 1.50; $p = 0.025$). Comparing both genotypes, intermediate and tubular mitochondria did not show any significant change (PINK1^{+/+} intermediate: 77.10% ± 1.94; PINK1^{-/-} intermediate: 84.69% ± 1.47; $p = 0.100$; PINK1^{+/+} tubular: 0.19% ± 0.09; PINK1^{-/-} tubular: 0.33% ± 0.14; $p = 0.075$; see also Tab. 6 and Fig. 20). This *in vivo* findings stand in clear contrast to previously published *in vitro* studies in mammals, where an increase in fragmentation was obtained. Thus, our results can be a first hint that compensatory mechanisms may take place in living animals.

percental mitochondrial length grouping in PINK1 ^{+/+} and PINK1 ^{-/-} mice		
	PINK1 ^{+/+}	PINK1 ^{-/-}
fragmented	22.71 ± 1.96	14.98 ± 1.50
p-value	0.025	
intermediate	77.10 ± 1.94	84.69 ± 1.47
p-value	0.100	
tubular	0.19 ± 0.09	0.33 ± 0.14
p-value	0.075	

Tab. 6: Mitochondrial length grouping of PINK1^{+/+} and PINK1^{-/-} striatal neurons. PINK1^{-/-} mice show a significant decrease in fragmented mitochondria. Red marked p-values indicate statistical significances, while blue ones show tendencies ($p = 0.05 - 0.1$).

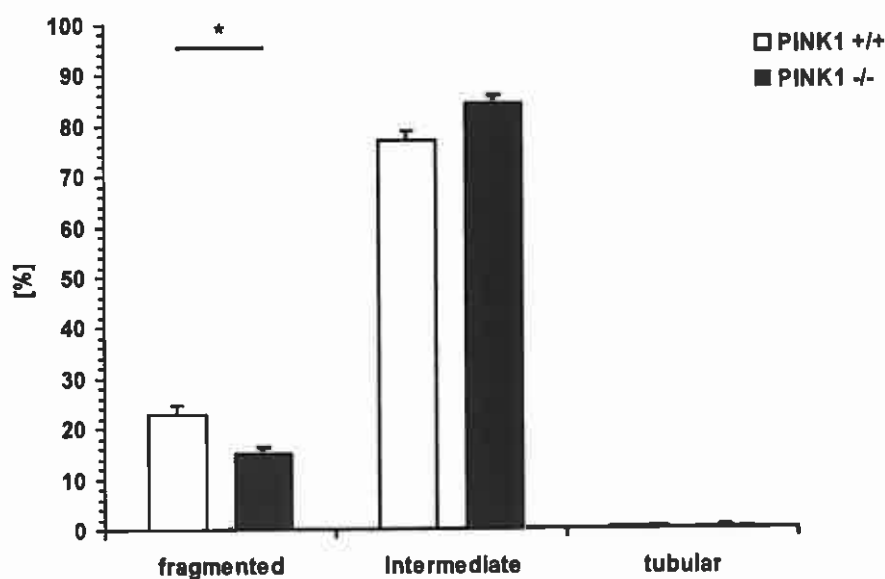


Fig. 20: Morphological quantification of PINK1^{+/+} and PINK1^{-/-} striatal neurons. At day 3 an obvious decrease of fragmented mitochondria in PINK1^{-/-} neurons is taking place.

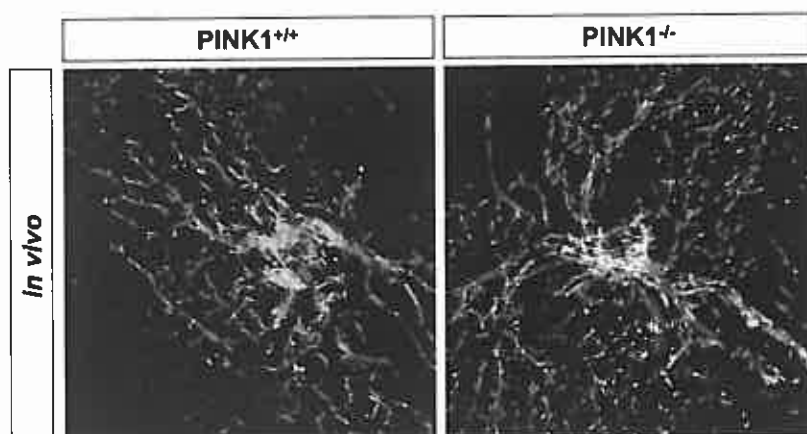


Fig. 21: 3D projection of neurons from PINK1^{+/+} and PINK1^{-/-} mice infected with a lentivirus expressing mito-eYFP.

4.1.1.2 Mitochondrial morphology in acute PINK1 knockdown cortex neurons at different time points *in vivo*

To obtain a possible transient compensatory mechanism, stereotactic injections into the cortex of C57/Bl6-J mice were performed to down regulate PINK1 acutely. Therefore a lentiviral shRNA vector (Pink1shRNA) carrying mito-eYFP, to detect mitochondria, was injected in one cortex hemisphere, while in the other hemisphere just mito-eYFP as control vector was injected. Three respectively five days after injection the mice were killed and the brains were prepared and further treated as described in chapter 2 of material and methods. Interestingly, no significant differences in mitochondrial length could be found at day 3 after acute knockdown *in vivo* (Tab. 7), neither in soma and processes nor in the whole neuron. However, at day 5 significant decrease in mitochondrial length of the processes and also of the entire neuron could be detected (control processes: $0.96 \mu\text{m} \pm 0.06$; Pink1sh processes: $0.78 \mu\text{m} \pm 0.03$; p-value processes = 0.010 / control whole cell: $0.90 \mu\text{m} \pm 0.05$; Pink1sh whole cell: $0.77 \mu\text{m} \pm 0.03$; p-value whole cell = 0.027). The soma itself shows no mitochondrial length changes (control soma: $0.80 \mu\text{m} \pm 0.06$; Pink1sh soma: $0.74 \mu\text{m} \pm 0.04$; p-value soma = 0.351). These ratios are clearly shown in Fig. 22. Our results show, that an acute knockdown of PINK1 *in vivo* influences mitochondrial length in a time-dependent manner.

mitochondrial length of acute PINK1 knockdown neurons				
	day 3		day 5	
	control	Pink1sh	control	Pink1sh
soma	1.05 ± 0.07	0.93 ± 0.06	0.80 ± 0.06	0.74 ± 0.04
p-value	0.197		0.351	
processes	1.05 ± 0.05	1.03 ± 0.09	0.96 ± 0.06	0.78 ± 0.03
p-value	0.798		0.010	
whole cell	1.06 ± 0.05	1.08 ± 0.13	0.90 ± 0.05	0.77 ± 0.03
p-value	0.877		0.027	

Tab. 7: Average mitochondrial length in Pink1sh cortex neurons and control neurons of *in vivo* injected mice after two different time points show a length decrease of mitochondria in the processes and entire neurons of PINK1-deficient cells at day 5 after acute knockdown.

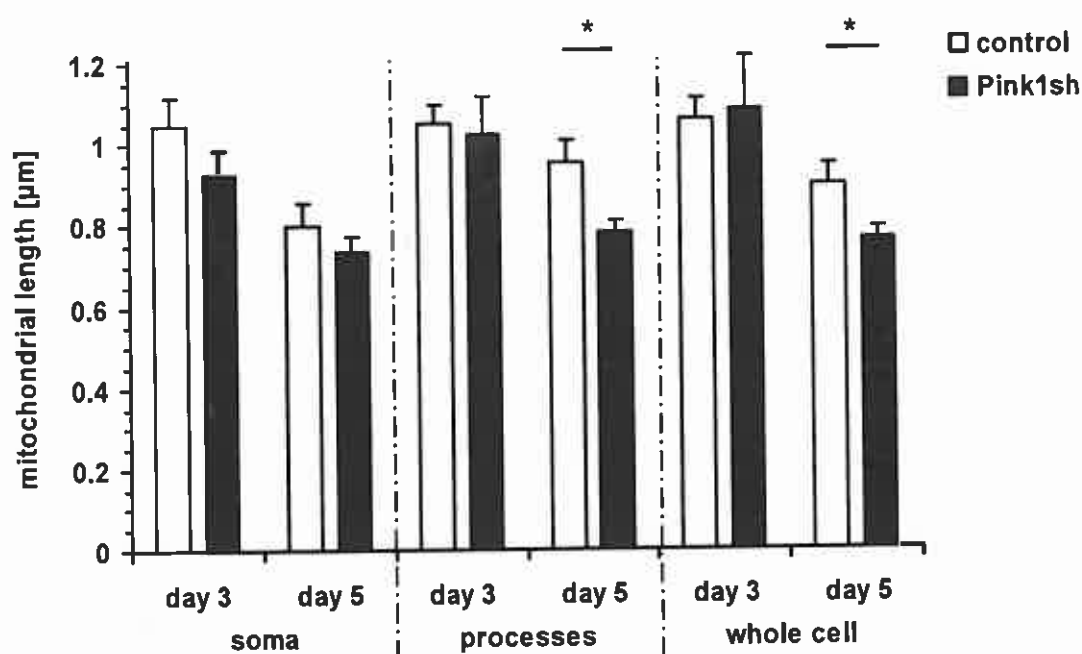


Fig. 22: Quantification of mitochondrial length in different neuron compartments and at different time points after acute knockdown of PINK1 via shRNA *in vivo*. A general decrease of mitochondrial length at day 5 compared to day 3 is obvious in both Pink1sh and control neurons. But no significant differences between both groups (control and Pink1sh) can be observed at day 3. Only at day 5 a significant decrease of mitochondrial length in processes and whole cell occurs.

The percentage values of the individual groups of fragmentation, intermediate (normal sized) and tubular (elongated) mitochondria show clearly no differences between PINK1-deficient neurons and wild type neurons, at both time points, day 3 and day 5 (Tab. 8 and Fig. 23). Interestingly, mitochondrial fragmentation increases in both control and PINK1 knockdown cells from day 3 to day 5 in the same proportion as intermediate mitochondria decreases. Even the elongated mitochondria are reduced from day 3 to day 5 in control and in Pink1sh neurons. Also the changes from day 3 to day 5 seem to be higher in PINK1-deficient neurons compared to control neurons, but yet they show no significances. Considering the changes of the individual phenotypes between day 3 and day 5, control mitochondria show less changes than PINK1 acute knockdown mitochondria (p-values of mitochondria changes between day 3 and day 5: fragmented control: 0.011; intermediate control: 0.011, tubular control: 0.347; fragmented Pink1sh: 1.3×10^{-5} ; intermediate Pink1sh: 1.3×10^{-5} ; tubular Pink1sh: 0.429). Our findings demonstrate that acute knockdown of PINK1 *in vivo* indeed influences mitochondrial morphology. However, it can be compensated in a yet unknown mechanism.

percental mitochondrial length grouping in acute PINK1 knockdown neurons				
	day 3		day 5	
	control	Pink1sh	control	Pink1sh
fragmented	9.74 $\pm$ 1.96	10.44 $\pm$ 1.90	22.54 $\pm$ 4.19	27.41 $\pm$ 2.59
p-value	0.800		0.332	
intermediate	90.13 $\pm$ 1.94	89.13 $\pm$ 1.78	77.43 $\pm$ 4.18	72.48 $\pm$ 2.58
p-value	0.708		0.324	
tubular	0.13 $\pm$ 0.09	0.43 $\pm$ 0.38	0.04 $\pm$ 0.04	0.11 $\pm$ 0.11
p-value	0.449		0.532	

Tab. 8: Percental distribution of mitochondria at different time points after stereotactical lentivirus injection. Acute knockdown of PINK1 shows no differences between control and PINK1-deficient mitochondria.

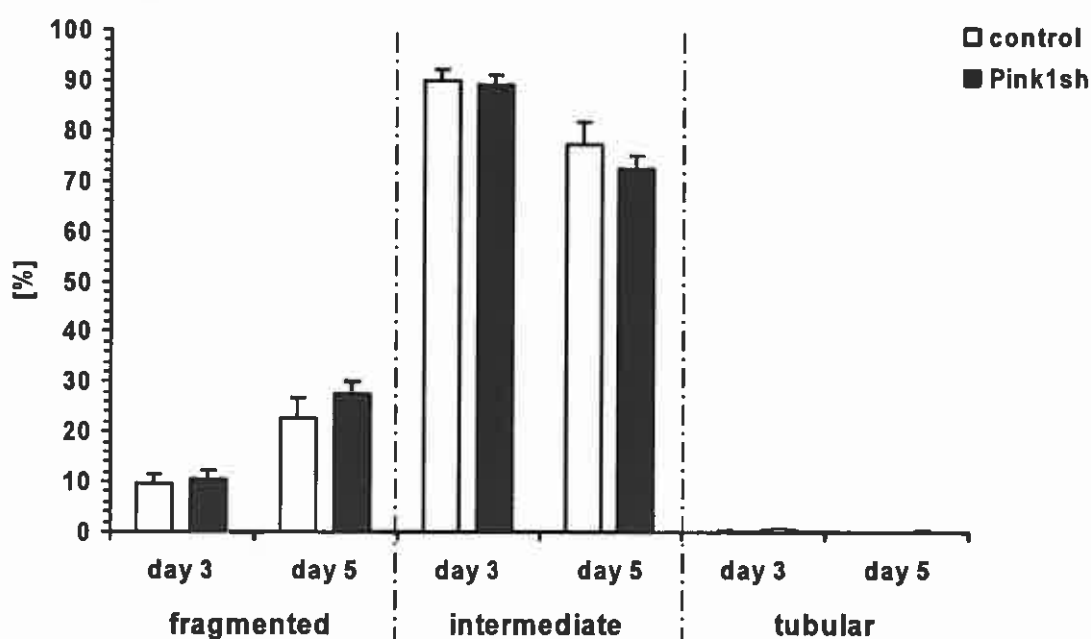


Fig. 23: Morphological quantification of *in vivo* injected cortex neurons with siRNA of PINK1 and appropriate control neurons. Fragmented mitochondria have a length $< 0.5 \mu\text{m}$, whereas intermediate mitochondria length reaches from 0.5 to $5 \mu\text{m}$ and tubular (elongated) mitochondria have a length over $5 \mu\text{m}$. In both neuron groups (control and Pink1sh) an increase in fragmentation is taking place. But this increase is approximately proportional, so that no differences between the groups can be detected there.

4.1.2 In vitro study of mitochondrial morphology

Mitochondrial morphology in primary hippocampal neurons was monitored by measuring mitochondrial length at three different time points (three, four and five days) after acute knockdown of PINK1 using lentiviral shRNA vectors (PINK1 shRNA) or control lentivectors (control), both of which carried a mito-EYFP to visualize mitochondria. Mitochondrial length of soma and processes of two different cultures with each 20 neurons were quantified via three-dimensional images (see Table 9 and Fig. 24). In addition mitochondria were classified into different morphological groups as followed: fragmented mitochondria ($< 0.5 \mu\text{m}$),

intermediate mitochondria ($0.5 - 5 \mu\text{m}$) and tubular mitochondria ($> 5 \mu\text{m}$) (Table 9 and Fig. 24). Thus, a total of about 5,000 mitochondria were analyzed.

At day 3 after virus transduction the average length of mitochondria in acute PINK1 knock down cells is significantly decreased in both soma and processes and hence in the whole neuronal cell. Where the control group shows a value of $0.83 \pm 0.01 \mu\text{m}$ in the soma, PINK1 knock down cells show a value of $0.68 \pm 0.06 \mu\text{m}$ ($p = 0.0001$), indicating highest significance. Such a difference is also reflected in the cell processes where the average mitochondrial length of control group is $0.16 \mu\text{m}$ bigger than PINK1-deficient neurons (control: $1.35 \pm 0.04 \mu\text{m}$; Pink1sh: 1.19 ± 0.04 ; $p = 0.0189$), but not as highly significant as in the soma. Consequently, this ratio is still present in the entire cell (control: $1.07 \pm 0.02 \mu\text{m}$; Pink1sh: $0.85 \pm 0 \mu\text{m}$; $p = 0.000002$). Only one day later, at day 4 after virus transduction the significant differences in mitochondrial length of control and PINK1-deficient neurons are only present in somata (control: $0.84 \pm 0.02 \mu\text{m}$; Pink1sh: 0.77 ± 0.02 ; $p = 0.0149$). Axons and dendrites as well as mitochondria in the whole cell show a tendency towards decreased mitochondrial length but these values are not anymore significant different from the control group (processes control: $1.48 \pm 0.07 \mu\text{m}$; processes Pink1sh: $1.3 \pm 0.06 \mu\text{m}$; processes $p = 0.0574$; whole cell control: $1.11 \pm 0.04 \mu\text{m}$; whole cell Pink1sh: $1.01 \pm 0.03 \mu\text{m}$; whole cell $p = 0.0737$). Another day later, at day 5 after virus transduction the difference in mitochondrial length of the somata has also disappeared and all mitochondria show more or less the same length (soma control: $0.83 \pm 0.03 \mu\text{m}$; soma Pink1sh: 0.75 ± 0.04 ; $p = 0.1233$; processes control: $1.15 \pm 0.05 \mu\text{m}$; processes Pink1sh: $1.05 \pm 0.05 \mu\text{m}$; processes $p = 0.1795$; whole cell control: $1.12 \pm 0.01 \mu\text{m}$; whole cell Pink1sh: $1.14 \pm 0.01 \mu\text{m}$; whole cell $p = 0.9271$). Taken together, these results show that the average mitochondrial length of PINK1-deficient neurons decreases three days after virus transduction, whereas at later time points no significant differences have been detected anymore.

mitochondrial length in hippocampal neurons after acute knockdown of PINK1						
	day 3		day 4		day 5	
	control	Pink1sh	control	Pink1sh	control	Pink1sh
soma	0.83 ± 0.01	0.68 ± 0.06	0.84 ± 0.02	0.77 ± 0.02	0.83 ± 0.03	0.75 ± 0.04
p-values	0.0001		0.0149		0.1233	
processes	1.35 ± 0.04	1.19 ± 0.04	1.48 ± 0.07	1.3 ± 0.06	1.15 ± 0.05	1.05 ± 0.05
p-values	0.0189		0.0574		0.1795	
whole cell	1.07 ± 0.02	0.85 ± 0	1.11 ± 0.04	1.01 ± 0.03	1.12 ± 0.01	1.14 ± 0.01
p-values	0.000002		0.0737		0.9271	

Table 9: Average mitochondrial length in primary hippocampal neurons at different time points after acute knockdown of PINK1 and associated controls.

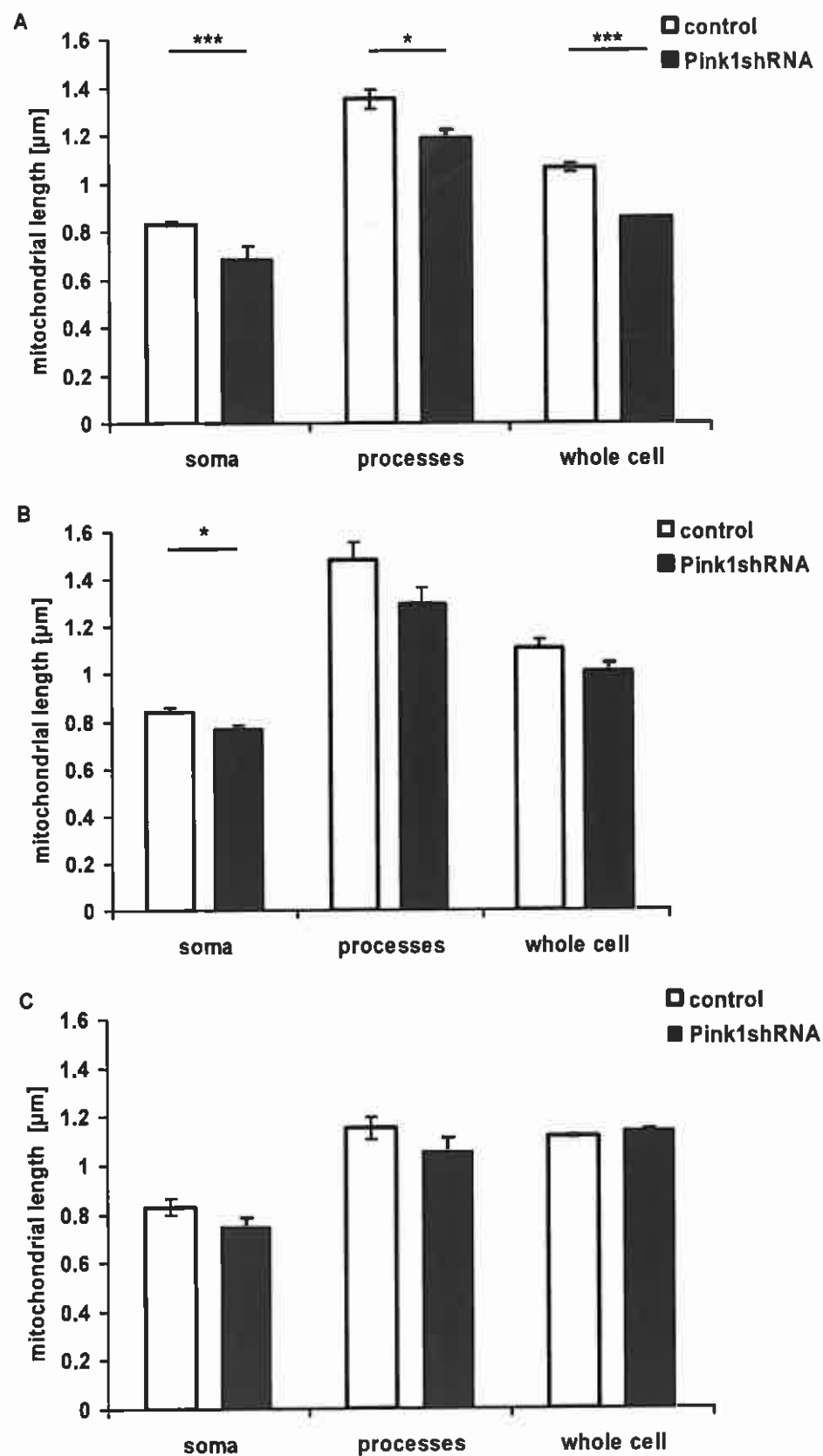


Fig. 24: Quantification of mitochondrial length in soma, cell processes and entire neuron of PINK1-deficient primary hippocampal neurons show shorter mitochondria at day 3 after virus transduction in cell

culture (A), whereas this phenotype has nearly vanished 4 days after transduction (B) and completely 5 days after transduction (C).

The distribution of fragmented, intermediate and tubular mitochondria (see Tab. 10 and Fig. 25) also shows high significant differences between acute PINK1 knockdown neurons and wild type neurons at day 3 after transduction. On day 4 and 5, these differences are gone, but still display a tendency, which however, also decreases. In detail, fragmentation increases in PINK1-deficient neurons compared to wild type neurons at day 3 (control: $9.64\% \pm 1.03$; Pink1sh: $14.96\% \pm 1.12$; $p = 0.0008$), which might be a hint to more dying neurons due to small fragmented mitochondria. This clear significance on day 3, however, is only visible as a trend on day 4 after virus transfection (control: $11.19\% \pm 1.05$; Pink1sh: $14.33\% \pm 1.25$; $p = 0.0581$), while it is almost entirely disappeared on day 5 after transfection (control: $12.82\% \pm 1.21$; Pink1sh: $15.70\% \pm 1.08$; $p = 0.0791$). In the same proportion as the increasing fragmentation of acute PINK1 knockdown cells, the percentage of intermediate and tubular mitochondria decreases in PINK1-deficient neurons (intermediate control at day 3: $89.71\% \pm 1.03$; intermediate Pink1sh at day 3: $84.92\% \pm 1.1$; intermediate p-value at day 3: 0.0021; intermediate control at day 4: $87.79\% \pm 1.04$; intermediate Pink1sh at day 4: $85.05\% \pm 1.21$; intermediate p-value at day 4: 0.0895; intermediate control at day 5: $86.59\% \pm 1.18$; intermediate Pink1sh at day 5: $83.97\% \pm 1.01$; intermediate p-value at day 5: 0.0952; tubular control at day 3: $0.65\% \pm 1.03$; tubular Pink1sh at day 3: $0.13\% \pm 0.07$; tubular p-value at day 3: 0.0114; tubular control at day 4: $1.02\% \pm 0.27$; tubular Pink1sh at day 4: $0.62\% \pm 0.15$; tubular p-value at day 4: 0.199; tubular control at day 5: $0.59\% \pm 0.13$ tubular Pink1sh at day 5: $0.33\% \pm 0.18$; tubular p-value at day 5: 0.2279).

percental mitochondrial length grouping in hippocampal neurons after acute knockdown of PINK1						
	day 3		day 4		day 5	
	control	Pink1sh	control	Pink1sh	control	Pink1sh
fragmented	9.64 ± 1.03	14.96 ± 1.12	11.19 ± 1.05	14.33 ± 1.25	12.82 ± 1.21	15.70 ± 1.08
p-values	0.0008		0.0581		0.0791	
intermediate	89.71 ± 1.03	84.92 ± 1.1	87.79 ± 1.04	85.05 ± 1.21	86.59 ± 1.18	83.97 ± 1.01
p-values	0.0021		0.0895		0.0952	
tubular	0.65 ± 1.03	0.13 ± 0.07	1.02 ± 0.27	0.62 ± 0.15	0.59 ± 0.13	0.33 ± 0.18
p-values	0.0114		0.199		0.2279	

Table 10: Mitochondrial percentage distribution of primary hippocampal neurons at different time points after acute knockdown of PINK1 and associated controls.

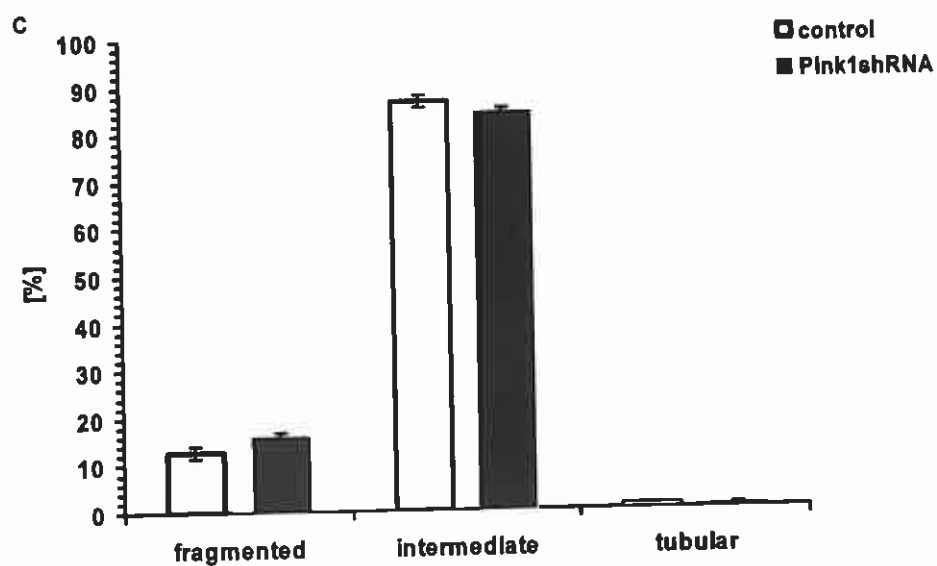
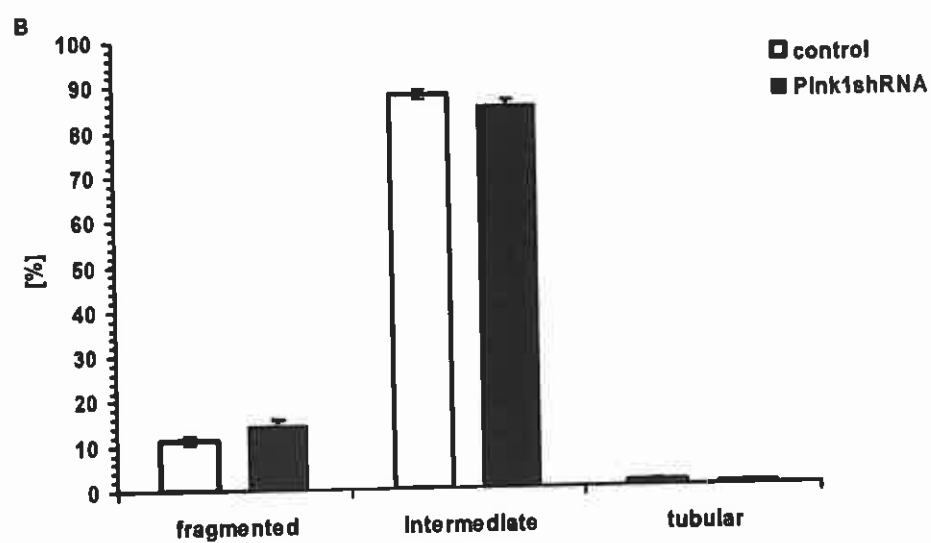
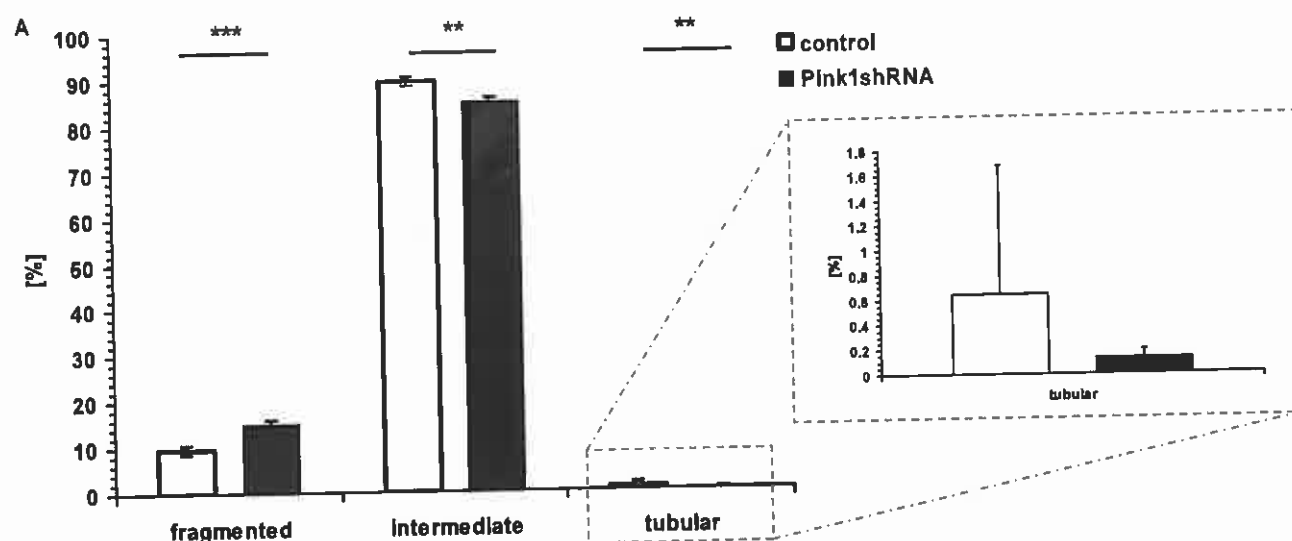


Fig. 25 previous page: Morphological grouping of PINK1-deficient primary hippocampal mitochondria, transduced with a mitoYFP expressing lentivirus and grouped in fragmented ($< 0.5 \mu\text{m}$) intermediate ($0.5 - 5 \mu\text{m}$) and tubular ($> 0.5 \mu\text{m}$) mitochondria. (A) Three days after virus transduction, mitochondrial fragmentation is induced by the acute knockdown, whereas intermediate and tubular mitochondria show a decrease. At day 4 (B) and day 5 (C) after transduction there is still a tendency but significant differences could not be seen here anymore.

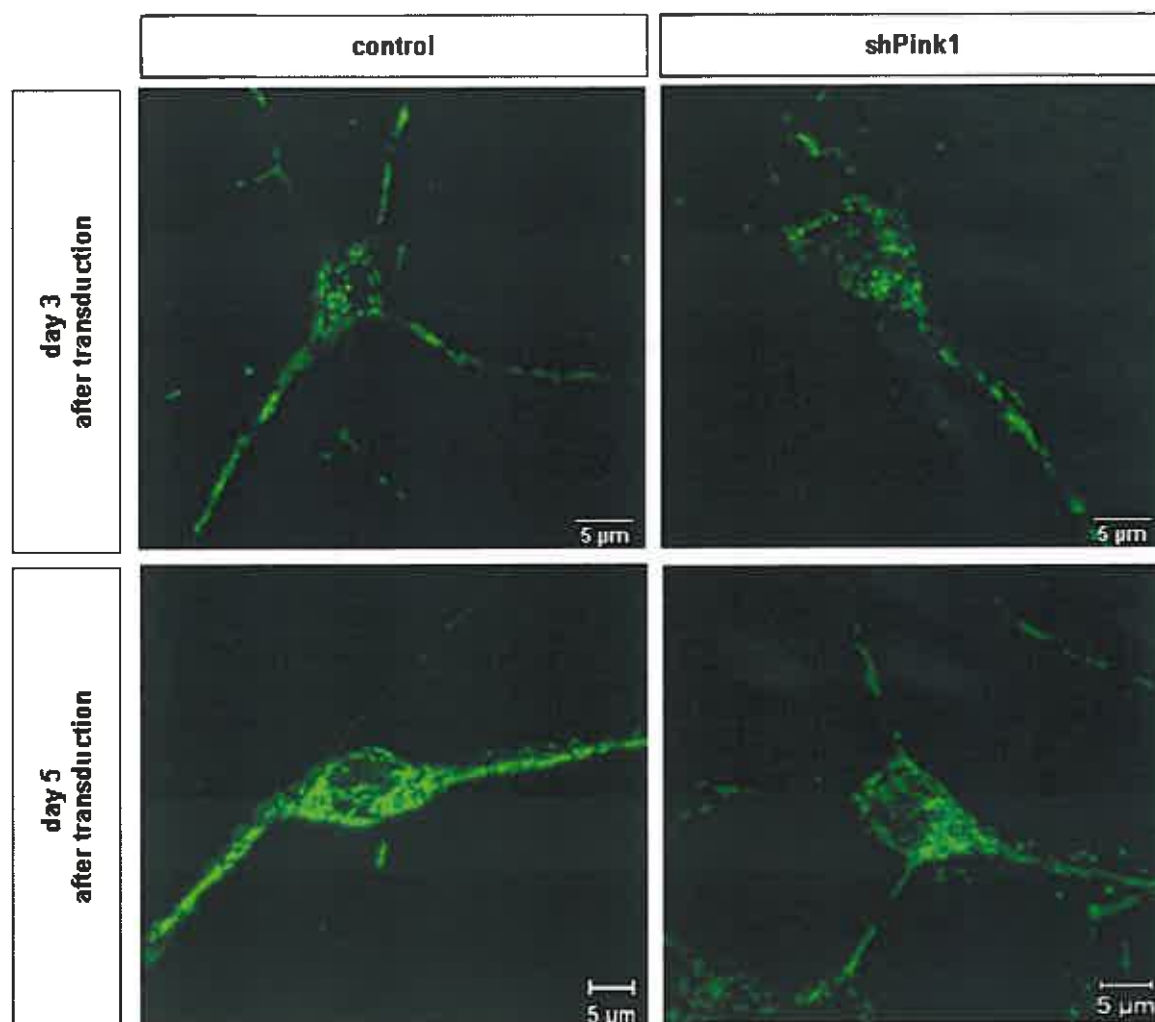


Fig. 26: *In vitro* transduction of C57/BL6-J hippocampal neurons with a lentivirus expressing mito-eYFP (control) or mito-eYFP with additional shRNA (shPink1), respectively. Images were taken three days and 5 days after transduction. Note that three day after virus transduction of neurons, shorter and more fragmented mitochondria appears in PINK1-deficient cells. This effect seems to be compensated over time and does not exist anymore at day 5.

The following table (Tab. 11) provides an overview of all mitochondria morphology results, which were carried out in this study. An increase is expressed as "↑", whereas "↓" represents a decrease and "=" means no changes. Significances are expressed with an asterisk, highly significances with two asterisks and highest significances with three asterisks.

mitochondrial morphology in PINK1-deficient neurons							
	time after virus transfection	average mitochondrial length			grouping in the whole cell		
		soma	processes	whole cell	fragmented	inter-mediate	tubular
<i>in vitro</i> - acute knockdown	day 3	↓ ***	↓ **	↓ ***	↑ ***	↓ **	↓ *
	day 4	↓ *	=	=	=	=	=
	day 5	=	=	=	=	=	=
<i>in vivo</i> - acute knockdown	day 3	=	=	=	=	=	=
	day 5	=	↓ *	↓ *	=	=	=
<i>in vivo</i> PINK1 ^{-/-} mice	~2 weeks	=	=	=	↓ *	=	=

Tab. 11: Overview of the mitochondrial morphology study results. Acute PINK1 knockdown *in vitro* shows highly significant changes in length and morphology at day 3 after virustransduction, whereas this effect has almost completely disappeared at day 4 and already compensated at day 5. *In vivo* acute knockdown at the same time points after virus administration shows only an effect in impaired mitochondrial length at day 5. In contrast, our constitutive mice showed a decrease in fragmented mitochondria two weeks after virus injection.

Thus, for the first time we could show that at least acute PINK1-deficiency is compensated over time *in vitro*. Several evidences suggest also compensatory mechanisms *in vivo* in acute and endogenous knockdown of PINK1.

4.2 mitochondrial trafficking

4.2.1 Mitochondrial trafficking in acute PINK1 knockdown primary neurons

Actual changes in mitochondrial transport of primary cortical neurons after acute PINK1 knockdown were investigated at different developmental states of neurons (young neurons: 4 days in culture before virustransfection, matured neurons: virustransfection on day 11 and aged neurons: virustransfection on day 18) to examine possible changes depending on the maturation status of neurons. Indeed it is known, that "older" neurons react differently to external changes like environmental toxins, stress etc. as the "younger" ones. At the time point when the trafficking experiments started we have already discovered the possibility of compensatory mechanisms in our morphology-study that might counteract the acute knockdown of PINK1. For that reason we in addition have examined changes in mitochondrial transport at two different time points after virustransfection. Detailed data from mitochondrial speed, mobility, direction changes, stop phases and covered distance were counted during live cell imaging. The average observing time of each cell and therefore of each mitochondria was 15 minutes. About 10,000 mitochondria were analyzed. Each section is followed by a summary table including all obtained values. Red labeled p-values indicate statistical significances, while blue marked p-values show tendencies ($p = 0.05 - 0.1$). Data are expressed as means $\pm$ S.E.M.. Statistical analyses of all data points were carried out using Students t-Test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4.2.1.1 Mitochondrial speed in acute PINK1 knockdown primary neurons

To analyze the speed of mitochondria within primary cortical neurons, different variables were measured, like the average speed of mitochondria (including also stop phases during motion), the average speed of mitochondria during anterograde movement and the average speed of mitochondria during retrograde movement. Speed data are displayed in $\mu\text{m}/\text{min}$. Neither in young neurons nor in matured or aged neurons changes in mitochondrial speed of PINK1-deficient cortex neurons could be detected at day 3 and day 5 except an speed increase at day 3 of aged neurons (young neurons day 3: control 0.027 ± 0.019 , Pink1sh 0.002 ± 0.001 , $p = 0.224$; young neurons day 5: control 0.004 ± 0.001 , Pink1sh 0.004 ± 0.002 , $p = 0.942$; matured neurons day 3: control 0.010 ± 0.003 , Pink1sh 0.006 ± 0.001 , $p = 0.227$; matured neurons day 5: control 0.002 ± 0.001 , Pink1sh 0.006 ± 0.002 , $p = 0.115$; aged neurons day 3: control 0.001 ± 0.000 , Pink1sh 0.004 ± 0.001 , $p = 0.027$; aged neurons day 5: control 0.001 ± 0.000 , Pink1sh 0.002 ± 0.001 , $p = 0.471$). A graphic representation of the average mitochondrial speed is shown in Fig. 27. Dividing mitochondrial trafficking furthermore into anterograde and retrograde direction, it is obvious that the speed of anterograde traveling mitochondria in motion (calculated without stop phases) and of

retrograde traveling mitochondria in motion shows a similar pattern of movement. There are no speed differences between anterograde and retrograde moving mitochondria neither in control nor in acute PINK1 knockdown neurons (see Fig. 28A for anterograde speed and Fig. 28B for retrograde speed). If we look at the speed values in detail we can also see no anterograde speed changes in Pink1sh versus control mitochondria at each time point and developmental state except at day 3 of aged neurons (young neurons day 3: control 0.031 ± 0.024 , Pink1sh 0.005 ± 0.001 , $p = 0.258$; young neurons day 5: control 0.005 ± 0.001 , Pink1sh 0.004 ± 0.001 , $p = 0.539$; matured neurons day 3: control 0.014 ± 0.004 , Pink1sh 0.010 ± 0.002 , $p = 0.368$; matured neurons day 5: control 0.004 ± 0.001 , Pink1sh 0.008 ± 0.002 , $p = 0.109$; aged neurons day 3: control 0.003 ± 0.000 , Pink1sh 0.006 ± 0.001 , $p = 0.027$; aged neurons day 5: control 0.003 ± 0.001 , Pink1sh 0.003 ± 0.001 , $p = 0.615$). Similar results be seen in the speed of retrograde moving mitochondria where at day 3 of aged neurons the speed increased in PINK1-deficient neurons (young neurons day 3: control 0.024 ± 0.017 , Pink1sh 0.003 ± 0.001 , $p = 0.240$; young neurons day 5: control 0.005 ± 0.001 , Pink1sh 0.005 ± 0.001 , $p = 0.907$; matured neurons day 3: control 0.015 ± 0.004 , Pink1sh 0.009 ± 0.002 , $p = 0.161$; matured neurons day 5: control 0.003 ± 0.001 , Pink1sh 0.007 ± 0.002 , $p = 0.061$; aged neurons day 3: control 0.003 ± 0.000 , Pink1sh 0.007 ± 0.001 , $p = 0.030$; aged neurons day 5: control 0.007 ± 0.004 , Pink1sh 0.003 ± 0.001 , $p = 0.321$). For a compact overview of the data please look at table 12.

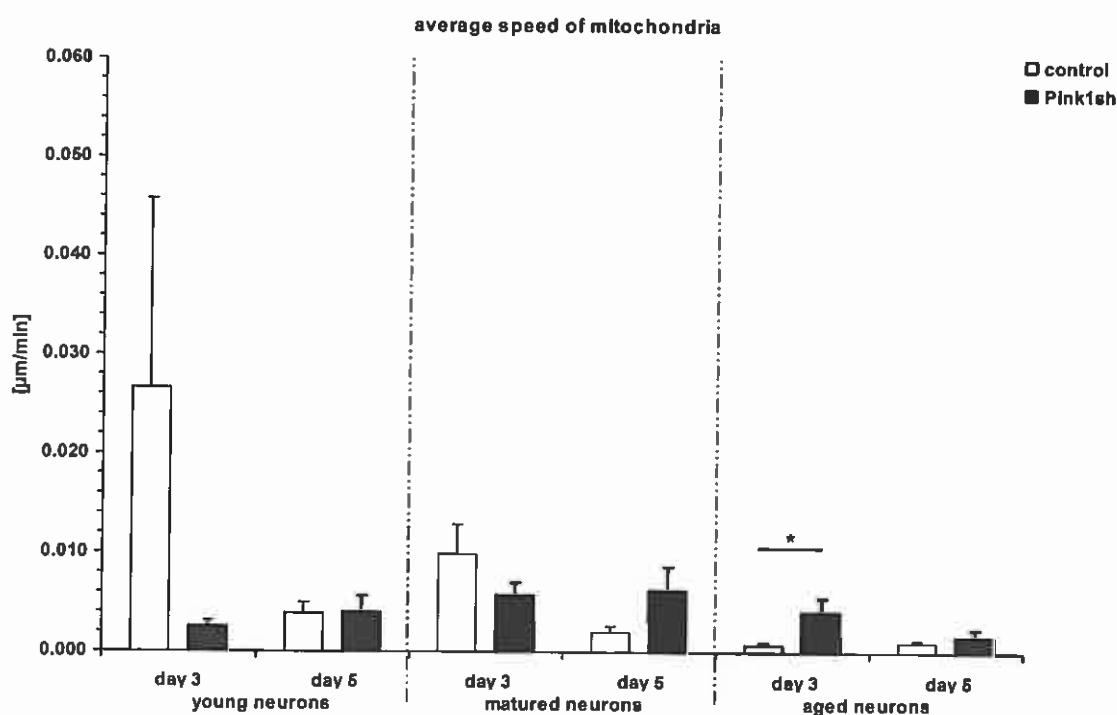


Fig. 27: Overall average speed of mitochondria including stop phases. Pink1sh mitochondria moving with the same speed like normal mitochondria except that there is a significant increase in speed of PINK1-deficient mitochondria at day 3 of aged neurons ($p = 0.027$).

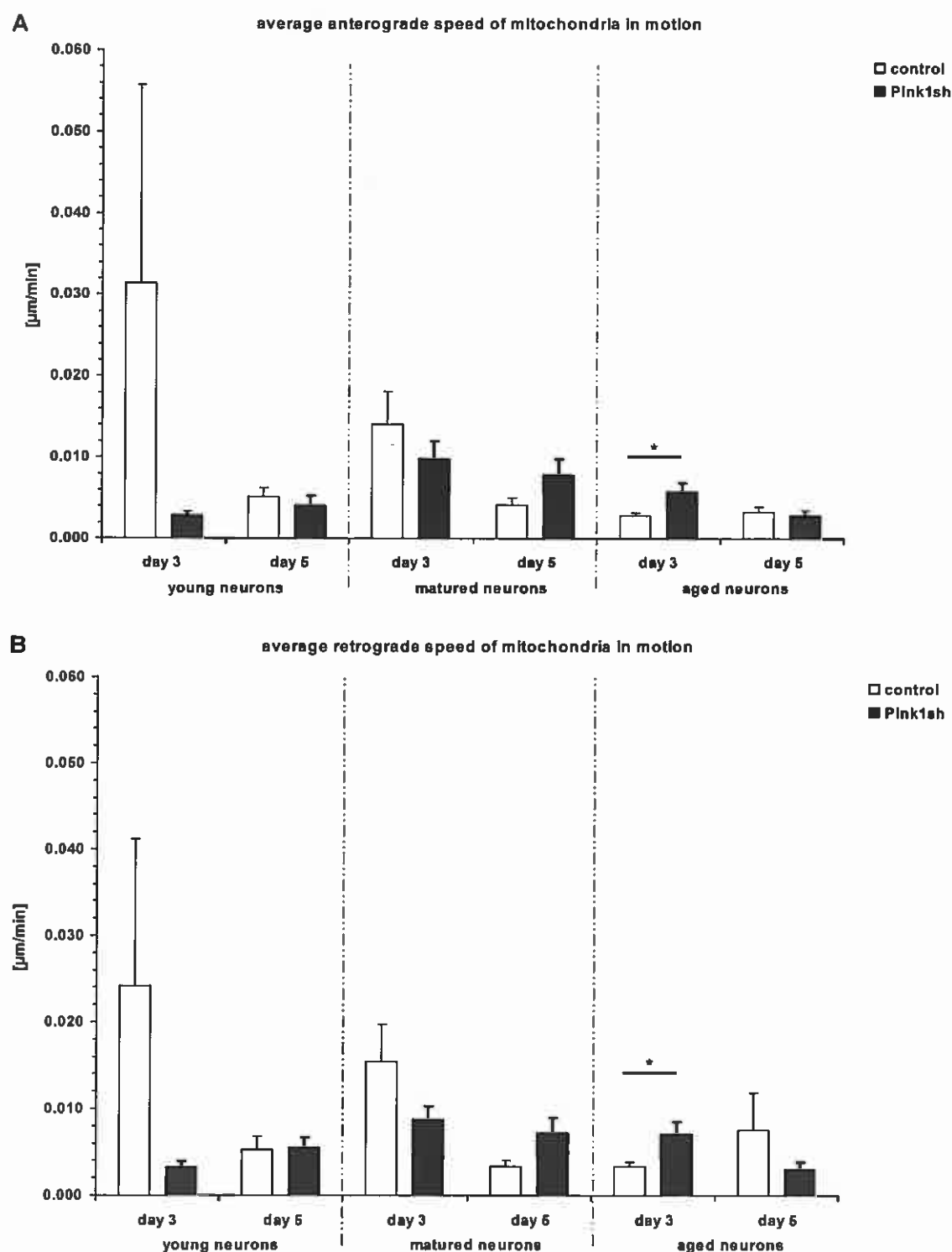


Fig. 28: Average speed of mitochondria during continuous anterograde (A) and retrograde (B) motion. (A) Anterograde speed of Pink1-deficient mitochondria shows no differences to control neurons except an increase at day 3 of aged neurons ($p = 0.027$). (B) Retrograde speed of Pink1-deficient mitochondria shows the same moving properties than anterograde moving mitochondria. No changes in retrograde speed between acute knockdown PINK1 neurons and control neurons are obvious except day 3 in aged neurons, where PINK1-deficient mitochondria are significant faster than their controls ($p = 0.030$).

Mitochondrial speed [$\mu\text{m}/\text{min}$] in acute PINK1 knockdown primary cortex neurons												
speed	young neurons				matured neurons				aged neurons			
	day 3		day 5		day 3		day 5		day 3		day 5	
	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh
average speed of mitochondria	0.027 $\pm$ 0.019	0.002 $\pm$ 0.001	0.004 $\pm$ 0.001	0.004 $\pm$ 0.002	0.010 $\pm$ 0.003	0.006 $\pm$ 0.001	0.002 $\pm$ 0.001	0.008 $\pm$ 0.002	0.001 $\pm$ 0.000	0.004 $\pm$ 0.001	0.001 $\pm$ 0.000	0.002 $\pm$ 0.001
p-value	0.224		0.942		0.227		0.115		0.027		0.471	
average anterograde speed of mitochondria in motion	0.031 $\pm$ 0.024	0.003 $\pm$ 0.000	0.005 $\pm$ 0.001	0.004 $\pm$ 0.001	0.014 $\pm$ 0.004	0.010 $\pm$ 0.002	0.004 $\pm$ 0.001	0.008 $\pm$ 0.002	0.003 $\pm$ 0.000	0.008 $\pm$ 0.001	0.003 $\pm$ 0.001	0.003 $\pm$ 0.001
p-value	0.268		0.539		0.368		0.109		0.027		0.515	
average retrograde speed of mitochondria in motion	0.024 $\pm$ 0.017	0.003 $\pm$ 0.001	0.005 $\pm$ 0.001	0.005 $\pm$ 0.001	0.015 $\pm$ 0.004	0.009 $\pm$ 0.002	0.003 $\pm$ 0.001	0.007 $\pm$ 0.002	0.003 $\pm$ 0.000	0.007 $\pm$ 0.001	0.007 $\pm$ 0.004	0.003 $\pm$ 0.001
p-value	0.240		0.907		0.181		0.061		0.030		0.321	

Tab. 12: Mitochondrial speed in acute PINK1 knockdown primary cortex neurons. While no changes in speed could be observed in young and matured neurons, there is a general increase in mitochondrial speed at day 3 after acute PINK1 knockdown. This speed increase is already cancelled two days later, on day 5 of aged neurons.

4.2.1.2 Mitochondrial mobility in acute PINK1 knockdown primary neurons

The transport and positioning of mitochondria along axons is essential for neuronal homeostasis and mitochondria perform rapid but intermittent transport in the anterograde as well as the retrograde direction [Chada and Hollenbeck, 2003]. To uncover differences in the mobility of neuronal mitochondria, which might be a first sign of possibly altered homeostasis, I measured the proportion of the stationary phase during motion, the number of anterograde phases as well as retrograde phases during motion and the distribution of immobile and mobile mitochondria. Mobility data are displayed in percentage.

For the stationary phase during motion significant changes can be seen at day 5 after Pink1sh virustransfection of young and matured neurons. In young Pink1-deficient neurons an increase in the stationary phase at day 5 is obvious ($p = 0.009$), whereas in matured Pink1-deficient neurons the stationary phases of mitochondria during motion decreases ($p = 0.040$). All other values of the remaining time points and developmental states are not changed (young neurons day 3: control 38.20 ± 4.85 , Pink1sh 51.23 ± 4.15 , $p = 0.051$; young neurons day 5: control 56.95 ± 5.84 , Pink1sh 79.44 ± 5.21 , $p = 0.009$; matured neurons day 3: control 63.10 ± 5.05 , Pink1sh 69.09 ± 6.07 , $p = 0.454$; matured neurons day 5: control 80.44 ± 3.92 , Pink1sh 66.74 ± 4.98 , $p = 0.040$; aged neurons day 3: control 72.89 ± 3.02 , Pink1sh 69.36 ± 5.32 , $p = 0.572$; aged neurons day 5: control 71.78 ± 4.35 , Pink1sh 83.72 ± 4.19 , $p = 0.058$).

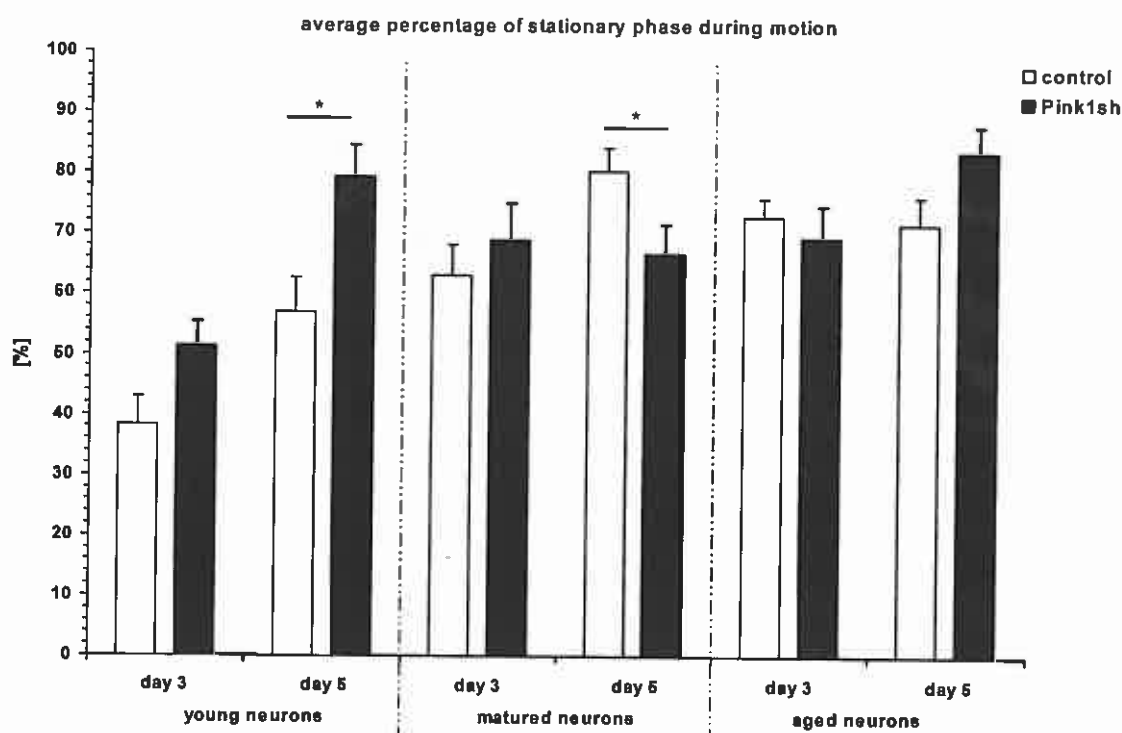


Fig. 29: Average percentage of stationary phase during motion. With increasing age, the stationary phase during mitochondrial motion also increases in healthy neurons and reach a plateau phase in aged neurons. Whereas PINK1-deficient neurons show a steep increase of the stationary phase only in young neurons and then appear to remain unchanged until the stationary phase slightly increases again at day 5 of aged neurons.

Mitochondrial motility is separated into anterograde and retrograde movement direction. The percental portion of anterograde phase during motion has not been changed in acute PINK1 knockdown neurons, except for day 5 after virustransduction in young neurons, where a decrease in anterograde movement can be seen (young neurons day 3: control 28.60 ± 3.49 , Pink1sh 24.21 ± 2.75 , $p = 0.331$; young neurons day 5: control 19.21 ± 3.38 , Pink1sh 7.50 ± 1.53 , $p = 0.006$; matured neurons day 3: control 20.39 ± 2.66 , Pink1sh 17.27 ± 3.71 , $p = 0.500$; matured neurons day 5: control 11.49 ± 2.46 , Pink1sh 18.66 ± 3.14 , $p = 0.084$; aged neurons day 3: control 16.20 ± 1.66 , Pink1sh 15.51 ± 2.88 , $p = 0.837$; aged neurons day 5: control 15.20 ± 2.63 , Pink1sh 8.15 ± 2.68 , $p = 0.071$).

Also the percental portion of retrograde phase during motion has not been changed significantly in PINK1-deficient neurons, except an increase of retrograde moving mitochondria in matured neurons 5 days after PINK1 down regulation (young neurons day 3: control 33.19 ± 3.68 , Pink1sh 24.56 ± 2.54 , $p = 0.065$; young neurons day 5: control 23.85 ± 3.58 , Pink1sh 13.07 ± 3.81 , $p = 0.052$; matured neurons day 3: control 16.50 ± 2.56 , Pink1sh 13.64 ± 3.14 , $p = 0.485$; matured neurons day 5: control 8.07 ± 1.71 , Pink1sh 14.65 ± 2.28 , $p = 0.030$; aged neurons day 3: control 10.87 ± 1.68 , Pink1sh 15.35 ± 2.87 , $p = 0.198$; aged neurons day 5: control 13.14 ± 2.14 , Pink1sh 8.13 ± 2.08 , $p = 0.105$). For a detailed overview please look at the attached table 13 as well as Figure 30.

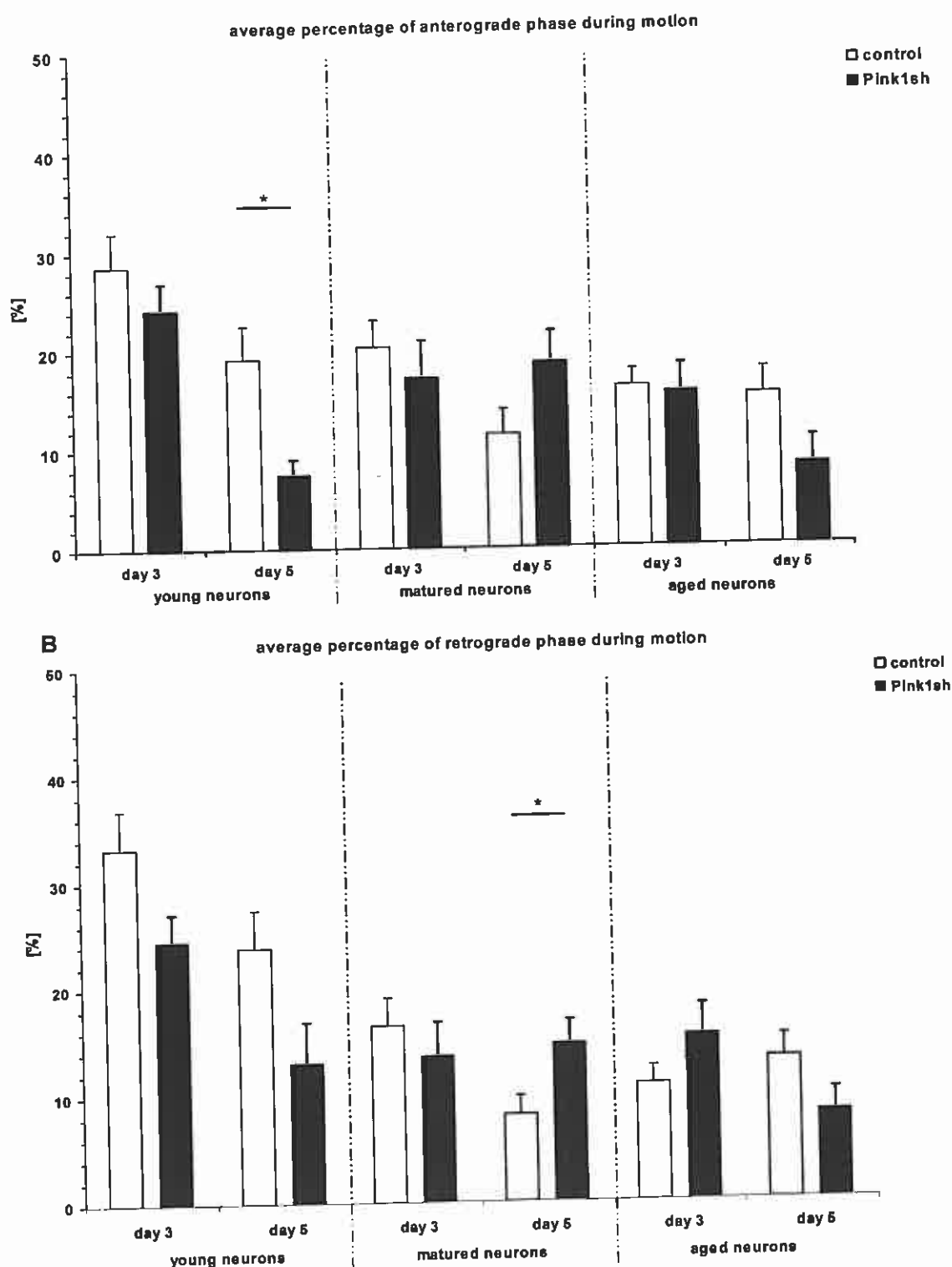


Fig. 30: Average percentage of anterograde phase (A) and retrograde phase (B) during motion. The percental proportions of anterograde and retrograde phase during mitochondrial motion have not been changed comparing PINK1-deficient neurons with neurons under normal condition, except a highly decrease of anterograde movement in young neurons with five days of PINK1 knockdown and an increase of PINK1-deficient neurons in matured cells at day 5.

To investigate mobility changes in Pink1-deficient neurons and to consider the natural variability in energy metabolism, cell growth and homeostasis, we first had to monitor the natural course of mitochondrial mobility in functional healthy neurons (control) within the different developmental states and time frames after lentivirustransfection. Considering the neurons under normal condition, it is obvious that in young neurons mitochondria are less immobile and much more mobile than in matured or aged neurons. Only ¼ of mitochondria are immobile at day 3 after virustransfection ($24.90 \% \pm 5.30$). Already at day 5 after virustransfection in young neurons this value rises up to 39 % ($38.85 \% \pm 8.11$; day 3 versus day 5: $p = 0.164$). In matured neurons nearly one half of the mitochondria are immobile at day 3 after virustransduction ($47.40 \% \pm 6.29$), whereas 67 % are immobile only two days later ($67.35 \% \pm 6.41$; day 3 versus day 5: $p = 0.035$). A balance also exists in aged neurons where ½ of the neurons are immobile whereas the other half was transported in the observed time period, three and five days after transduction (immobility of aged healthy neurons at day 3: $56.37 \% \pm 4.81$ and day 5: $54.85 \% \pm 6.19$; day 3 versus day 5: $p = 0.848$). Considering now the PINK1 acute knockdown neurons, already at day 3 after acute knockdown of PINK1 in young neurons the mitochondria are less mobile than their fully functional counterparts (mobile young neurons day 3: control 75.10 ± 5.30 , Pink1sh 62.07 ± 4.74 , $p = 0.077$; immobile young neurons day 3: control 24.90 ± 5.30 , Pink1sh 37.93 ± 4.74 , $p = 0.077$). This effect highly increases only two days later, where there is a significant difference between the motility of mitochondria of Pink1sh and control neurons (mobile young neurons day 5: control 61.15 ± 8.11 , Pink1sh 35.82 ± 7.94 , $p = 0.036$; immobile young neurons day 5: control 38.85 ± 8.11 , Pink1sh 64.18 ± 7.94 , $p = 0.036$). The balance of half mobile and half immobile mitochondria within a given time point, which can be seen in aged control neurons, can also be found in neurons with acute knockdown of PINK1. But within these neurons the plateau phase already exists in matured neurons, beginning from day 3 after acute PINK1 knockdown (PINK1sh day 3 versus day 5: in young neurons $p = 0.012$; in matured neurons $p = 0.892$; in aged neurons $p = 0.042$). A detailed overview of the individual values is illustrated in table 13. Another interesting point to be mentioned is the high and significant increase of immobile mitochondria in aged neurons at day 5 after PINK1 knockdown. Up to ¾ of these mitochondria are immobile (control 54.85 ± 6.19 , Pink1sh 74.18 ± 5.45 , $p = 0.026$; mobile aged neurons day 5: control 45.15 ± 6.19 , Pink1sh 25.82 ± 5.45 , $p = 0.026$). The values are shown graphically in Fig. 31 for a better understanding.

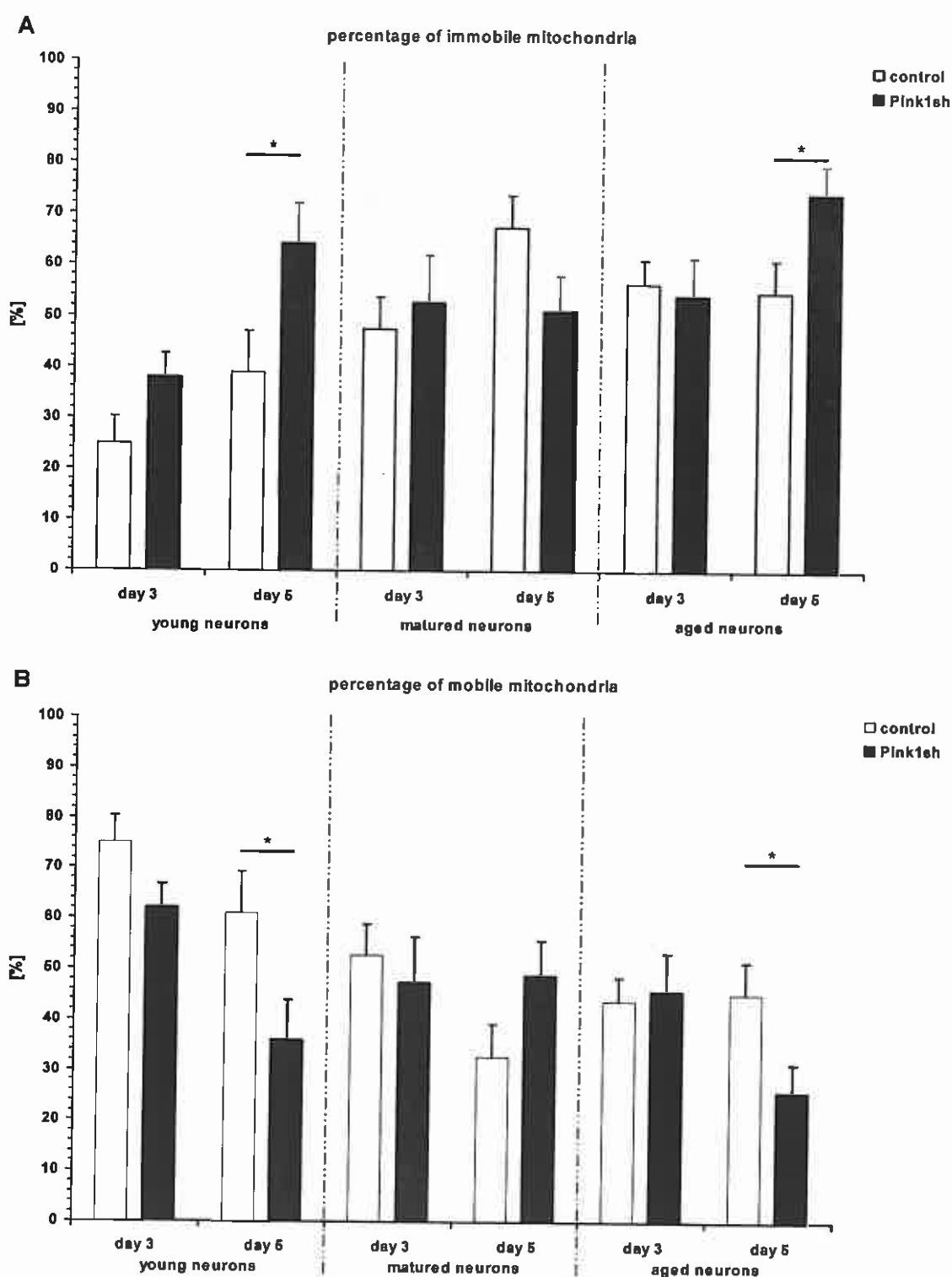


Fig. 31: Average percentage of Immobile (A) and mobile (B) mitochondria. The number of immobile mitochondria in PINK1-deficient neurons increases significantly at day 5 of young and aged neurons, whereas all other time points seem not to be affected. Consequently, in the same proportional ratio the number of mobile mitochondria is decreasing in young and aged neurons at day 5 after acute PINK1 knockdown.

Mitochondrial mobility in acute PINK1 knockdown primary cortex neurons [%]												
motility	young neurons				matured neurons				aged neurons			
	day 3		day 5		day 3		day 5		day 3		day 5	
	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh
stationary phase during motion	38.20 [±] 4.85	51.23 [±] 4.15	58.95 [±] 5.84	79.44 [±] 5.21	83.10 [±] 5.05	69.08 [±] 6.07	80.44 [±] 3.92	66.74 [±] 4.98	72.89 [±] 3.02	69.36 [±] 5.32	71.78 [±] 4.35	83.72 [±] 4.19
p-value	0.051		0.005		0.454		0.040		0.572		0.055	
anterograde phase during motion	28.60 [±] 3.49	24.21 [±] 2.75	19.21 [±] 3.38	7.50 [±] 1.53	20.39 [±] 2.66	17.27 [±] 3.71	11.46 [±] 2.46	18.66 [±] 3.14	16.20 [±] 1.66	15.51 [±] 2.88	15.20 [±] 2.83	8.15 [±] 2.68
p-value	0.331		0.005		0.500		0.004		0.837		0.071	
retrograde phase during motion	33.19 [±] 3.68	24.56 [±] 2.54	23.85 [±] 3.58	13.07 [±] 3.81	18.50 [±] 2.58	13.64 [±] 3.14	8.07 [±] 1.71	14.65 [±] 2.28	10.87 [±] 1.66	15.35 [±] 2.87	13.14 [±] 2.14	8.13 [±] 2.08
p-value	0.056		0.082		0.485		0.030		0.183		0.108	
immobile mitochondria	24.80 [±] 5.30	37.83 [±] 4.74	38.85 [±] 8.11	64.18 [±] 7.94	47.40 [±] 6.29	52.74 [±] 9.14	67.35 [±] 6.41	51.17 [±] 6.84	56.37 [±] 4.81	54.24 [±] 7.29	54.85 [±] 6.18	74.18 [±] 5.45
p-value	0.077		0.034		0.835		0.096		0.810		0.026	
mobile mitochondria	75.10 [±] 5.30	62.07 [±] 4.74	61.15 [±] 8.11	35.82 [±] 7.94	52.60 [±] 6.29	47.26 [±] 9.14	32.65 [±] 6.41	48.83 [±] 6.84	43.63 [±] 4.82	45.76 [±] 7.29	45.15 [±] 6.18	25.82 [±] 5.45
p-value	0.077		0.034		0.835		0.096		0.810		0.026	

Tab. 13: Mitochondrial mobility in acute PINK1 knockdown primary cortex neurons.

4.2.1.3 Mitochondrial direction changes in acute PINK1 knockdown primary neurons

During organelle trafficking it very often occur, that mitochondria change their direction. To take a closer look on this generally recognized but not further investigated phenomenon we distinguished between percentage of mitochondria with direction changes during motion, number of direction changes per 10 frames (meaning number of direction changes per 30 seconds), number of speed changes during motion and number of speed changes during anterograde and retrograde motion. Measurements of mitochondria with direction changes during motion are displayed in percentage whereas the other values in this group are displayed as average number.

No differences of percental proportion of mitochondria with direction changes during motion could be found in neurons with an acute PINK1-deficiency at each time point after lentivirustransduction and developmental cell state (young neurons day 3: control 32.63 ± 6.08, Pink1sh 29.31 ± 3.51, $p = 0.640$; young neurons day 5: control 40.27 ± 8.25, Pink1sh 21.54 ± 4.70, $p = 0.063$; matured neurons day 3: control 36.02 ± 5.48, Pink1sh 33.25 ± 8.69, $p = 0.790$; matured neurons day 5: control 20.38 ± 4.43, Pink1sh 33.30 ± 5.73, $p = 0.086$; aged neurons day 3: control 27.57 ± 3.77, Pink1sh 30.75 ± 6.10, $p = 0.664$; aged neurons day 5: control 27.74 ± 5.21, Pink1sh 16.07 ± 3.46, $p = 0.073$), see Figure 32 and table 14. Note that there is always a bigger but not significant difference between control and Pink1sh neurons at day 5 in young, matured and aged neurons compared to day 3.

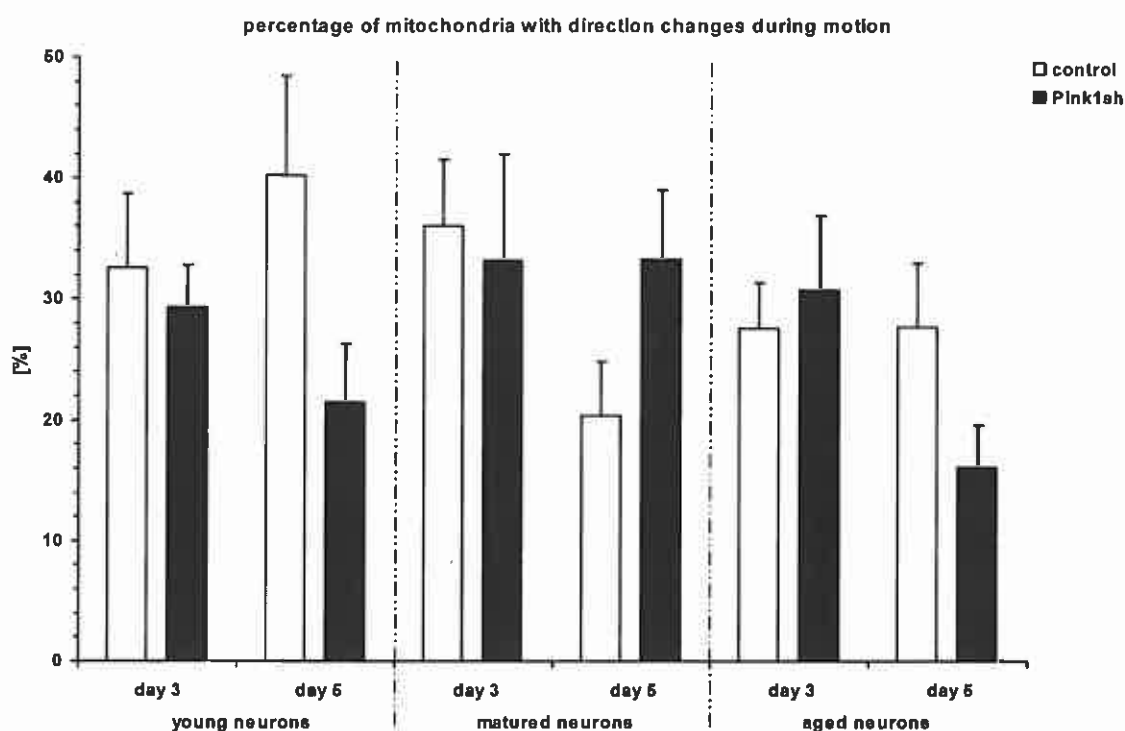


Fig. 32: Percentage of mitochondria with direction changes during motion. Although some distinct trends are obvious, especially at day 5 of each developmental state, no significant changes were calculated.

Furthermore, if we have a closer look to the average number of direction changes of mitochondria during motion, based and calculated per 10 frames, no differences could be found between healthy primary cortex neurons and PINK1-deficient neurons at each time point after lentivirustransfection (young neurons day 3: control 0.14 ± 0.04 , Pink1sh 0.09 ± 0.01 , $p = 0.275$; young neurons day 5: control 0.15 ± 0.04 , Pink1sh 0.08 ± 0.02 , $p = 0.135$; matured neurons day 3: control 0.11 ± 0.02 , Pink1sh 0.10 ± 0.03 , $p = 0.942$; matured neurons day 5: control 0.03 ± 0.01 , Pink1sh 0.08 ± 0.02 , $p = 0.035$; aged neurons day 3: control 0.04 ± 0.01 , Pink1sh 0.06 ± 0.01 , $p = 0.308$; aged neurons day 5: control 0.05 ± 0.01 , Pink1sh 0.03 ± 0.01 , $p = 0.137$). However, a significant increase of mitochondrial direction changes during mitochondrial motion is obvious at day 5 of matured PINK1-deficient neurons. Note that the number of direction changes per 10 timeframes is generally decreasing in older neurons compared to younger ones. Interestingly, this decrease can be seen earlier in our healthy control neurons, whereas the average number of direction changes in acute PINK1 knockdown neurons decreases only gradually.

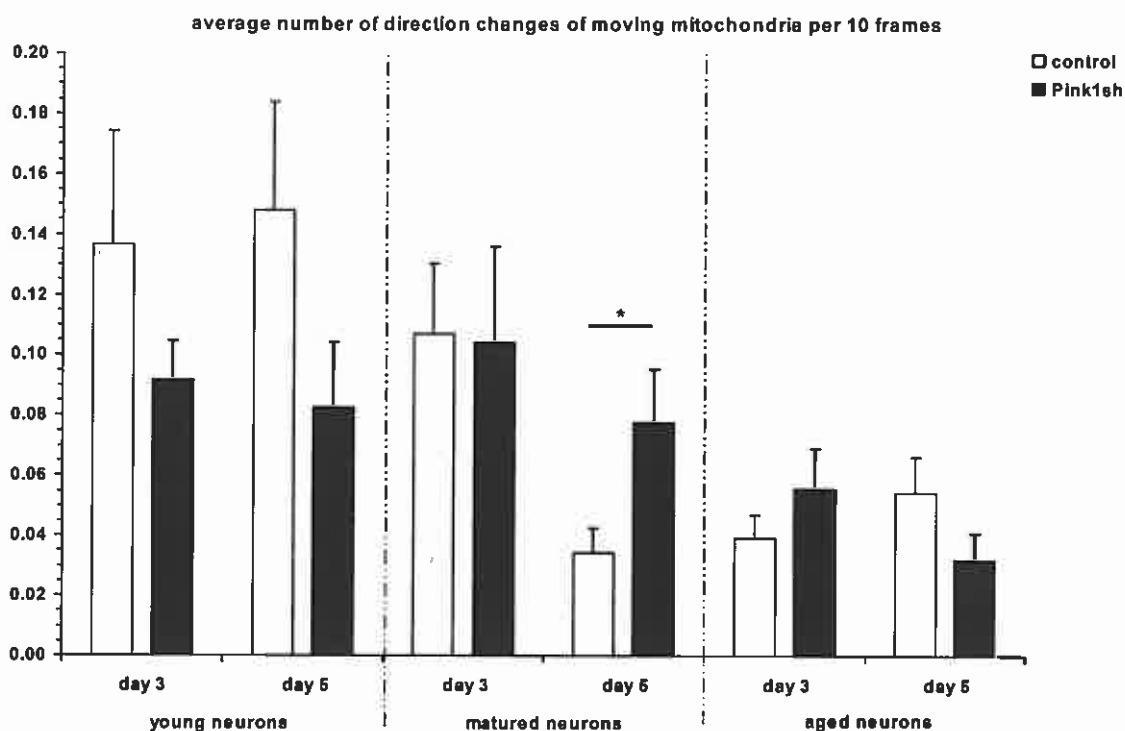


Fig. 33: Average number of direction changes of mitochondria per 30 seconds. Day 5 of matured neurons shows a clear increase of direction changes in PINK1sh neurons compared to healthy control neurons. Specifically, this difference seems to appear from a delayed decrease of direction changes within PINK1-deficient neurons in an older developmental state.

Not only the direction of mitochondria but also the speed is an indicator of energy balance and requirement of the cell. Therefore changes in speed might be an important hint to malfunction of a neuron.

In our acute PINK1 knockdown neurons an increase in the number of speed changes during mitochondrial motion again at day 5 of matured neurons could be observed (control 0.27 ± 0.06 , Pink1sh 0.53 ± 0.10 , $p = 0.043$). At all other time points, no differences could be detected (young neurons day 3: control 0.60 ± 0.12 , Pink1sh 0.50 ± 0.07 , $p = 0.483$; young neurons day 5: control 0.67 ± 0.12 , Pink1sh 0.39 ± 0.12 , $p = 0.106$; matured neurons day 3: control 0.92 ± 0.19 , Pink1sh 0.67 ± 0.16 , $p = 0.324$; aged neurons day 3: control 0.31 ± 0.06 , Pink1sh 0.43 ± 0.11 , $p = 0.350$; aged neurons day 5: control 0.31 ± 0.06 , Pink1sh 0.24 ± 0.09 , $p = 0.564$; see table 14). Interestingly, if we look at the graphic in Fig. 34, we can see quite obvious a natural occurring increase of speed changes during mitochondrial motion in efficient working young neurons to matured neurons, and rapid decrease in mitochondrial speed changes at day 5, which then settles down to a plateau phase in aged neurons. For PINK1-deficient mitochondria, this decrease seems rather delayed and decelerated.

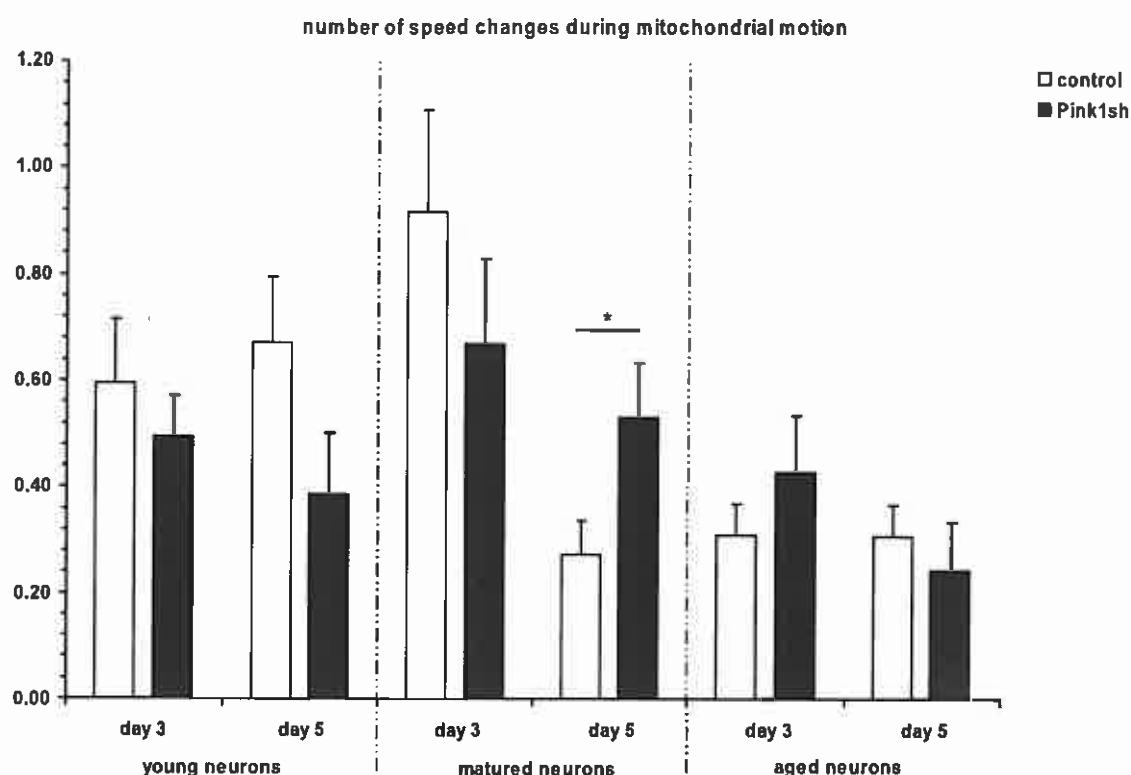


Fig. 34: Average number of speed changes during mitochondrial motion. The average number of speed changes at day 5 of matured neurons is about twice as high in PINK1-deficient neurons as in comparable wild type neurons (control 0.27 ± 0.06 , Pink1sh 0.53 ± 0.10 , $p = 0.043$). In all other developmental states and time points there are no genetic differences.

Interesting to see, that the number of speed changes during anterograde phase and retrograde phase seem to be well-balanced. For the number of speed changes during anterograde motion no changes between Pink1sh and control neurons can be found except a significant decrease at day 5 of young neurons (young neurons day 3: control 0.26 ± 0.05 , Pink1sh 0.24 ± 0.05 , $p = 0.818$; young neurons day 5: control 0.31 ± 0.07 , Pink1sh 0.11 ± 0.02 , $p = 0.018$; matured neurons day 3: control 0.49 ± 0.12 , Pink1sh 0.39 ± 0.10 , $p = 0.517$; matured neurons day 5: control 0.17 ± 0.04 , Pink1sh 0.31 ± 0.06 , $p = 0.088$; aged neurons day 3: control 0.17 ± 0.03 , Pink1sh 0.18 ± 0.06 , $p = 0.853$; aged neurons day 5: control 0.16 ± 0.03 , Pink1sh 0.14 ± 0.06 , $p = 0.791$). The same applies to the number of speed changes during retrograde motion. Here, only a significant difference regarding an increase of Pink1sh mitochondria speed changes at day 5 of matured neurons occurs (young neurons day 3: control 0.34 ± 0.07 , Pink1sh 0.25 ± 0.05 , $p = 0.344$; young neurons day 5: control 0.36 ± 0.06 , Pink1sh 0.28 ± 0.10 , $p = 0.470$; matured neurons day 3: control 0.43 ± 0.09 , Pink1sh 0.28 ± 0.08 , $p = 0.244$; matured neurons day 5: control 0.10 ± 0.03 , Pink1sh 0.22 ± 0.04 , $p = 0.024$; aged neurons day 3: control 0.15 ± 0.03 , Pink1sh 0.25 ± 0.06 , $p = 0.118$; aged neurons day 5: control 0.15 ± 0.04 , Pink1sh 0.10 ± 0.03 , $p = 0.374$). For a detailed overview of the individual values please look at table 14 and Fig. 35.

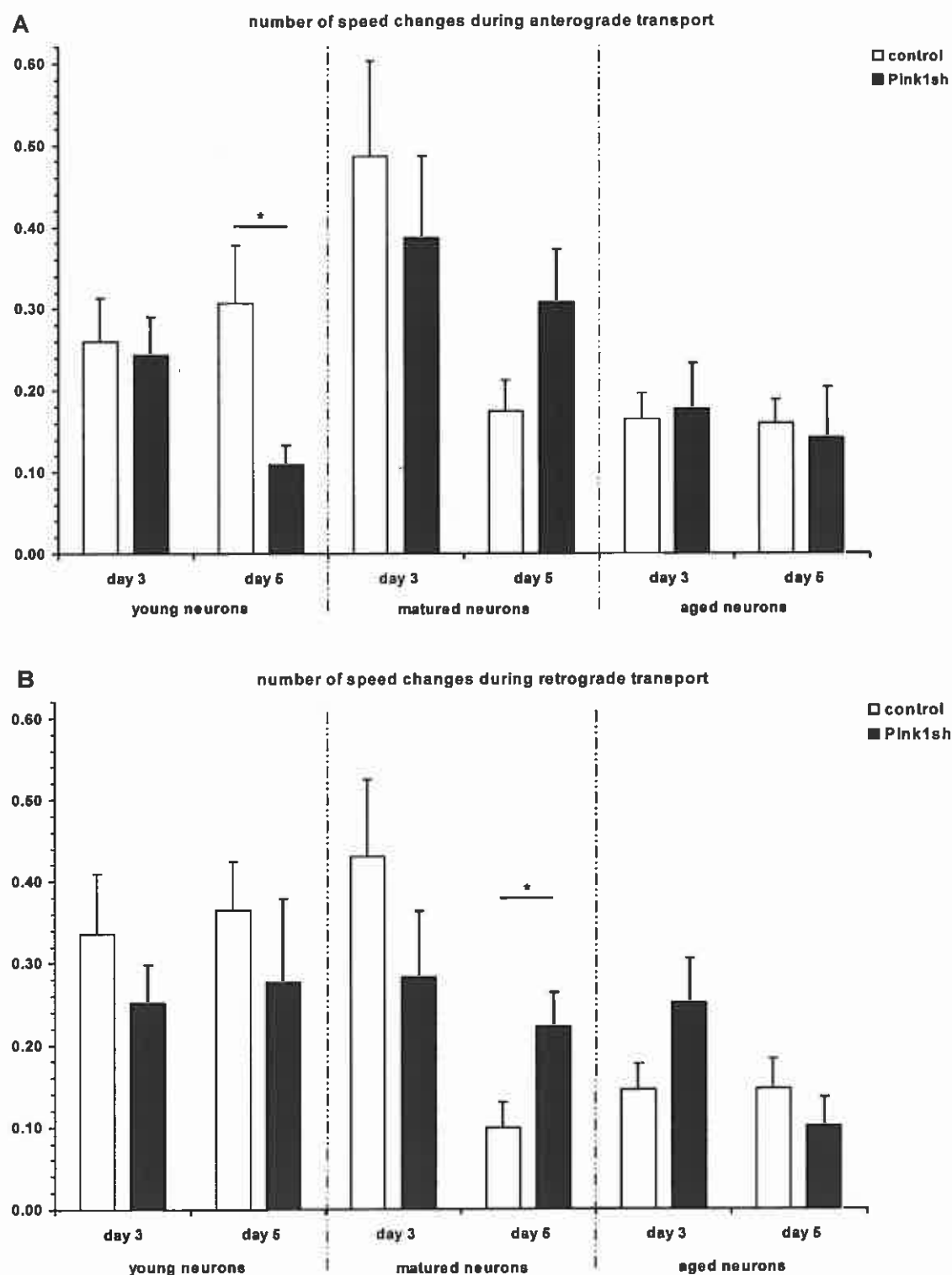


Fig. 35: Average number of speed changes during anterograde (A) and retrograde (B) transport. The number of speed changes of mitochondria during anterograde and retrograde motion is in both cases higher in PINK1-deficient neurons at day 5 of matured neurons. But only in retrograde transport (B) this difference reached significance. However, in anterograde transport (A), the number of speed changes of neurons with acute knockdown of PINK1 is significantly decreased at day 5 of the young neurons.

Mitochondrial direction changes in acute PINK1 knockdown primary cortex neurons												
direction changes	young neurons				matured neurons				aged neurons			
	day 3		day 5		day 3		day 5		day 3		day 5	
	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh
percentage of mitochondria with direction changes during motion (independent of holding phase)	32.63 ± 6.08	29.31 ± 3.51	40.27 ± 8.25	21.54 ± 4.70	36.02 ± 5.48	33.25 ± 8.6939753	20.38 ± 4.43	33.30 ± 5.73	27.57 ± 3.77	30.75 ± 6.10	27.74 ± 5.21	16.07 ± 3.46
p-value	0.640		0.063		0.790		0.088		0.664		0.073	
number of direction changes during motion	1.20 ± 0.34	0.83 ± 0.14	1.48 ± 0.36	0.80 ± 0.25	1.49 ± 0.27	1.40 ± 0.47	0.60 ± 0.15	1.16 ± 0.24	0.69 ± 0.13	0.91 ± 0.22	0.99 ± 0.21	0.49 ± 0.15
p-value	0.319		0.135		0.681		0.089		0.380		0.066	
number of direction changes during motion / 10 frames	0.14 ± 0.04	0.09 ± 0.01	0.15 ± 0.04	0.08 ± 0.02	0.11 ± 0.02	0.10 ± 0.03	0.03 ± 0.01	0.08 ± 0.02	0.04 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.03 ± 0.01
p-value	0.276		0.136		0.942		0.035		0.308		0.157	
number of speed changes during mitochondrial motion	0.60 ± 0.12	0.50 ± 0.07	0.87 ± 0.12	0.39 ± 0.12	0.92 ± 0.19	0.67 ± 0.16	0.27 ± 0.06	0.53 ± 0.10	0.31 ± 0.06	0.43 ± 0.11	0.31 ± 0.06	0.24 ± 0.09
p-value	0.283		0.196		0.324		0.043		0.380		0.684	
number of speed changes during anterograde phase	0.26 ± 0.05	0.24 ± 0.05	0.31 ± 0.07	0.11 ± 0.02	0.48 ± 0.12	0.39 ± 0.10	0.17 ± 0.04	0.31 ± 0.06	0.17 ± 0.03	0.19 ± 0.06	0.16 ± 0.03	0.14 ± 0.06
p-value	0.818		0.018		0.517		0.088		0.863		0.781	
number of speed changes during retrograde phase	0.34 ± 0.07	0.25 ± 0.05	0.36 ± 0.06	0.28 ± 0.10	0.43 ± 0.09	0.28 ± 0.08	0.10 ± 0.03	0.22 ± 0.04	0.15 ± 0.03	0.25 ± 0.06	0.15 ± 0.04	0.10 ± 0.03
p-value	0.344		0.470		0.244		0.024		0.118		0.374	

Tab. 14: Mitochondrial direction changes in acute PINK1 knockdown primary cortex neurons.

4.2.1.4 Mitochondrial stop phases in acute PINK1 knockdown primary neurons

"A mitochondrion can either move continually over a long distance at a relatively constant speed, or pause sporadically and start again with a different speed or direction" [Lovas and Wang; 2012]. "In neurites approximately 30-40 % of total mitochondria are engaged in salutatory movement at any given time" [Wang and Schwarz et al., 2009; Lovas and Wang; 2012] and mitochondria undergo rapid but intermittent transport [Chada and Hollenbeck, 2003]. As known so far, the stop phases is expected to perform mitofusion processes, mutual exchange of information or waiting for new instructions from the cell, etc.. Some publications even describe an up to 80 % immobile phase during the observation period [Baloh, 2008; Cai and Sheng, 2009]. For that reason and particularly with regard to our interest relating to mitofission and -fusion processes, I additionally investigated the percentage of mitochondria with holding stops during motion, as well as the average duration of stop time, the number of mitochondrial stops and the number of stops after anterograde and retrograde motion.

There were no differences between the average percentage of mitochondria with holding stops during motion in young and matured neurons and also not at aged neurons three days after lentivirustransduction (young neurons day 3: control 22.85 ± 4.21 , Pink1sh 22.92 ± 3.31 , $p = 0.989$; young neurons day 5: control 26.72 ± 4.99 , Pink1sh 15.43 ± 3.95 , $p = 0.090$; matured neurons day 3: control 25.43 ± 3.57 , Pink1sh 19.03 ± 4.26 , $p = 0.259$;

matured neurons day 5: control 13.62 ± 2.73 , Pink1sh 21.75 ± 3.88 , $p = 0.099$; aged neurons day 3: control 16.51 ± 2.86 , Pink1sh 16.65 ± 2.97 , $p = 0.974$). Only in aged neurons 5 days after acute Pink1 knockdown a significant decrease of mitochondria with holding stops occur (aged neurons day 5: control 21.19 ± 3.39 , Pink1sh 10.55 ± 3.15 , $p = 0.029$). But if we have a closer look at the individual values (Tab. 15) and especially at the graph (Fig. 36), it can be recognized that the values are inversely proportional. Whenever the distribution of mitochondria with stop phases during motion increases in control mitochondria from day 3 to day 5, it decreases in Pink1sh mitochondria and vice versa.

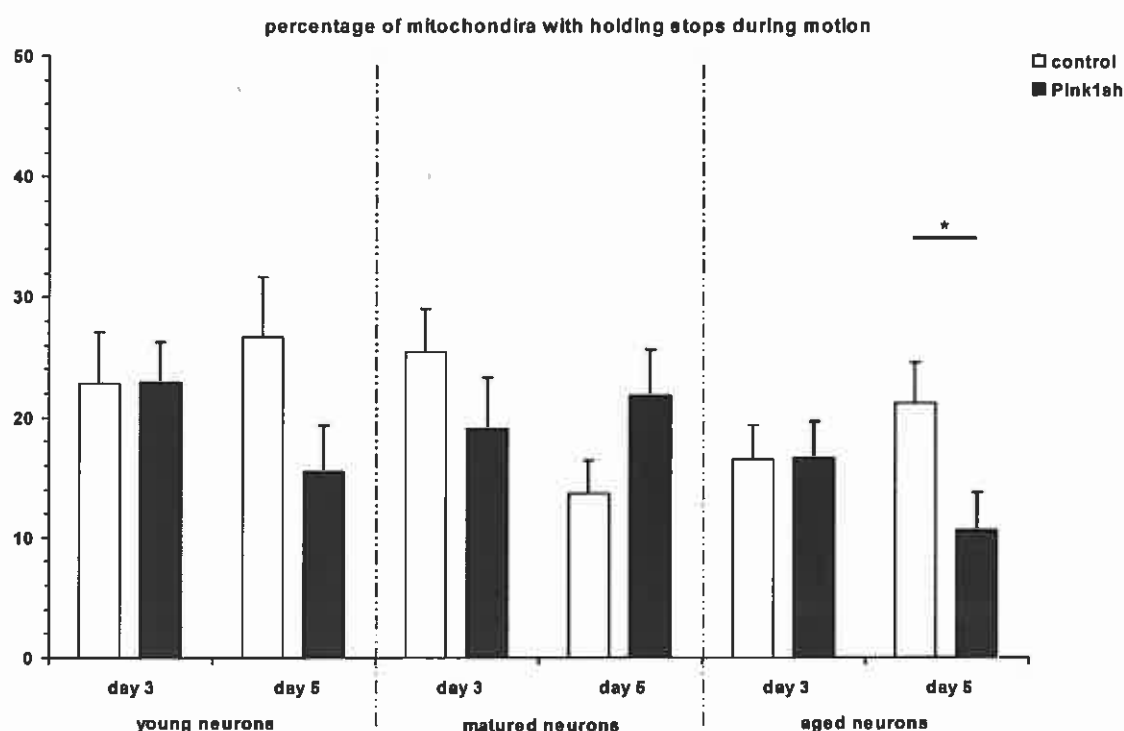


Fig. 36: Percentage of mitochondria with holding stops during motion. On day five of each developmental state, there are differences between both genotypes (Pink1sh and control), but only in aged neurons this differences are significant. On day 3, however, the percentage of mitochondria with holding stops during motion is almost the same in each developmental state.

The measurements of the average duration of mitochondrial stop time are specified in seconds. The time when mitochondria stay in a sporadic break increases with the continuous differentiation and networking of the neurons, in both, in the controls, as in the PINK1-deficient neurons. During a observation period of 10 minutes in which mitochondria under normal conditions are moving, they remain approximately 2 minutes in rest within young neurons *in vitro* (young control neurons: day 3 120.10 ± 9.84 ; day 5 125.94 ± 10.87). With increasing differentiation the break time also increases up to 5 minutes (matured control neurons day 3: 212.81 ± 17.89 ; day 5: 252.61 ± 30.49 ; aged control neurons day 3: 307.64 ± 24.59 ; day 5: 289.94 ± 15.75). This means that mitochondria of the aged neurons

in vitro remain resting half of their time in which they perform a movement. Comparing the acute PINK1 knockdown neurons with control ones at each time point after virustransfection and differentiation state (see Tab.15 and Fig. 37), no changes could be found except a significant increase ($p = 0.021$) of stop time duration in PINK1-deficient neurons at day 5 after acute knockdown in aged neurons, where they remain for more than 6 minutes at rest (aged neurons day 5: Pink1sh 372.10 ± 28.52).

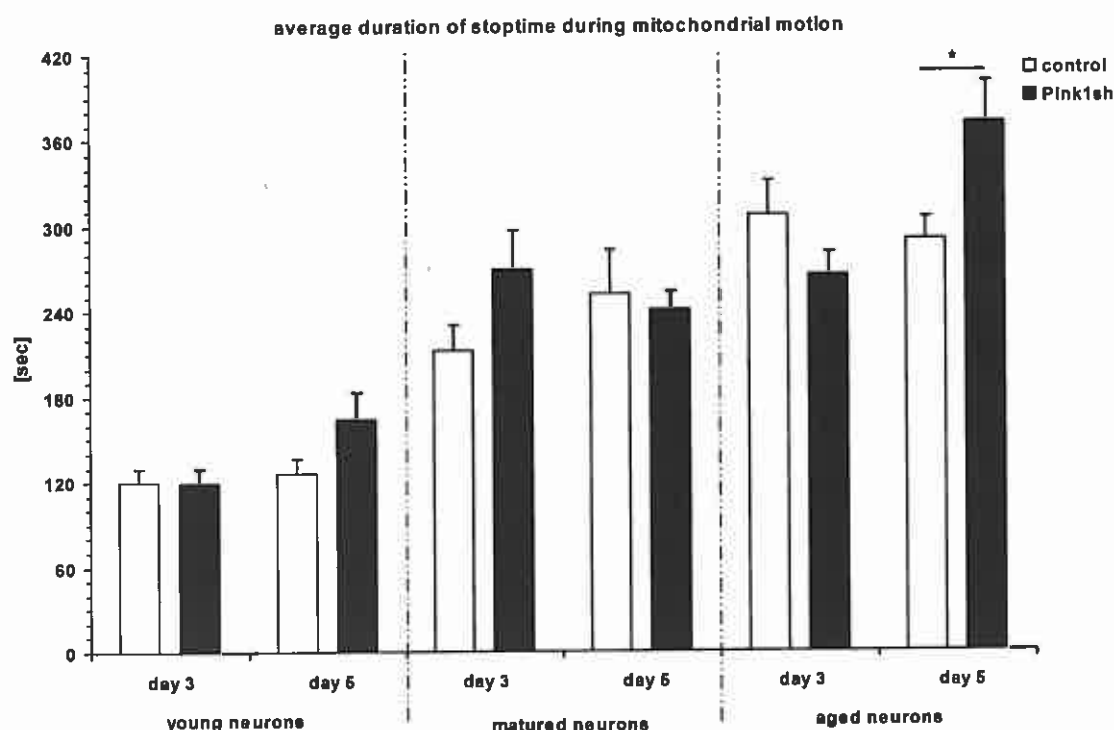


Fig. 37: Average duration of stop time during mitochondrial motion. With increasing developmental state and networking of neurons, the stoptime of mitochondria during motion also increases.

No significant changes were found for the average number of stops which mitochondria perform during their movement through the neuronal processes (young neurons day 3: control 0.34 ± 0.07 , Pink1sh 0.30 ± 0.04 , $p = 0.686$; young neurons day 5: control 0.49 ± 0.10 , Pink1sh 0.33 ± 0.07 , $p = 0.218$; matured neurons day 3: control 0.45 ± 0.07 , Pink1sh 0.43 ± 0.12 , $p = 0.833$; matured neurons day 5: control 0.23 ± 0.05 , Pink1sh 0.40 ± 0.08 , $p = 0.083$; aged neurons day 3: control 0.28 ± 0.05 , Pink1sh 0.31 ± 0.07 , $p = 0.709$; aged neurons day 5: control 0.39 ± 0.07 , Pink1sh 0.19 ± 0.07 , $p = 0.059$; Tab.15), see Fig.38. Although day 3 at each developmental state *in vitro* is very consistent between healthy and Pink1-deficient mitochondria, it is always possible to detect an interesting tendency of difference at day 5.

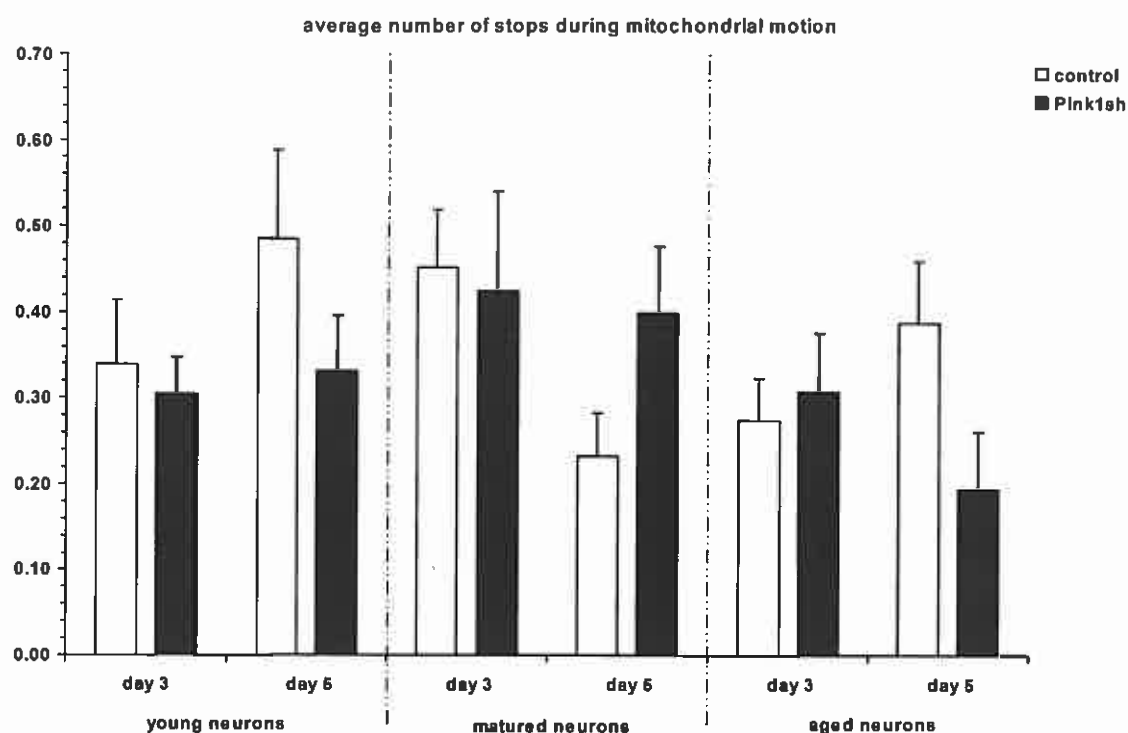


Fig. 38: Average number of stops during mitochondrial motion shows no changes for Pink1sh neurons at each developmental state *in vitro*.

Furthermore, if the average number of stops in mitochondrial transport under normal conditions will be divided in anterograde (Fig. 39A) and retrograde (Fig. 39B) movement, no changes in the anterograde motion is obvious at each differentiation and transduction time point, but a significant decrease of stops after retrograde movement of mitochondria occur in aged neurons 5 days after acute knockdown of PINK1 ($p = 0.019$). An overview of all values can be seen in Tab. 15.

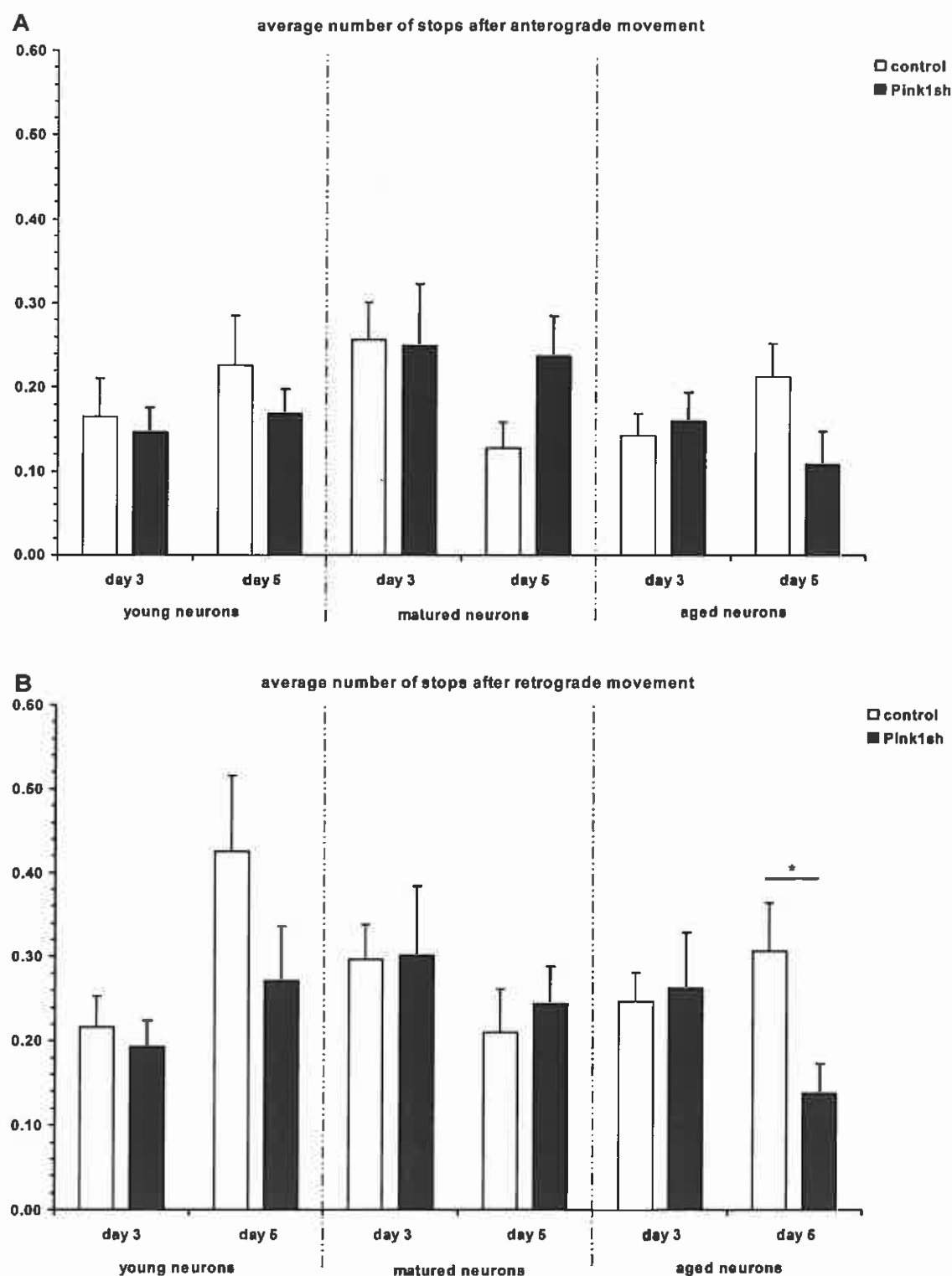


Fig. 39: Average number of stops after anterograde (A) and retrograde (B) movement. Nearly no obvious changes in the number of stops after anterograde or retrograde motion are detectable, except a significant reduction of stop numbers within the retrograde motion of aged PINK1-deficient cells five days after acute knockdown.

Mitochondrial stop phases in acute PINK1 knockdown primary cortex neurons												
holding stops	young neurons				matured neurons				aged neurons			
	day 3		day 5		day 3		day 5		day 3		day 5	
	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh
percentage of mitochondria with holding stops during motion	22.85 ± 4.21	22.82 ± 3.31	26.72 ± 4.99	15.43 ± 3.95	25.43 ± 3.57	19.03 ± 4.26	13.62 ± 2.73	21.75 ± 3.88	16.51 ± 2.86	16.65 ± 2.97	21.19 ± 3.39	10.55 ± 3.15
p-value	0.989		0.080		0.259		0.099		0.974		0.029	
average duration of stop time during mitochondrial motion	120.10 ± 9.84	119.59 ± 10.07	125.94 ± 10.87	164.19 ± 18.64	212.81 ± 17.89	269.30 ± 27.768	252.61 ± 30.49	241.04 ± 13.31	307.64 ± 24.59	265.51 ± 15.87	289.94 ± 15.75	372.10 ± 28.52
p-value	0.971		0.097		0.100		0.732		0.166		0.021	
number of mitochondrial stops during motion	0.34 ± 0.07	0.30 ± 0.04	0.49 ± 0.10	0.33 ± 0.07	0.45 ± 0.07	0.43 ± 0.12	0.23 ± 0.05	0.40 ± 0.08	0.28 ± 0.05	0.31 ± 0.07	0.39 ± 0.07	0.18 ± 0.07
p-value	0.688		0.218		0.833		0.083		0.709		0.069	
number of stops after anterograde motion	0.16 ± 0.05	0.15 ± 0.03	0.23 ± 0.06	0.17 ± 0.03	0.26 ± 0.04	0.25 ± 0.07	0.13 ± 0.03	0.24 ± 0.05	0.14 ± 0.03	0.16 ± 0.03	0.21 ± 0.04	0.11 ± 0.04
p-value	0.747		0.401		0.853		0.066		0.867		0.069	
number of stops after retrograde motion	0.22 ± 0.04	0.19 ± 0.03	0.43 ± 0.09	0.27 ± 0.07	0.30 ± 0.04	0.30 ± 0.08	0.21 ± 0.05	0.24 ± 0.04	0.25 ± 0.035	0.26 ± 0.07	0.31 ± 0.06	0.14 ± 0.03
p-value	0.824		0.179		0.987		0.818		0.827		0.075	

Tab. 15: Mitochondrial stop phases in acute PINK1 knockdown primary cortex neurons.

4.2.1.5 Mitochondrial traveled distance in acute PINK1 knockdown primary neurons

Not only impaired mobility but also altered distribution of mitochondria may play a role in Parkinson's disease as it was shown in a wide range of other neurodegenerative diseases [Baloh et al., 2007; De Vos et al., 2007; Atkin et al., 2011; Wang et al., 2011; Lovas and Wang, 2012]. Distribution of mitochondria varies in response to multiple factors such as energy requirements, growth factors or the membrane potential of the mitochondria [Morris and Hollenbeck, 1993; Chada and Hollenbeck, 2004; Miller and Sheetz, 2004; Rice and Gelfand, 2006]. Changes in the distance traveled may give a first hint of different mitochondria distribution within neurons. Therefore, I searched for changes in the traveled distance from the origin point of the measurement, the whole traveled distance (meaning anterograde and retrograde distance summarized), the distance traveled into anterograde direction and the distance traveled into retrograde direction. The unit of the distance is given in μm . Retrograde distances are specified as negative numbers, whereas anterograde distances are given as positive numbers.

At the checkpoint "traveled distance from origin point" the position of the mitochondria are localized at the beginning of the observed timeframe and at the end. Thus, the net movement was measured. Anterograde and retrograde traveled path from a bidirectional movement may cancel each other in this measurement. The p-value of the Student's *t*-test is not calculable due to the different algebraic sign. Interestingly, both the control and the Pink1sh mitochondria moving more retrograde in young neurons *in vitro* (young neurons day 3: control -0.41 ± 0.19 , Pink1sh -0.18 ± 0.23 ; young neurons day 5: control -0.10 ± 0.40 ,

Pink1sh -0.19 ± 0.26), whereas both moving more anterograde in matured neurons (matured neurons day 3: control 0.14 ± 0.24 , Pink1sh 0.15 ± 0.25 ; matured neurons day 5: control 0.30 ± 0.19 , Pink1sh 0.23 ± 0.1). Also in aged neurons, mitochondria travel more retrograde, except the control ones at day 3, which were slightly moving anterograde (aged neurons day 3: control 0.04 ± 0.09 , Pink1sh 0.07 ± 0.12 ; matured neurons day 5: control 0.09 ± 0.08 , Pink1sh 0.04 ± 0.07), see Fig. 40 and table 16.

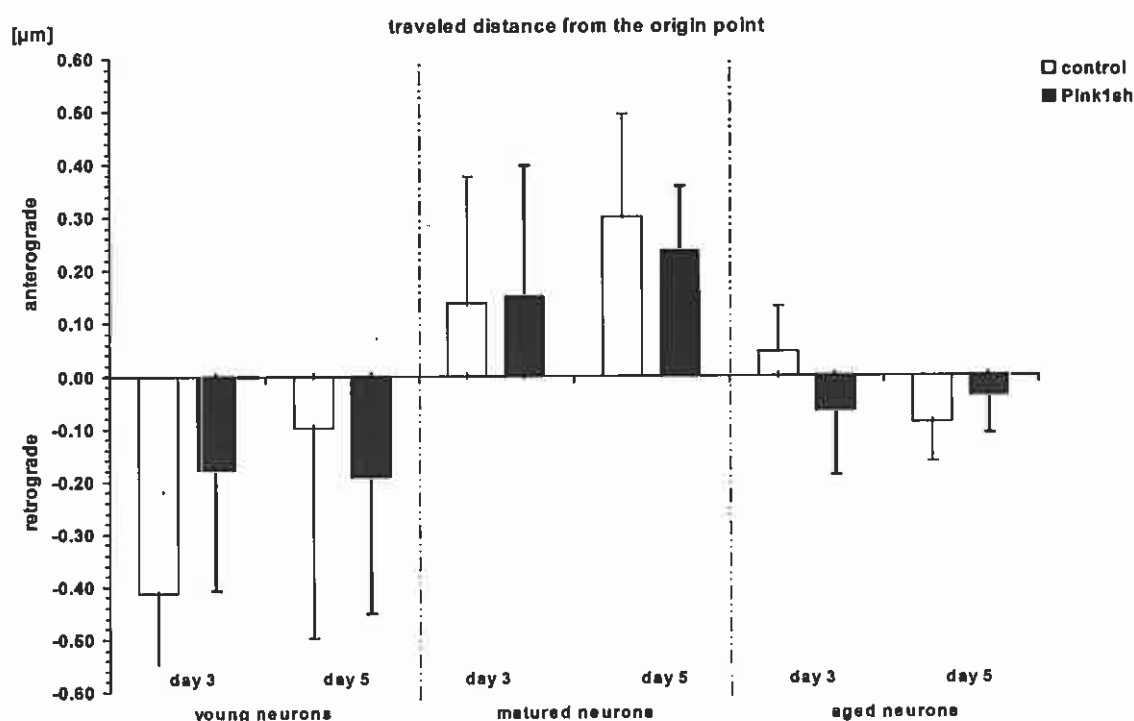


Fig. 40: Mitochondria traveled distance from the origin point in acute PINK1- deficient primary neurons show similar patterns to mitochondria under normal conditions.

Gross movement of mitochondria is given in the data point "whole traveled distance", where the total distance traveled is represented (see Tab. 16 and Fig. 41). Although mitochondria in healthy neurons of young and day 3 in matured stages have a tendency to travel longer distances than their comparable PINK1sh representatives, there are obviously no significant differences (young neurons day 3: control 2.75 ± 0.47 , Pink1sh 1.86 ± 0.29 , $p = 0.120$; young neurons day 5: control 3.24 ± 0.64 , Pink1sh 2.02 ± 0.43 , $p = 0.132$; matured neurons day 3: control 5.38 ± 1.30 , Pink1sh 3.19 ± 0.64 , $p = 0.145$). The general distance traveled is decreasing in aged neurons in both groups (matured neurons day 5: control 1.66 ± 0.35 , Pink1sh 3.48 ± 0.97 , $p = 0.096$; aged neurons day 3: control 1.09 ± 0.14 , Pink1sh 2.17 ± 0.55 , $p = 0.085$; aged neurons day 5: control 1.45 ± 0.32 , Pink1sh 1.33 ± 0.50 , $p = 0.840$). But even here there are no significant differences. Again, a slight delay and lower response is visible in PINK1-deficient neurons compared to control ones.

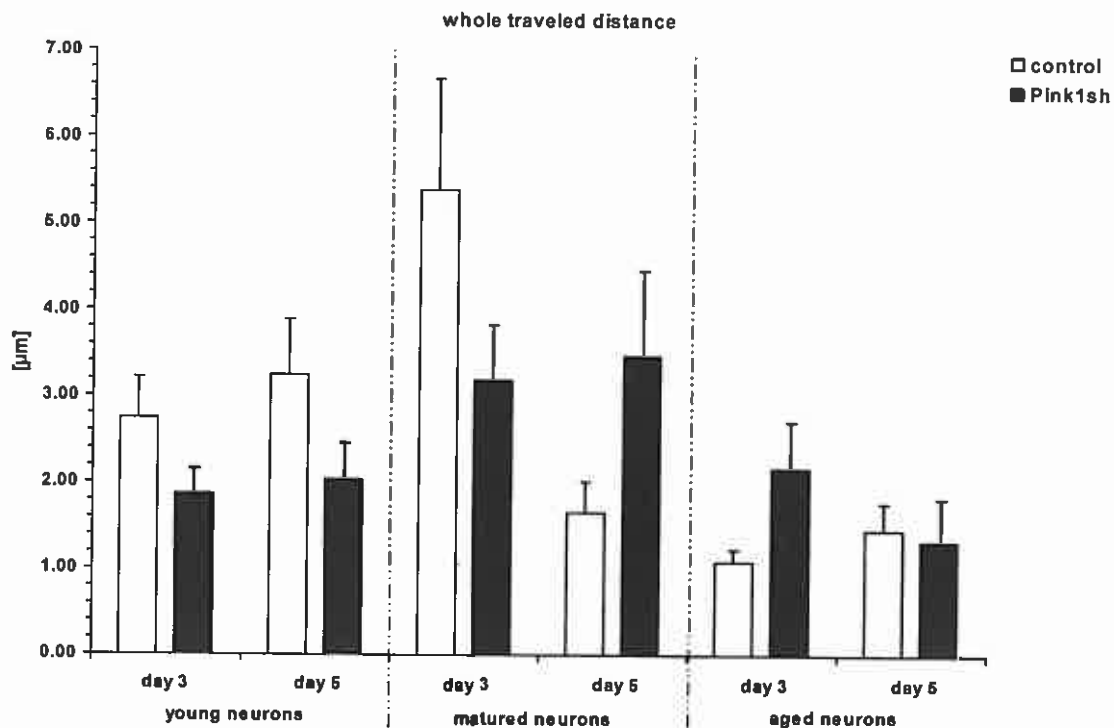


Fig. 41: Whole traveled distance of mitochondria within an observation time of 10 to 15 minutes.

The distance traveled in only anterograde moving mitochondria show a similar trend towards an increase of the distance in young and matured mitochondria with a decrease in older ones (young neurons day 3: control 1.17 ± 0.21 , Pink1sh 0.84 ± 0.16 , $p = 0.225$; young neurons day 5: control 1.57 ± 0.37 , Pink1sh 0.92 ± 0.29 , $p = 0.175$; matured neurons day 3: control 2.76 ± 0.69 , Pink1sh 1.67 ± 0.37 , $p = 0.178$; matured neurons day 5: control 0.98 ± 0.22 , Pink1sh 1.86 ± 0.48 , $p = 0.115$; aged neurons day 3: control 0.57 ± 0.07 , Pink1sh 1.05 ± 0.27 , $p = 0.112$; aged neurons day 5: control 0.68 ± 0.14 , Pink1sh 0.65 ± 0.26 , $p = 0.906$) as we can see it in the whole traveled distance. But even here there are no significant differences between control and PINK1-deficient mitochondria at the corresponding days after virus transfection (see Tab. 16, Fig. 42A).

Similar observations can be made in the retrograde distance traveled (young neurons day 3: control -1.58 ± 0.29 , Pink1sh -1.02 ± 0.21 , $p = 0.128$; young neurons day 5: control -1.67 ± 0.39 , Pink1sh -1.11 ± 0.21 , $p = 0.222$; matured neurons day 3: control -2.62 ± 0.63 , Pink1sh -1.52 ± 0.32 , $p = 0.133$; matured neurons day 5: control -0.68 ± 0.18 , Pink1sh -1.62 ± 0.50 , $p = 0.093$; aged neurons day 3: control -0.52 ± 0.09 , Pink1sh -1.12 ± 0.29 , $p = 0.079$; aged neurons day 5: control -0.77 ± 0.19 , Pink1sh -0.68 ± 0.25 , $p = 0.783$; Tab.16, Fig.42B).

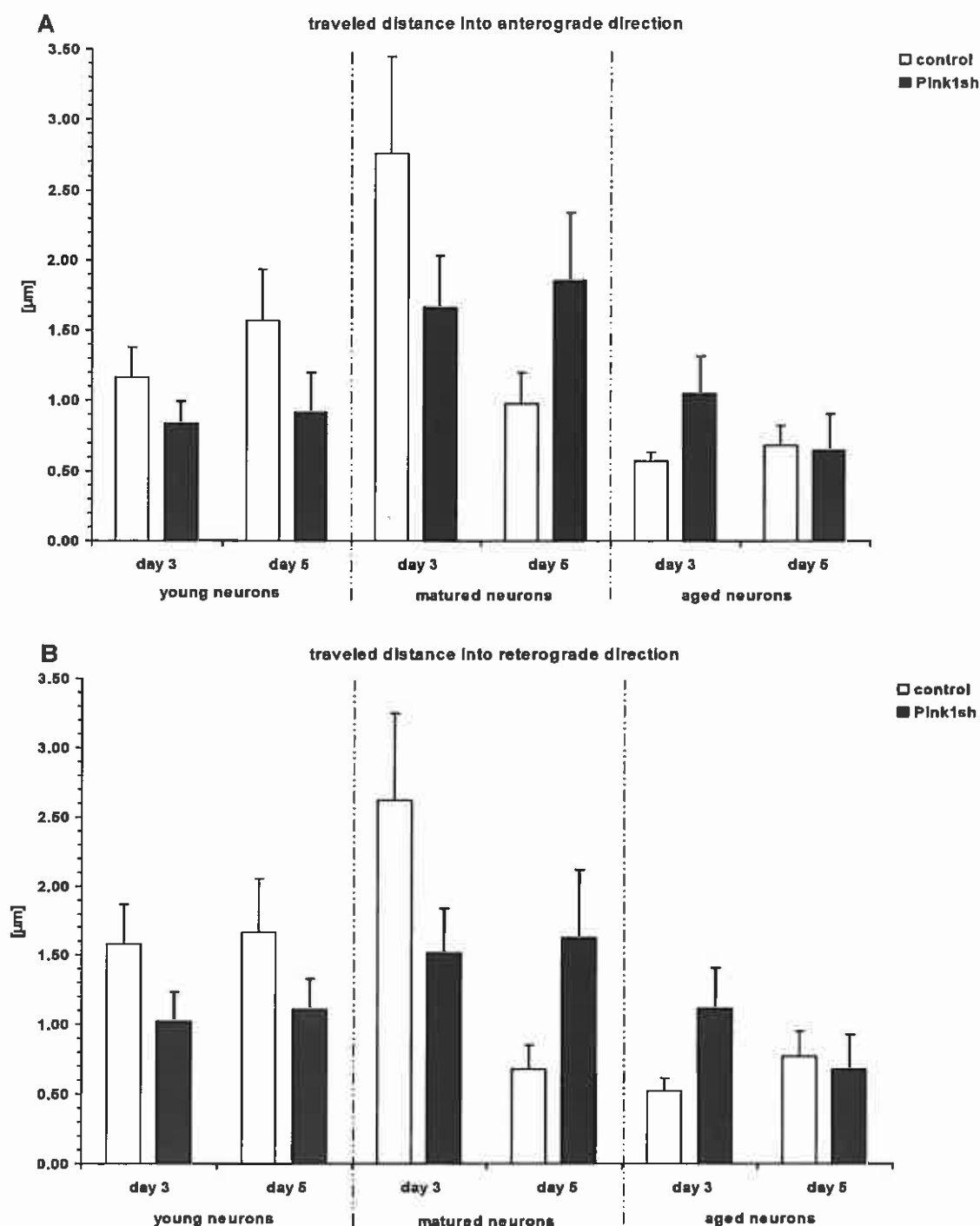


Fig. 42: Traveled distance into anterograde (A) and retrograde (B) direction. In young neurons mitochondria under normal conditions travel a longer distance than the mitochondria of acute PINK1 knockdown neurons, whereas in older neurons this ratio turns. Here, the PINK1-deficient neurons are the ones, in which mitochondria travel the longest distance.

Mitochondrial traveled distance in acute PINK1 knockdown primary cortex neurons												
traveled distance	young neurons				matured neurons				aged neurons			
	day 3		day 5		day 3		day 5		day 3		day 5	
	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh
traveled distance from origin point	-0.41 [±] 0.18	-0.18 [±] 0.23	-0.10 [±] 0.40	-0.19 [±] 0.26	0.14 [±] 0.24	0.15 [±] 0.25	0.30 [±] 0.19	0.23 [±] 0.1	0.04 [±] 0.09	-0.07 [±] 0.12	-0.09 [±] 0.08	-0.04 [±] 0.07
p-value	not calculable		not calculable		not calculable		not calculable		not calculable		not calculable	
whole traveled distance	2.75 [±] 0.47	1.88 [±] 0.29	3.24 [±] 0.64	2.02 [±] 0.43	5.38 [±] 1.30	3.19 [±] 0.64	1.66 [±] 0.35	3.48 [±] 0.97	1.09 [±] 0.14	2.17 [±] 0.55	1.45 [±] 0.32	1.33 [±] 0.50
p-value	0.120		0.132		0.145		0.096		0.086		0.840	
traveled distance into anterograde direction	1.17 [±] 0.21	0.84 [±] 0.16	1.57 [±] 0.37	0.92 [±] 0.29	2.76 [±] 0.89	1.67 [±] 0.37	0.88 [±] 0.22	1.86 [±] 0.48	0.57 [±] 0.07	1.05 [±] 0.27	0.68 [±] 0.14	0.65 [±] 0.26
p-value	0.225		0.178		0.178		0.114		0.112		0.808	
traveled distance into retrograde direction	-1.58 [±] 0.29	-1.02 [±] 0.21	-1.67 [±] 0.39	-1.11 [±] 0.21	-2.62 [±] 0.63	-1.52 [±] 0.32	-0.68 [±] 0.18	-1.82 [±] 0.50	-0.52 [±] 0.09	-1.12 [±] 0.26	-0.77 [±] 0.19	-0.68 [±] 0.25
p-value	0.128		0.222		0.133		0.093		0.079		0.783	

Tab. 16: Mitochondrial traveled distance in acute PINK1 knockdown primary cortex neurons.

4.2.1.6 Complete overview of mitochondrial trafficking in acute PINK1 knockdown primary neurons

An overview of all mitochondrial trafficking values I measured in acute PINK1 knockdown primary cortex neurons is shown in the following table (Tab. 17). The abbreviation n.s. means that there was no significant change, whereas an upward-pointing arrow with asterisk represents an increase in PINK1 acute knockdown mitochondria compared to mitochondria under normal conditions. In turn, a downward-pointing arrow with asterisk represents a decrease in PINK1 acute knockdown mitochondria. The number of asterisk indicates the strength of significance. One asterisk expresses significant of $p \leq 0.05$, two asterisks represent a p-value between 0.01 – 0.001, and three asterisks exhibit highest significances of $p < 0.001$.

Mitochondria trafficking in acute PINK1 knockdown primary cortex neurons												
	young neurons				matured neurons				aged neurons			
	day 3		day 5		day 3		day 5		day 3		day 5	
	control	Pink1 sh	control	Pink1 sh	control	Pink1 sh	control	Pink1 sh	control	Pink1 sh	control	Pink1 sh
average speed of mitochondria	n.s.		n.s.		n.s.		n.s.				n.s.	
average anterograde speed of mitochondria in motion	n.s.		n.s.		n.s.		n.s.				n.s.	
average retrograde speed of mitochondria in motion	n.s.		n.s.		n.s.		n.s.				n.s.	
stationary phase during motion	n.s. but tendency ↑				n.s.				n.s.		n.s.	
anterograde phase during motion	n.s.				n.s.		n.s.		n.s.		n.s.	
retrograde during motion	n.s.		n.s. but tendency ↓		n.s.				n.s.		n.s.	
immobile mitochondria	n.s.				n.s.		n.s.		n.s.			
mobile mitochondria	n.s.				n.s.		n.s.		n.s.			
percentage of mitochondria with direction changes during motion	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.	
number of direction changes during motion	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.	
number of direction changes during motion / 10 frames	n.s.		n.s.		n.s.				n.s.		n.s.	
number of speed changes during mitochondrial motion	n.s.		n.s.		n.s.				n.s.		n.s.	
number of speed changes during anterograde phase	n.s.				n.s.		n.s.		n.s.		n.s.	
number of speed changes during retrograde phase	n.s.		n.s.		n.s.				n.s.		n.s.	
percentage of mitochondria with holding stops during motion	n.s.		n.s.		n.s.		n.s.		n.s.			
average duration of stop time during mitochondrial motion	n.s.		n.s.		n.s.		n.s.		n.s.			
number of mitochondrial stops during motion	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.	
number of stops after anterograde motion	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.	
number of stops after retrograde motion	n.s.		n.s.		n.s.		n.s.		n.s.			
traveled distance from origin point	not calculable		not calculable		not calculable		not calculable		not calculable		not calculable	
whole traveled distance	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.	
traveled distance into anterograde direction	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.	
traveled distance into retrograde direction	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.	

Tab. 17: Mitochondrial trafficking in primary cortex neurons at different time points after acute knockdown of PINK1 and at different developmental states of neurons *in vitro*.

4.2.1.7 Summarized overview of mitochondrial trafficking in acute PINK1-deficient primary neurons at different developmental states and time points after PINK1 down regulation

Given the variety of the observed and calculated measurement points described in detail in the previous sections, the focus here should be only on major and highly compressed results of mitochondrial speed, mobility, direction changes, stop phases and traveled distance, to ensure a better overview. Therefore I created an overview table (see table 18) where measurement points without any changes in acute PINK1-deficient neurons compared to normal PINK1 expressing neurons are presented in gray boxes. Up to three main significant changes are illustrated in orange fields, while red fields indicate values with more than three significant changes. Speed data consist initially of three different values (average speed of mitochondria, average speed of mitochondria during anterograde and retrograde movement). Here changes in all three values are already marked with a red box. All other groups have at least four different values (see also the color code in table 18).

Particular at day 5 significant changes in mitochondria trafficking of acute PINK1-deficient neurons are taking place and almost no change on day 3 after lentivirus transduction. The mobility and direction changes are impaired in young and matured neurons with acute knockdown of PINK1. In aged neurons the mobility is also changed in PINK1-deficient cells, but no differences in direction changes occur. Interestingly, changes of the stop phases come into effect. In addition, changes in mitochondrial speed occur at day 3 of aged PINK1-deficient neurons.

	young neurons		matured neurons		aged neurons	
	day3	day5	day3	day5	day3	day5
speed					= 3	
mobility		> 3		≤ 3		≤ 3
direction changes		≤ 3		≤ 3		
stop phases						≤ 3
traveled distance						

Tab. 18: Summarized results of mitochondria trafficking in acute PINK1-deficient primary cortex neurons at different developmental states of the cells and different time points after virus transduction. Mitochondrial trafficking of PINK1-deficient neurons is changed at day 5 after PINK1 down regulation in all developmental states of the neurons. Aged neurons additionally receive a change on day 3.

4.2.2 Mitochondrial trafficking in acute PINK1-deficient primary neurons during constant stress application

Many studies in recent years showed that Parkinson's disease is a multifactorial disease which is caused by the sum of several factors. Therefore, in addition to the genetic defect I decided to stress the neurons to achieve an even stronger effect in our PINK1-deficient cells. Due to the fact that calcium is a major intracellular messenger and mediates many physiological responses of neurons to chemical and electrical stimulations and that neurons are extremely sensitive to changes in intracellular calcium, I investigated mitochondrial trafficking under constant calcium elevation in the cell culture medium. Therefore, neurons were stressed with two different calcium concentrations (1% and 10% calcium increase in medium) and mitochondrial trafficking was measured regarding speed, mobility, direction changes, stop phases and covered distance. The average observed time of each cell and therefore of each mitochondria was again 10 - 15 minutes. To detect a possible occurring compensatory mechanism, I have also used two different time points after acute knockdown of PINK1, namely day 3 and day 5. Due to the stress indication of 10 % calcium most of the neurons die at day 5 after lentivirus transduction. Therefore, no reproducible data points were collected at this time point and we neglect this data. All other data are expressed as means $\pm$ S.E.M.. Statistical analysis of all data points were carried out using Two-Way ANOVA and Bonferroni posttests. If Two-Way ANOVA shows no interaction ($p > 0.005$) p -values of the ANOVA reveal genotype differences with * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. If Two-Way ANOVA reveal a significant interaction of treatment ($p \geq 0.05$) Bonferroni posttest was performed. The Bonferroni posttest of the analysis software Graph Pad does not provide exact p -values. It only displays if there is a significance or not (ns = not significant). About 5,000 mitochondria during stress application were analyzed.

4.2.2.1 Mitochondrial speed in acute PINK1-deficient primary neurons during constant stress application

To detect speed changes of mitochondria due to neuronal stressors like high calcium overload of the cells, I measured the average speed of mitochondria (including stop phases and mitochondria traveling in both directions, anterograde and retrograde, respectively). All speed data are displayed in $\mu\text{m}/\text{min}$. The average speed of mitochondria is not changed in PINK1-deficient neurons at day 3 post virus transduction (w/o control 0.027 ± 0.019 ; w/o Pink1sh 0.002 ± 0.001 ; 1 % Ca^{2+} control: 0.0010 ± 0.0004 ; 1 % Ca^{2+} Pink1sh: 0.0060 ± 0.0025 ; 10 % Ca^{2+} control: 0.0020 ± 0.0007 ; 10 % Ca^{2+} Pink1sh: 0.0010 ± 0.0004 ; Genotype $\times$ Treatment interaction: 0.2530; $p = 0.3836$; Fig. 43). Whereas at day 5 a significant decrease of mitochondrial speed in Pink1sh neurons is taking place in 1 % calcium stressed

cells (w/o control 0.004 ± 0.001 ; w/o Pink1sh 0.004 ± 0.002 ; 1 % Ca^{2+} control: 0.0101 ± 0.0020 ; 1 % Ca^{2+} Pink1sh: 0.0033 ± 0.0013 ; Genotype x Treatment interaction: 0.0260; Bonferroni p-value: control p = ns; Pink1sh p = **; Fig. 43). The separation into anterograde and retrograde speed of mitochondria also reveals no genotype dependent changes at day 3 within permanent stressed neurons (average anterograde speed of mitochondria in motion at day 3: w/o control 0.031 ± 0.024 ; w/o Pink1sh 0.003 ± 0.000 ; 1 % Ca^{2+} control 0.0014 ± 0.0005 ; 1 % Ca^{2+} Pink1sh: 0.0071 ± 0.0029 ; 10 % Ca^{2+} control: 0.0028 ± 0.0008 ; 10 % Ca^{2+} Pink1sh: 0.0012 ± 0.0003 ; Genotype x Treatment interaction: 0.372; p = 0.5591; average retrograde speed of mitochondria in motion at day 3: w/o control 0.024 ± 0.017 ; w/o Pink1sh 0.003 ± 0.001 ; 1 % Ca^{2+} control 0.0015 ± 0.0005 ; 1 % Ca^{2+} Pink1sh: 0.0062 ± 0.0022 ; 10 % Ca^{2+} control: 0.0029 ± 0.0009 ; 10 % Ca^{2+} Pink1sh: 0.0015 ± 0.0005 ; Genotype x Treatment interaction: 0.2755; p = 0.4000; Fig. 44A+B, table 19). The anterograde speed of mitochondrial transport stays unchanged at 1 % stressed neurons on day 5 after lentivirus transduction (average anterograde speed of mitochondria in motion at day 3: w/o control 0.005 ± 0.001 ; w/o Pink1sh 0.004 ± 0.001 ; 1 % Ca^{2+} control 0.0094 ± 0.0034 ; 1 % Ca^{2+} Pink1sh: 0.0050 ± 0.0013 ; Genotype x Treatment interaction: 0.3831; p = 0.1613), whereas the retrograde speed shows a reduction in speed of PINK1-deficient mitochondria (average retrograde speed of mitochondria in motion at day 3: w/o control 0.005 ± 0.001 ; w/o Pink1sh 0.005 ± 0.001 ; 1 % Ca^{2+} control 0.0123 ± 0.0018 ; 1 % Ca^{2+} Pink1sh: 0.0049 ± 0.0015 ; Genotype x Treatment interaction: 0.0174; Bonferroni p-value of control: p = ns; of Pink1sh p = **; Fig. 44A+B, table 19). At day 3 no significant changes between Pink1sh and control neurons occur via increased stress application of neuron, neither in average speed, nor in anterograde or retrograde speed of mitochondria. For a compact overview of the data please look at table 19.

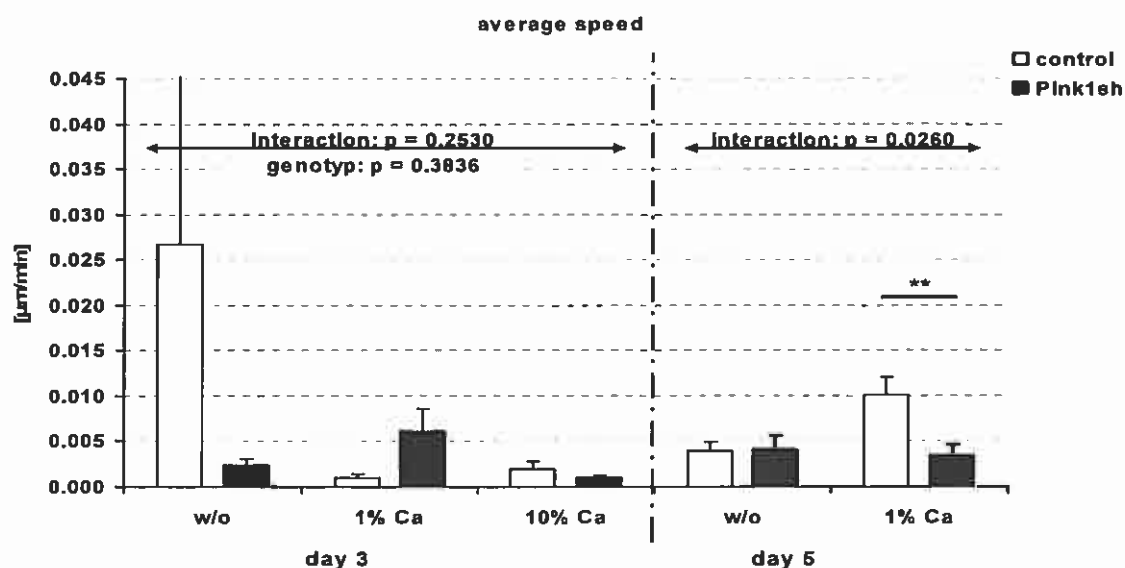


Fig. 43: Average speed of mitochondria during constant stress application. Obviously, there are no significant speed differences of mitochondria between acute PINK1-deficient neurons and controls, except a

decrease in mitochondrial speed of Pink1sh neurons with 1 % permanent calcium treated cells five days after acute knockdown of PINK1.

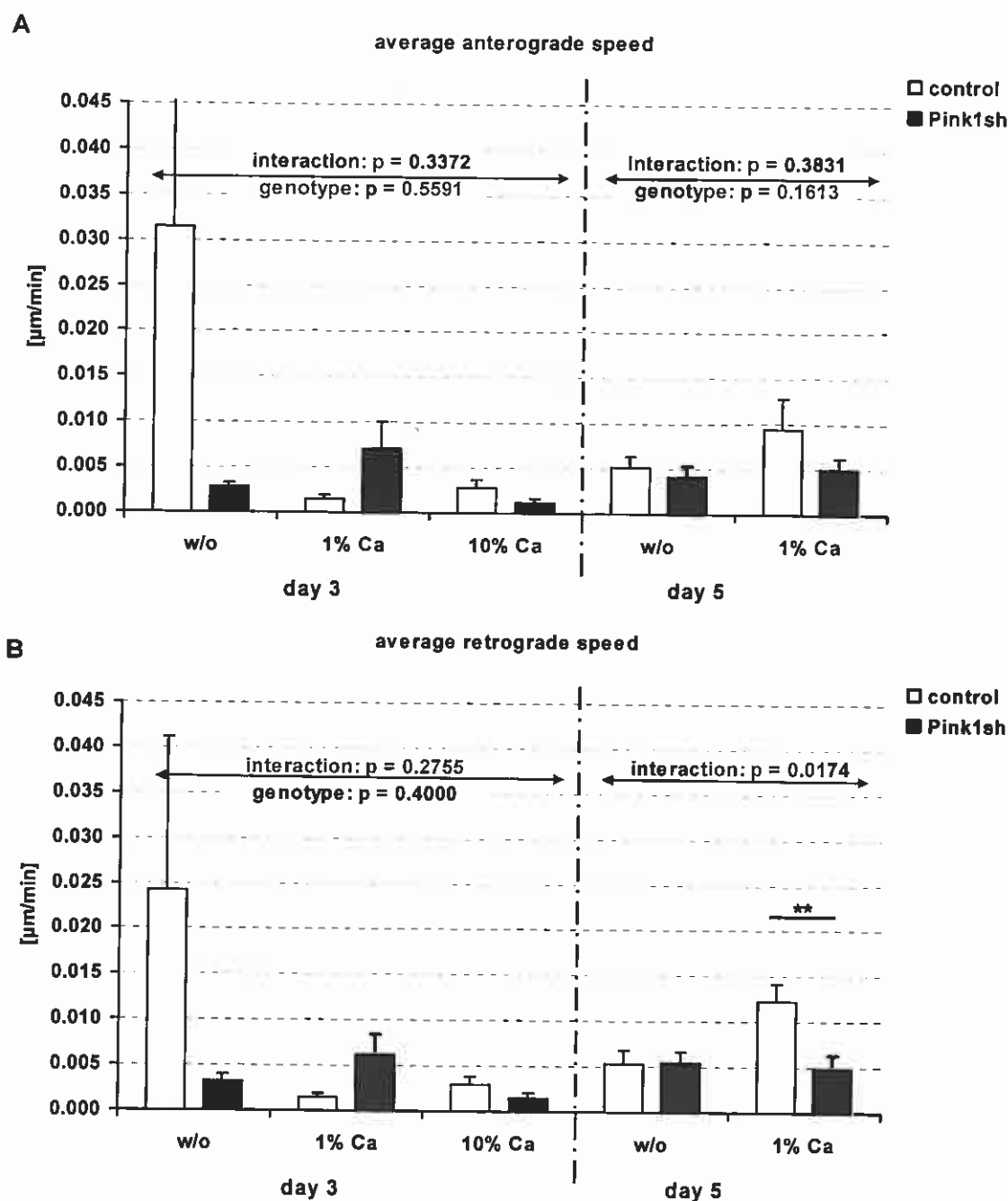


Fig. 44: Average anterograde (A) and retrograde (B) speed of moving mitochondria during constant stress application exhibits also no changes in the mitochondrial speed of acute PINK1-deficient neurons compared to neurons under normal condition (control), except a decrease of retrograde speed in PINK1-deficient neurons in 1 % calcium stressed cells 5 days after lentivirus transduction.

Mitochondrial speed [$\mu\text{m}/\text{min}$] in acute PINK1 knockdown primary cortex neurons during constant stress application										
young neurons	day 3						day 5			
	w/o		1 % Ca^{2+}		10 % Ca^{2+}		w/o		1 % Ca^{2+}	
	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh
average speed of mitochondria	0.027 $\pm$ 0.019	0.002 $\pm$ 0.001	0.0010 $\pm$ 0.0004	0.0060 $\pm$ 0.0025	0.0020 $\pm$ 0.0007	0.0010 $\pm$ 0.0004	0.004 $\pm$ 0.001	0.004 $\pm$ 0.002	0.0101 $\pm$ 0.0020	0.0033 $\pm$ 0.0013
Interaction value	0.2530						0.0260			
p-value	0.3836						ns			
average anterograde speed of mitochondria in motion	0.031 $\pm$ 0.024	0.003 $\pm$ 0.000	0.0014 $\pm$ 0.0005	0.0071 $\pm$ 0.0029	0.0028 $\pm$ 0.0008	0.0012 $\pm$ 0.0003	0.005 $\pm$ 0.001	0.004 $\pm$ 0.001	0.0094 $\pm$ 0.0034	0.0050 $\pm$ 0.0013
Interaction value	0.372						0.3831			
p-value	0.5591						0.1613			
average retrograde speed of mitochondria in motion	0.024 $\pm$ 0.017	0.003 $\pm$ 0.001	0.0015 $\pm$ 0.0005	0.0062 $\pm$ 0.0022	0.0029 $\pm$ 0.0009	0.0015 $\pm$ 0.0005	0.005 $\pm$ 0.001	0.005 $\pm$ 0.001	0.0123 $\pm$ 0.0018	0.0048 $\pm$ 0.0015
Interaction value	0.2755						0.0174			
p-value	0.4000						ns			

Tab. 19: Mitochondrial speed in acute PINK1 knockdown primary cortex neurons during constant stress application via calcium increased environment.

4.2.2.2 Mitochondrial mobility in acute PINK1 knockdown primary neurons during constant stress application

The mobility of mitochondria, which serves as an indicator of energy demand at different sections of the nerve cell and contributes to cell homeostasis, was measured also in acute PINK1 knockdown primary neurons during constant stress application with 1 % and 10 % calcium increased culture medium. Therefore, the following measuring points were recorded: the proportion of the stationary phase during motion (i.e. the relation of movement and stop phases within a mitochondria), the number of anterograde phases as well as retrograde phases during motion and the distribution of immobile and mobile mitochondria. All data are represented in percentage. Although there is a strong increase in the calcium treated neurons at day 3 after lentivirus transfection, no obvious significant changes between acute PINK1 knockdown neurons and neurons under normal condition could be detected (average percentage of stationary phase of mitochondria at day 3: w/o control 38.20 ± 4.85 ; w/o Pink1sh 51.23 ± 4.15 ; 1 % Ca^{2+} control 84.69 ± 3.75 ; 1 % Ca^{2+} Pink1sh: 69.38 ± 6.23 ; 10 % Ca^{2+} control: 68.63 ± 6.97 ; 10 % Ca^{2+} Pink1sh: 82.99 ± 5.01 ; Genotype x Treatment interaction: 0.0151; Bonferroni p-values of w/o, 1 % Ca^{2+} and 10 % Ca^{2+} = ns). Interesting to see is the continuously rise of the stationary phase during mitochondrial transport with elevated calcium concentration in Pink1sh neurons at day 3 after PINK1 post virus

transduction. On day 5 after lentivirus transduction, the situation looks quite different. Mitochondria without calcium treatment (w/o) have per se a higher rate of stationary phases with significant differences between Pink1sh and control neurons. At this time point a significant increase of stationary phase in PINK1-deficient neurons can be obtained dependent on calcium stress (average percentage of stationary phase of mitochondria at day 5: w/o control 56.95 ± 5.84 ; w/o Pink1sh 79.44 ± 5.21 ; 1 % Ca^{2+} control 49.86 ± 3.74 ; 1 % Ca^{2+} Pink1sh: 62.98 ± 5.09 ; Genotype x Treatment interaction: 0.3846; $p = 0.0018$). Another interesting and remarkable fact is, that PINK1-deficient neurons always (except day 3 with 1 % calcium stressed cells) have a higher proportion of stationary phases compared to neurons with normally functioning PINK1 at both time points after lentivirus transduction. Similar trends, but thus, anti-proportional can be seen in anterograde phase and retrograde phase during motion. Here, no genotype effect due to stress application could be observed at day 3 post virus transduction (anterograde phase of mitochondria at day 3: w/o control 28.60 ± 3.49 ; w/o Pink1sh 24.21 ± 2.75 ; 1 % Ca^{2+} control 6.03 ± 1.55 ; 1 % Ca^{2+} Pink1sh: 14.81 ± 3.41 ; 10 % Ca^{2+} control 11.04 ± 3.39 ; 10 % Ca^{2+} Pink1sh: 5.7 ± 1.58 ; Genotype x Treatment interaction: 0.0439; Bonferroni p-values of w/o, 1 % Ca^{2+} and 10 % Ca^{2+} = ns; retrograde phase of mitochondria at day 3: w/o control 33.19 ± 3.68 ; w/o Pink1sh 24.56 ± 2.54 ; 1 % Ca^{2+} control 9.28 ± 2.45 ; 1 % Ca^{2+} Pink1sh: 15.48 ± 3.26 ; 10 % Ca^{2+} control 13.2 ± 2.72 ; 10 % Ca^{2+} Pink1sh: 8.43 ± 1.98 ; Genotype x Treatment interaction: 0.0453; Bonferroni p-values of w/o, 1 % Ca^{2+} and 10 % Ca^{2+} = ns; Fig. 45). But at day 5 PINK1-deficient cells show significant impairment in the anterograde and retrograde phase during mitochondrial trafficking (anterograde phase of mitochondria at day 5: w/o control 19.21 ± 3.38 ; w/o Pink1sh 7.50 ± 1.53 ; 1 % Ca^{2+} control 24.24 ± 2.35 ; 1 % Ca^{2+} Pink1sh: 16.87 ± 2.50 ; Genotype x Treatment interaction: 0.4405; $p = 0.0015$; retrograde phase of mitochondria at day 5: w/o control 23.85 ± 3.58 ; w/o Pink1sh 13.07 ± 3.81 ; 1 % Ca^{2+} control 25.81 ± 2.12 ; 1 % Ca^{2+} Pink1sh: 19.98 ± 2.82 ; Genotype x Treatment interaction: 0.4611; $p = 0.0167$; Fig. 45).

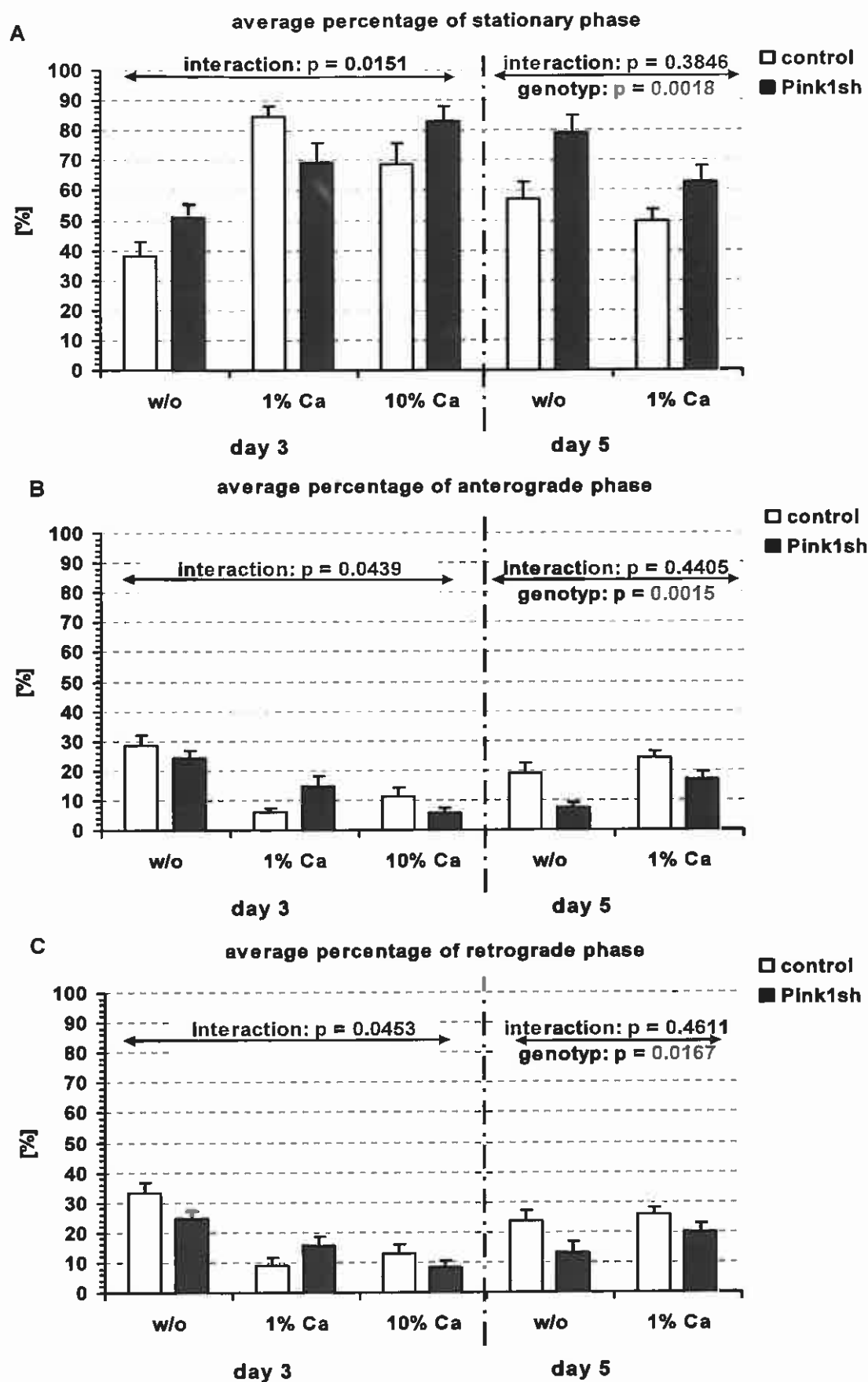


Fig. 45 previous page: Average percentage of stationary (A), anterograde (B) and retrograde (C) phase during mitochondrial movement with permanent stress application via calcium increased environment at day 3 and 5 after lentivirus transduction in young neurons.

Although these trends exist, no significant changes could be obtained in immobile and mobile mitochondria at day 3, except a significant increase of mitochondrial mobile phase of PINK1-deficient neurons compared to controls in 1 % calcium applicated neurons (immobile mitochondria at day 3: w/o control 24.90 ± 5.30 ; w/o Pink1sh 37.93 ± 4.74 ; 1 % Ca^{2+} control 77.68 ± 5.01 ; 1 % Ca^{2+} Pink1sh: 55.51 ± 8.13 ; 10 % Ca^{2+} control 58.13 ± 7.41 ; 10 % Ca^{2+} Pink1sh: 77.83 ± 4.64 ; Genotype x Treatment interaction: 0.0027; Bonferroni p-values of w/o, 1 % Ca^{2+} and 10 % Ca^{2+} = ns; mobile mitochondria at day 3: w/o control 75.10 ± 5.30 ; w/o Pink1sh 62.07 ± 4.74 ; 1 % Ca^{2+} control 22.32 ± 5.01 ; 1 % Ca^{2+} Pink1sh: 44.49 ± 8.13 ; 10 % Ca^{2+} control 34.74 ± 6.49 ; 10 % Ca^{2+} Pink1sh: 22.17 ± 4.64 ; Genotype x Treatment interaction: 0.0056; Bonferroni p-values of w/o, and 10 % Ca^{2+} = ns; p-values 1% Ca^{2+} = *; Fig. 46). Again, day 5 post virus transduction reveals a significant genotype effect of immobile and mobile mitochondria, comparing Pink1sh neurons and normal Pink1 expressing neurons und increased permanent stressor (immobile mitochondria at day 5: w/o control 38.85 ± 8.11 ; w/o Pink1sh 64.18 ± 7.94 ; 1 % Ca^{2+} control 32.42 ± 5.27 ; 1 % Ca^{2+} Pink1sh: 43.07 ± 7.50 ; Genotype x Treatment interaction: 0.3434; p = 0.0237; mobile mitochondria at day 5: w/o control 61.15 ± 8.11 ; w/o Pink1sh 35.82 ± 7.94 ; 1 % Ca^{2+} control 67.58 ± 5.27 ; 1 % Ca^{2+} Pink1sh: 56.93 ± 7.50 ; Genotype x Treatment interaction: 0.3434; p = 0.0237; Fig. 46).

The general trend of an increase of stationary phase within PINK1-deficient neurons and increased permanent stressor on day 3 and a decrease on day 5 is also reflected in the percentage of immobile mitochondria. I.e. that not only the number of mitochondria which make frequent breaks during their movement rises, also the proportion of moving mitochondria is much smaller within calcium stressed cells at day 3 after lentivirus transduction.

These results reveal, that under permanent calcium stress, PINK1-deficient mitochondria 5 days after acute knockdown of PINK1, behave different than their "healthy" counterparts. This genotypic effect could not be detected on day 3 post virus transduction.

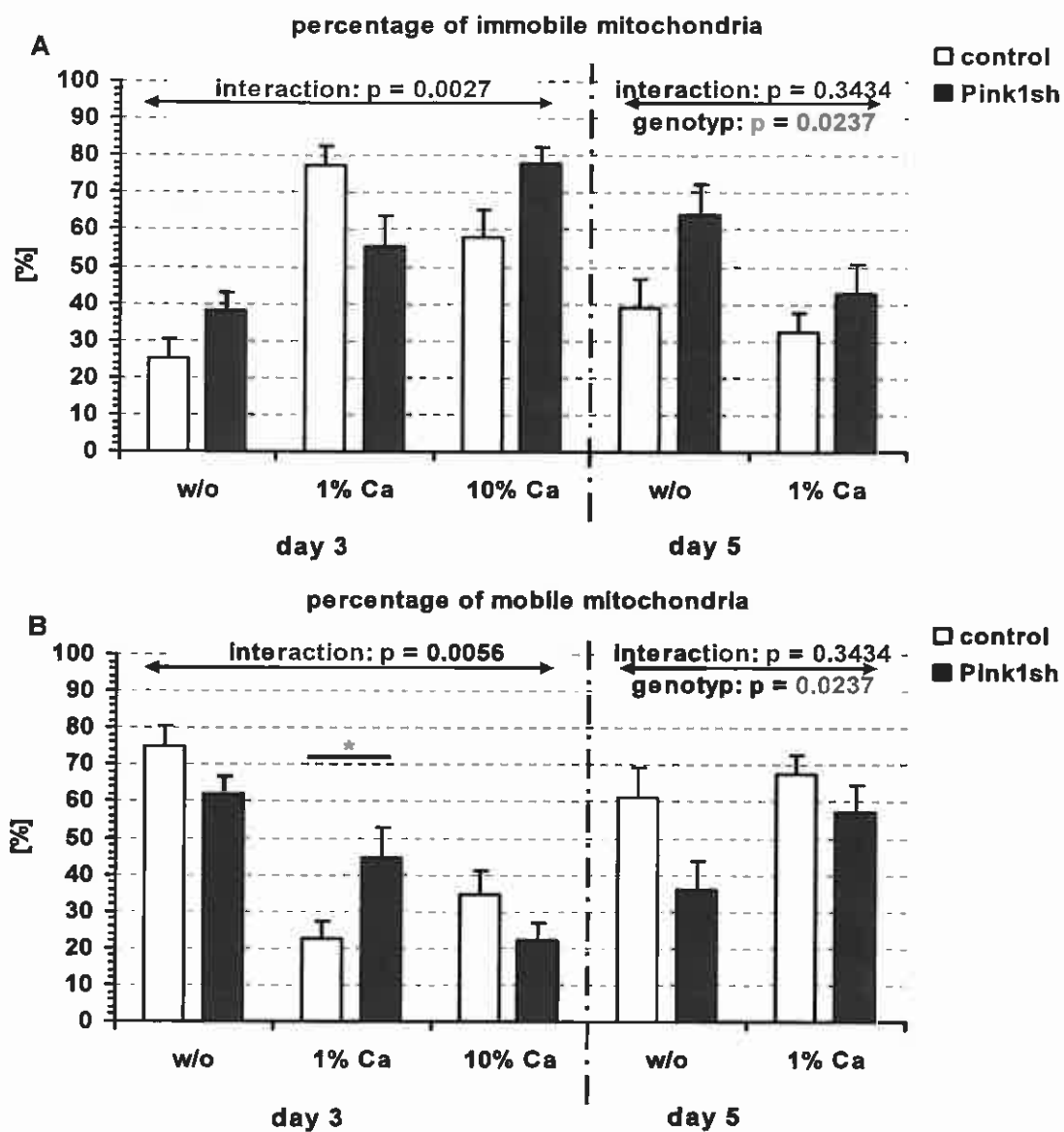


Fig. 46: Average percentage of immobile (A) and mobile (B) mitochondria with permanent stress application via calcium increased environment.

Mitochondrial mobility in acute PINK1 knockdown primary cortex neurons during constant stress application										
young neurons	day 3						day 5			
	w/o		1 % Ca ²⁺		10 % Ca ²⁺		w/o		1 % Ca ²⁺	
	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh
stationary phase during motion	38.20 ± 4.85	51.23 ± 4.15	84.69 ± 3.75	69.38 ± 6.23	68.63 ± 6.97	82.99 ± 5.01	56.95 ± 5.84	79.44 ± 5.21	49.86 ± 3.74	62.98 ± 5.09
Interaction value	0.0151						0.3846			
p-value	ns		ns		ns		0.0015			
anterograde phase during motion	28.60 ± 3.49	24.21 ± 2.75	6.03 ± 1.55	14.81 ± 3.41	11.04 ± 3.39	6.7 ± 1.58	19.21 ± 3.38	7.50 ± 1.53	24.24 ± 2.35	16.87 ± 2.50
Interaction value	0.0439						0.4405			
p-value	ns		ns		ns		0.0015			
retrograde phase during motion	33.19 ± 3.68	24.56 ± 2.54	9.28 ± 2.45	15.48 ± 3.26	13.2 ± 2.72	8.43 ± 1.98	23.85 ± 3.58	13.07 ± 3.81	25.81 ± 2.12	19.98 ± 2.82
Interaction value	0.0453						0.4611			
p-value	ns		ns		ns		0.0167			
immobile mitochondria	24.90 ± 5.30	37.93 ± 4.74	77.68 ± 5.01	55.51 ± 8.13	58.13 ± 7.41	77.83 ± 4.64	36.85 ± 8.11	64.18 ± 7.94	32.42 ± 5.27	43.07 ± 7.50
Interaction value	0.0027						0.3434			
p-value	ns		ns		ns		0.0237			
mobile mitochondria	75.10 ± 5.30	62.07 ± 4.74	22.32 ± 5.01	44.49 ± 8.13	34.74 ± 6.49	22.17 ± 4.64	61.15 ± 8.11	35.82 ± 7.94	67.58 ± 5.27	56.93 ± 7.50
Interaction value	0.0056						0.3434			
p-value	ns		ns		ns		0.0237			

Tab. 20: Mitochondria mobility in acute PINK1 knockdown primary cortex neurons during constant stress application via calcium increased environment.

4.2.2.3 Mitochondrial direction changes in acute PINK1 knockdown primary neurons during constant stress application

Comparing PINK1-deficient neurons with neurons expressing normal PINK1 levels (controls) under increased permanent stress application, no changes due to genotype can be found, in percentage of mitochondria with direction changes and average number of direction changes per 10 frames, neither in day 3 after lentivirus transduction nor at day 5 (percentage of mitochondria with direction changes during motion at day 3: w/o control 32.63 ± 6.08; w/o Pink1sh 29.31 ± 3.51; 1 % Ca²⁺ control 9.95 ± 3.23; 1 % Ca²⁺ Pink1sh: 27.33 ± 7.69; 10 % Ca²⁺ control 19.53 ± 5.108; 10 % Ca²⁺ Pink1sh: 12.87 ± 3.43; Genotype x Treatment interaction: 0.0596; p = 0.5567; percentage of mitochondria with direction changes during motion at day 5: w/o control 40.27 ± 8.25; w/o Pink1sh 21.54 ± 4.70; 1 % Ca²⁺ control 45.97 ± 5.08; 1 % Ca²⁺ Pink1sh: 44.19 ± 6.74; Genotype x Treatment interaction: 0.2293; p = 0.1477; average number of direction changes per 10 frames at day 3: w/o control 0.14 ± 0.04; w/o Pink1sh 0.09 ± 0.01; 1 % Ca²⁺ control 0.03 ± 0.01; 1 % Ca²⁺

Pink1sh: 0.11 ± 0.05 ; 10 % Ca^{2+} control 0.08 ± 0.02 ; 10 % Ca^{2+} Pink1sh: 0.04 ± 0.01 ; Genotype x Treatment interaction: 0.0485; Bonferroni p-values of w/o, 1 % Ca^{2+} and 10 % Ca^{2+} = ns; average number of direction changes per 10 frames at day 5: w/o control 0.15 ± 0.04 ; w/o Pink1sh 0.08 ± 0.02 ; 1 % Ca^{2+} control 0.20 ± 0.03 ; 1 % Ca^{2+} Pink1sh: 0.18 ± 0.04 ; Genotype x Treatment interaction: 0.5308; $p = 0.1997$, Fig. 47A+B, table 21).

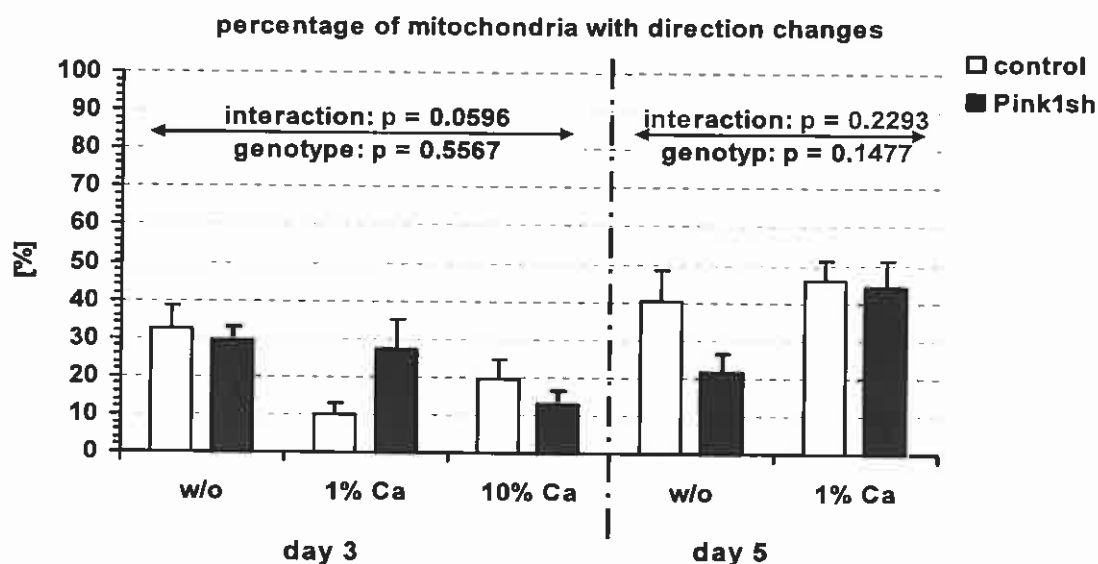


Fig. 47: Percentage of mitochondria with direction changes during motion in acute PINK1 knockdown neurons during constant stress application via calcium increased environment (1 % and 10 % calcium Increase). No obvious changes can be observed between Pink1sh and control neurons at day 3 or day 5. The strength of the stressor as well as the time frame does not seem to affect this value.

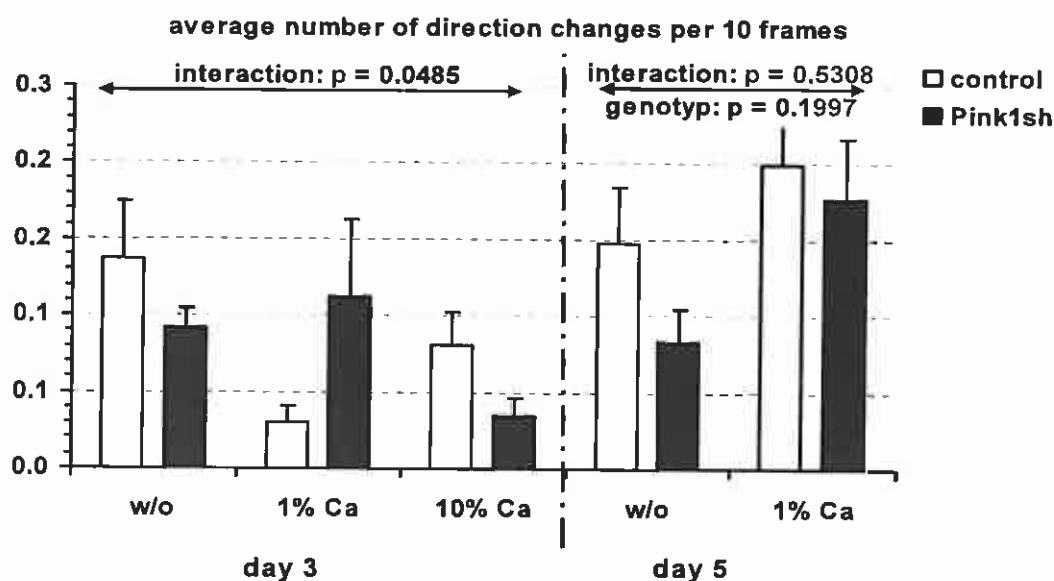


Fig. 48: Average number of direction changes during motion per 10 frames motion in acute PINK1 knockdown neurons during constant stress application via calcium increased environment.

As mentioned before, not only the direction changes of mitochondria but also the number of mitochondrial speed changes could be a key indicator of dysfunctional energy balance. The number of general speed changes, as well as anterograde and retrograde speed changes during mitochondrial trafficking, decreases in acute PINK1 down regulated neurons at day 3 with increased stress, compared to healthy control neurons. But no genotypic effect due to calcium treatment could be detected at day 3 (number of speed changes during mitochondrial motion at day 3: w/o control 0.60 ± 0.12 ; w/o Pink1sh 0.50 ± 0.07 ; 1 % Ca^{2+} control 0.21 ± 0.10 ; 1 % Ca^{2+} Pink1sh: 0.35 ± 0.12 ; 10 % Ca^{2+} control 0.27 ± 0.07 ; 10 % Ca^{2+} Pink1sh: 0.09 ± 0.03 ; Genotype x Treatment interaction: 0.2449; $p = 0.5489$; number of speed changes during anterograde phase at day 3: w/o control 0.26 ± 0.05 ; w/o Pink1sh 0.24 ± 0.05 ; 1 % Ca^{2+} control 0.07 ± 0.03 ; 1 % Ca^{2+} Pink1sh: 0.17 ± 0.06 ; 10 % Ca^{2+} control 0.14 ± 0.05 ; 10 % Ca^{2+} Pink1sh: 0.04 ± 0.01 ; Genotype x Treatment interaction: 0.1170; $p = 0.7907$; number of speed changes during retrograde phase at day 3: w/o control 0.34 ± 0.07 ; w/o Pink1sh 0.25 ± 0.05 ; 1 % Ca^{2+} control 0.14 ± 0.08 ; 1 % Ca^{2+} Pink1sh: 0.19 ± 0.06 ; 10 % Ca^{2+} control 0.13 ± 0.03 ; 10 % Ca^{2+} Pink1sh: 0.06 ± 0.03 ; Genotype x Treatment interaction: 0.4714; $p = 0.4283$). Interestingly, again at day 5 a significant genotype effect occur for the number of general speed changes and the average number of speed changes in anterograde transport, whereas the average number of speed changes during retrograde transport reveal no genotype effect (number of speed changes during mitochondrial motion at day 5: w/o control 0.67 ± 0.12 ; w/o Pink1sh 0.39 ± 0.12 ; 1 % Ca^{2+} control 0.93 ± 0.15 ; 1 % Ca^{2+} Pink1sh: 0.48 ± 0.11 ; Genotype x Treatment interaction: 0.5174; $p = 0.0061$; number of speed changes during anterograde phase at day 5: w/o control 0.31 ± 0.07 ; w/o Pink1sh 0.11 ± 0.02 ; 1 % Ca^{2+} control 0.41 ± 0.05 ; 1 % Ca^{2+} Pink1sh: 0.19 ± 0.05 ; Genotype x Treatment interaction: 0.8412; $p = 0.0007$; number of speed changes during retrograde phase at day 5: w/o control 0.36 ± 0.06 ; w/o Pink1sh 0.28 ± 0.10 ; 1 % Ca^{2+} control 0.52 ± 0.10 ; 1 % Ca^{2+} Pink1sh: 0.29 ± 0.10 ; Genotype x Treatment interaction: 0.3951; $p = 0.0628$). For a detailed overview of the individual values please look at figure 49 and table 21.

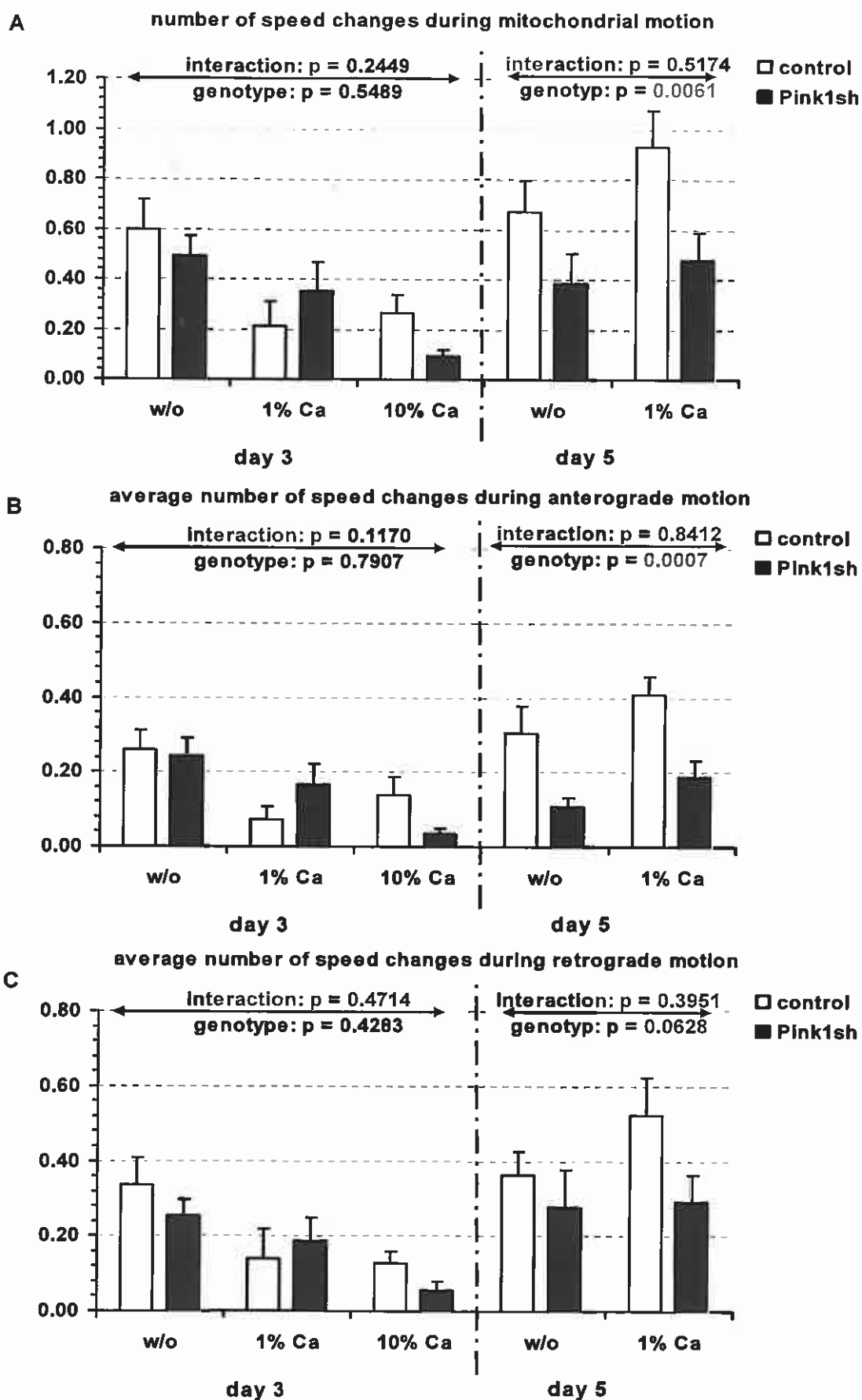


Fig. 49 previous page: Average number of speed changes during general motion (A), anterograde phase (B) and retrograde phase (C) in acute PINK1 knockdown neurons during constant stress application via calcium increased environment.

Mitochondrial direction changes in acute PINK1 knockdown primary Cortex neurons with Calcium increased environment										
young neurons	day 3						day 5			
	w/o		1 % Ca ²⁺		10 % Ca ²⁺		w/o		1 % Ca ²⁺	
	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh
% of mitochondria with direction changes during motion	32.63 ± 6.08	29.31 ± 3.51	9.95 ± 3.23	27.33 ± 7.69	19.53 ± 5.108	12.87 ± 3.43	40.27 ± 8.25	21.54 ± 4.70	45.97 ± 5.08	44.19 ± 6.74
Interaction value	0.0596						0.2293			
p-value	0.5567						0.1477			
number of direction changes during motion / 10 frames	0.14 ± 0.04	0.09 ± 0.01	0.03 ± 0.01	0.11 ± 0.05	0.08 ± 0.02	0.04 ± 0.01	0.15 ± 0.04	0.08 ± 0.02	0.20 ± 0.03	0.18 ± 0.04
Interaction value	0.0485						0.5308			
p-value	ns		ns		ns		0.1997			
number of speed changes during mitochondrial motion	0.60 ± 0.12	0.50 ± 0.07	0.21 ± 0.10	0.35 ± 0.12	0.27 ± 0.07	0.09 ± 0.03	0.67 ± 0.12	0.39 ± 0.12	0.93 ± 0.15	0.46 ± 0.11
Interaction value	0.2449						0.5174			
p-value	0.5488						0.0061			
number of speed changes during anterograde phase	0.26 ± 0.05	0.24 ± 0.05	0.07 ± 0.03	0.17 ± 0.06	0.14 ± 0.05	0.04 ± 0.01	0.31 ± 0.07	0.11 ± 0.02	0.41 ± 0.05	0.19 ± 0.05
Interaction value	0.1170						0.8412			
p-value	0.7907						0.0007			
number of speed changes during retrograde phase	0.34 ± 0.07	0.25 ± 0.05	0.14 ± 0.08	0.19 ± 0.06	0.13 ± 0.03	0.06 ± 0.03	0.36 ± 0.06	0.28 ± 0.10	0.52 ± 0.10	0.29 ± 0.10
Interaction value	0.4714						0.3951			
p-value	0.4283						0.0628			

Tab. 21: Mitochondria direction changes in acute PINK1 knockdown primary Cortex neurons with calcium increased environment.

4.2.2.4 Mitochondrial stop phases in acute PINK1 knockdown primary neurons during constant stress application

The percentage of mitochondria with holding stops in acute PINK1 knockdown primary neurons in response to permanent stress application were measured as well as the average duration of stop time, the number of mitochondrial stops and the number of stops after anterograde and retrograde motion.

During motion the percentage of mitochondrial holding stops in an calcium increased environment do not change in PINK1-deficient neurons compared with their control counterpart, neither at day 3 nor at day 5 post virus transduction (percentage of

mitochondria with holding stops during motion at day 3: w/o control 22.85 ± 4.21 ; w/o Pink1sh 22.92 ± 3.31 ; 1 % Ca^{2+} control 11.01 ± 3.02 ; 1 % Ca^{2+} Pink1sh: 15.51 ± 4.16 ; 10 % Ca^{2+} control 14.75 ± 3.07 ; 10 % Ca^{2+} Pink1sh: 6.96 ± 2.22 ; Genotype x Treatment interaction: 0.2219; $p = 0.7075$; at day 5: w/o control 26.72 ± 4.99 ; w/o Pink1sh 15.43 ± 3.95 ; 1 % Ca^{2+} control 25.37 ± 3.75 ; 1 % Ca^{2+} Pink1sh: 26.39 ± 4.33 ; Genotype x Treatment interaction: 0.1833; $p = 0.2653$; figure 50; table 22).

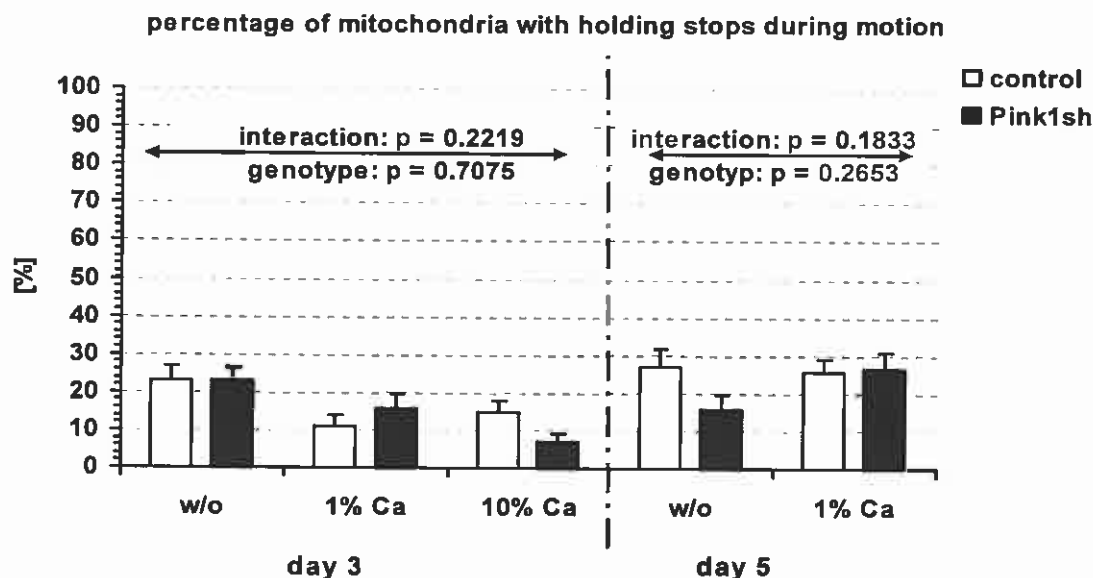


Fig. 50: Percentage of mitochondria with holding stops during motion in acute PINK1 knockdown neurons during constant stress application via calcium increased environment. In highly stressed neurons less mitochondria make holding stops three days after acute knockdown of PINK1.

Also no genotypic abnormalities could be detected in the average duration of stop time during mitochondrial trafficking, as well as no obvious changes in the average number of stops, the average number of stops after anterograde and retrograde motion (average duration of stop time during motion at day 3: w/o control 120.1 ± 9.84 ; w/o Pink1sh 119.5 ± 10.07 ; 1 % Ca^{2+} control 127.0 ± 20.72 ; 1 % Ca^{2+} Pink1sh: 171.0 ± 12.81 ; 10 % Ca^{2+} control 124.4 ± 19.49 ; 10 % Ca^{2+} Pink1sh: 158.4 ± 22.81 ; Genotype x Treatment interaction: 0.3599; $p = 0.0605$; at day 5: w/o control 125.9 ± 10.87 ; w/o Pink1sh 164.1 ± 18.64 ; 1 % Ca^{2+} control 142.0 ± 8.03 ; 1 % Ca^{2+} Pink1sh: 149.0 ± 9.03 ; Genotype x Treatment interaction: 0.2129; $p = 0.0753$; figure 51; number of mitochondrial stops during motion at day 3: w/o control 0.34 ± 0.07 ; w/o Pink1sh 0.30 ± 0.04 ; 1 % Ca^{2+} control 0.18 ± 0.06 ; 1 % Ca^{2+} Pink1sh: 0.26 ± 0.08 ; 10 % Ca^{2+} control 0.25 ± 0.05 ; 10 % Ca^{2+} Pink1sh: 0.08 ± 0.26 ; Genotype x Treatment interaction: 0.1107; $p = 0.3881$; at day 5: w/o control 0.49 ± 0.10 ; w/o Pink1sh 0.33 ± 0.07 ; 1 % Ca^{2+} control 0.43 ± 0.07 ; 1 % Ca^{2+} Pink1sh: 0.43 ± 0.08 ; Genotype x Treatment interaction: 0.3754; $p = 0.3986$; figure 52 A; number of stops after anterograde motion at day 3: w/o control 0.16 ± 0.05 ; w/o Pink1sh 0.15 ± 0.03 ; 1 % Ca^{2+} control 0.08 ± 0.03 ; 1 % Ca^{2+}

Pink1sh: 0.14 ± 0.05 ; 10 % Ca^{2+} control 0.12 ± 0.03 ; 10 % Ca^{2+} Pink1sh: 0.04 ± 0.01 ; Genotype x Treatment interaction: 0.1714; $p = 0.7325$; at day 5: w/o control 0.23 ± 0.06 ; w/o Pink1sh 0.17 ± 0.03 ; 1 % Ca^{2+} control 0.20 ± 0.04 ; 1 % Ca^{2+} Pink1sh: 0.21 ± 0.04 ; Genotype x Treatment interaction: 0.5345; $p = 0.5896$; figure 52 B; number of stops after retrograde motion at day 3: w/o control 0.22 ± 0.04 ; w/o Pink1sh 0.19 ± 0.03 ; 1 % Ca^{2+} control 0.14 ± 0.04 ; 1 % Ca^{2+} Pink1sh: 0.20 ± 0.05 ; 10 % Ca^{2+} control 0.17 ± 0.04 ; 10 % Ca^{2+} Pink1sh: 0.06 ± 0.02 ; Genotype x Treatment interaction: 0.1000; $p = 0.4239$; at day 5: w/o control 0.43 ± 0.09 ; w/o Pink1sh 0.27 ± 0.07 ; 1 % Ca^{2+} control 0.33 ± 0.05 ; 1 % Ca^{2+} Pink1sh: 0.35 ± 0.07 ; Genotype x Treatment interaction: 0.2568; $p = 0.3932$; figure 52 C; table 22).

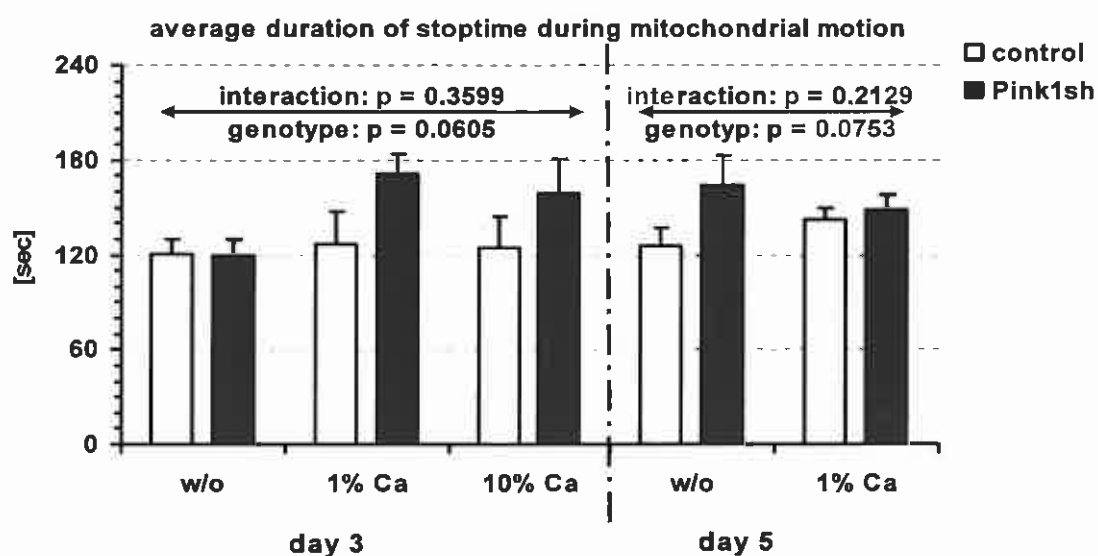


Fig. 51: Average duration of stop time during mitochondrial motion in acute PINK1 knockdown neurons during permanent stress application show no abnormalities.

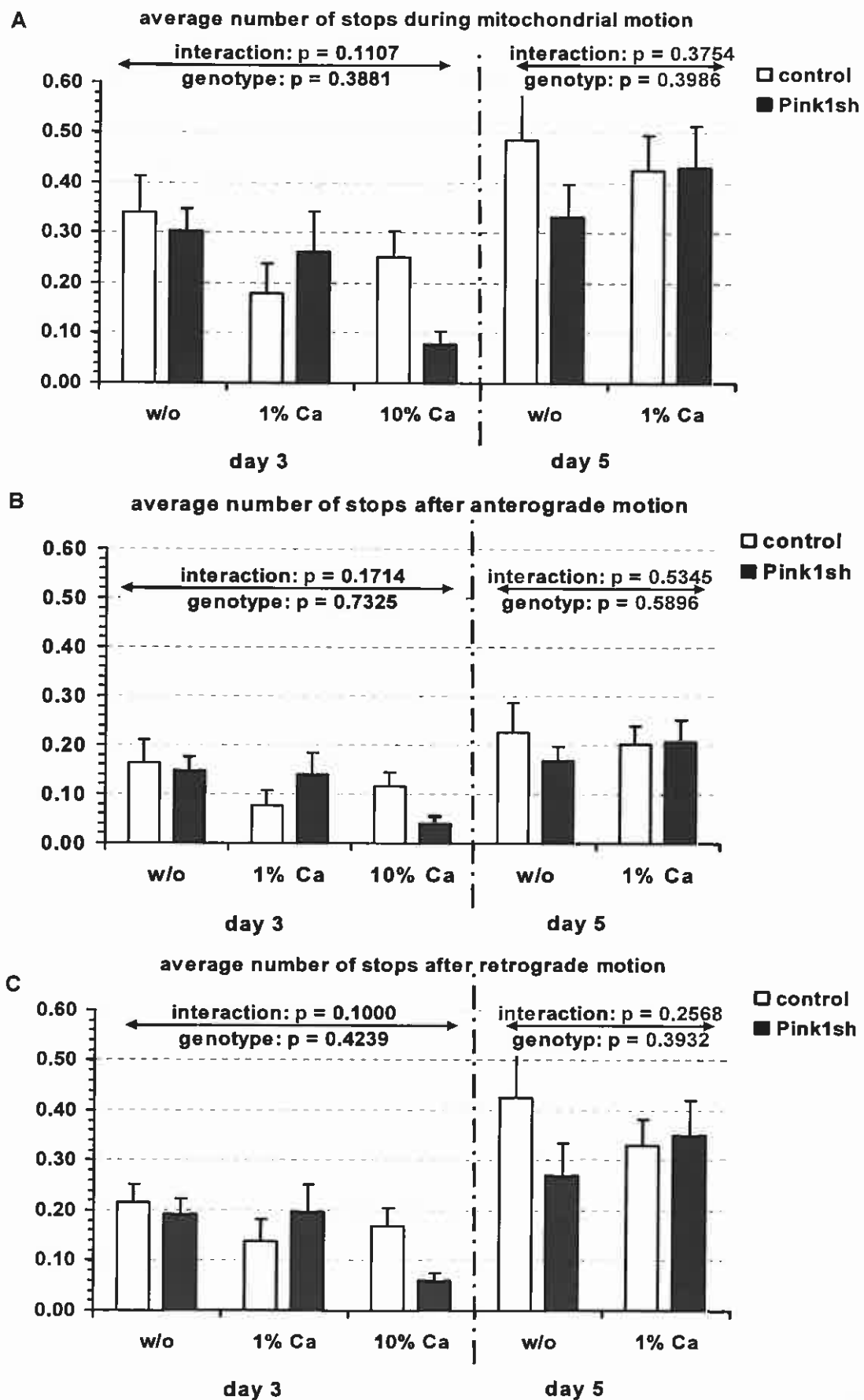


Fig. 52 previous page: Average number of stops during general mitochondrial motion (A), anterograde motion (B) and retrograde motion (C) in acute PINK1 knockdown neurons during constant stress application via calcium increased environment. Significant changes are obvious on day 3 after PINK1-deficiency of the highly stressed neurons (10 % calcium application).

Mitochondria stop phases in acute PINK1 knockdown primary Cortex neurons with Calcium increased environment										
young neurons	day 3						day 5			
	w/o		1 % Ca ²⁺		10 % Ca ²⁺		w/o		1 % Ca ²⁺	
	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh
% of mitochondria with holding stops during motion	22.85 ± 4.21	22.92 ± 3.31	11.01 ± 3.02	15.51 ± 4.16	14.75 ± 3.07	6.96 ± 2.22	26.72 ± 4.99	15.43 ± 3.95	25.37 ± 3.75	26.39 ± 4.33
Interaction value	0.2219						0.1833			
p-value	0.7075						0.2653			
average duration of stop time during motion	120.1 ± 9.84	119.5 ± 10.07	127.0 ± 20.72	171.0 ± 12.81	124.4 ± 19.49	158.4 ± 22.81	125.9 ± 10.87	164.1 ± 18.64	142.0 ± 8.03	149.0 ± 9.03
Interaction value	0.3599						0.2129			
p-value	0.0605						0.0753			
number of mitochondrial stops during motion	0.34 ± 0.07	0.30 ± 0.04	0.18 ± 0.06	0.26 ± 0.08	0.25 ± 0.05	0.08 ± 0.26	0.49 ± 0.10	0.33 ± 0.07	0.43 ± 0.07	0.43 ± 0.08
Interaction value	0.1107						0.3754			
p-value	0.3881						0.3986			
number of stops after anterograde motion	0.16 ± 0.05	0.15 ± 0.03	0.08 ± 0.03	0.14 ± 0.05	0.12 ± 0.03	0.04 ± 0.01	0.23 ± 0.06	0.17 ± 0.03	0.20 ± 0.04	0.21 ± 0.04
Interaction value	0.1714						0.5345			
p-value	0.7325						0.5896			
number of stops after retrograde motion	0.22 ± 0.04	0.19 ± 0.03	0.14 ± 0.04	0.20 ± 0.05	0.17 ± 0.04	0.06 ± 0.02	0.43 ± 0.09	0.27 ± 0.07	0.33 ± 0.05	0.35 ± 0.07
Interaction value	0.1000						0.2568			
p-value	0.4239						0.3932			

Tab. 22: Mitochondria stop phases in acute PINK1 knockdown primary Cortex neurons with Calcium increased environment.

4.2.2.5 Mitochondrial traveled distance in acute PINK1 knockdown primary neurons during constant stress application

Changes in the net and gross distance traveled by mitochondria may show further important evidence of dysfunctional transport or indicate an altered energy demand. Net movement means traveled distance from origin point. In this context, an anterograde and retrograde motion may cancel each other with the traveled anterograde movement specified as positive values and retrograde traveled distance as negative values. Gross movement describes the total distance traveled. Distances are given in μm .

Compared to neurons without stressors (w/o) mitochondria in neurons with constant stressors, either 1 % or 10 % calcium overload, show in general less net movement (Fig. 53)

regardless of which genotype they are. All movements are more into the retrograde direction and again both the PINK1-deficient neurons as well as neurons with normal functional PINK1 show the same tendencies. No genotype effect occur in net movement on day 3 and day 5 after acute knockdown of PINK1 (net mitochondrial movement at day 3: w/o control -0.41 ± 0.19 ; w/o Pink1sh -0.18 ± 0.23 ; 1 % Ca^{2+} control -0.08 ± 0.02 ; 1 % Ca^{2+} Pink1sh: -0.01 ± 0.16 ; 10 % Ca^{2+} control -0.03 ± 0.10 ; 10 % Ca^{2+} Pink1sh: -0.17 ± 0.12 ; Genotype x Treatment interaction: 0.4967; $p = 0.6961$; at day 5: w/o control -0.10 ± 0.40 ; w/o Pink1sh -0.19 ± 0.26 ; 1 % Ca^{2+} control -0.34 ± 0.32 ; 1 % Ca^{2+} Pink1sh: -0.09 ± 0.09 ; Genotype x Treatment interaction: 0.6031; $p = 0.8132$; figure 53; table 23).

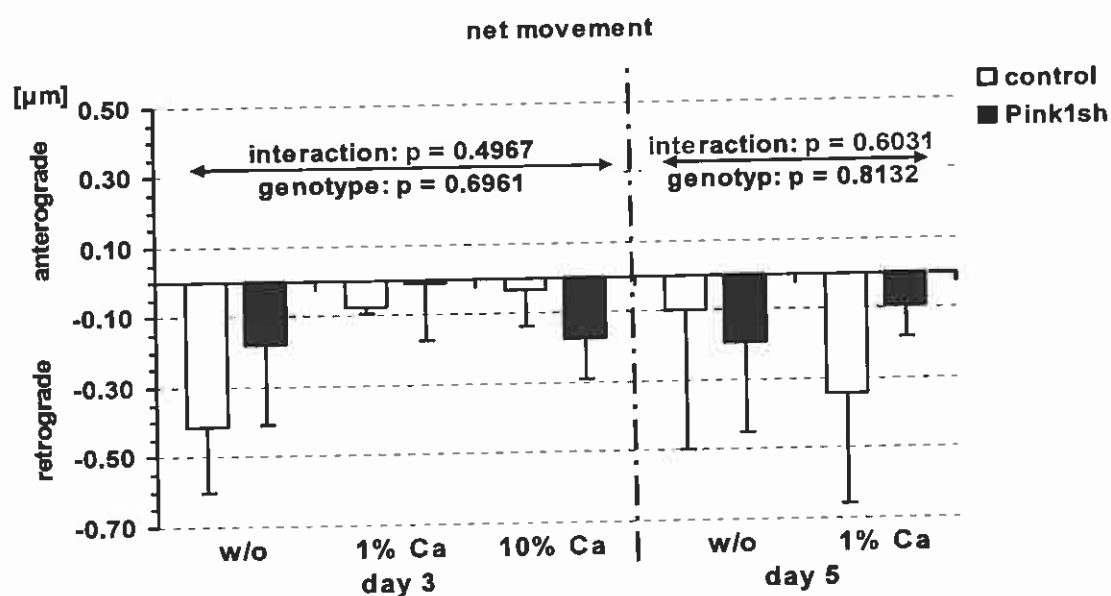


Fig. 53: Traveled distance of mitochondria from origin point, i.e. net movement, with and without constant stress application via calcium increased environment. The final net motions are nearly all directed in a retrograde movement, while on day 5 of the stressed cells with high concentrations of calcium, both groups (Pink1sh and control) travel a larger distance into anterograde direction.

The totally traveled distance of mitochondria, the gross movement, is represented in Fig. 54. However, no significant changes in acute PINK1-deficient neurons compared to controls are taking place under permanent cell stress at day 3 of acute PINK1-deficiency (w/o control 2.75 ± 0.47 ; w/o Pink1sh 1.86 ± 0.29 ; 1 % Ca^{2+} control 0.56 ± 0.15 ; 1 % Ca^{2+} Pink1sh: 1.88 ± 0.70 ; 10 % Ca^{2+} control 1.08 ± 0.34 ; 10 % Ca^{2+} Pink1sh: 0.62 ± 0.21 ; Genotype x Treatment interaction: 0.0220; Bonferroni p-values of w/o, 1 % Ca^{2+} and 10 % Ca^{2+} : $p = \text{ns}$). Again only on day 5 significant differences between both genotypes (control versus Pink1sh) can be seen under permanent stress application (w/o control 3.24 ± 0.64 ; w/o Pink1sh 2.02 ± 0.43 ; 1 % Ca^{2+} control 2.63 ± 0.53 ; 1 % Ca^{2+} Pink1sh: 1.44 ± 0.36 ; Genotype x Treatment interaction: 0.9828; $p = 0.0342$).

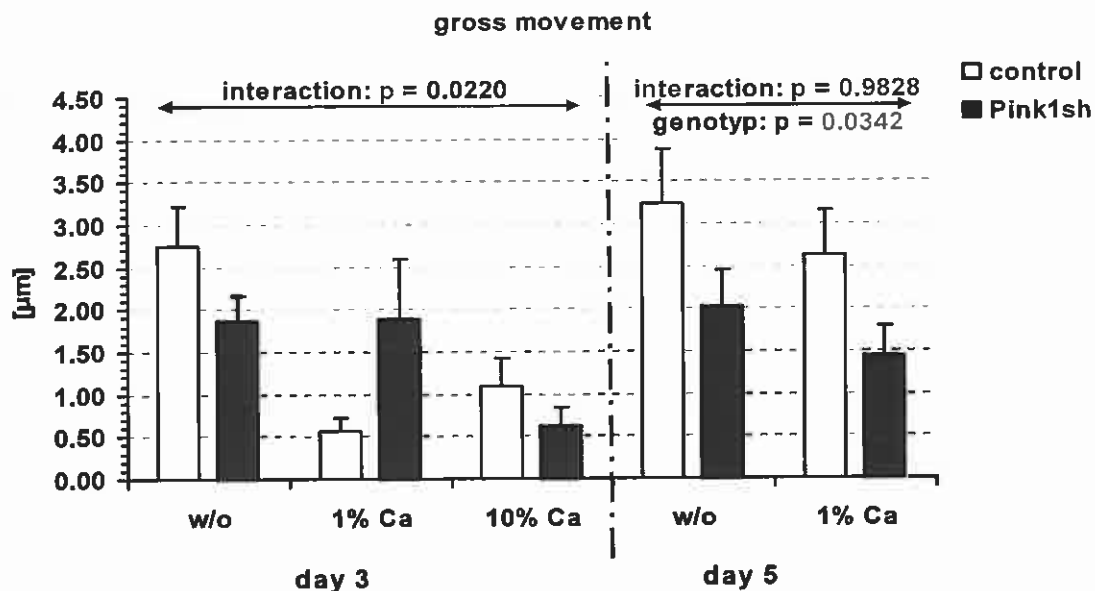


Fig. 54: Totally traveled distance of mitochondria, gross movement, with and without constant stress application via calcium increased environment.

Looking at only the differences in the net movement or gross motion, we get some additional information. But comparing them with each other provides much more meaningful results. In the gross movement calculation it is obvious that mitochondria travel a certain distance, but in the net movement they make seemingly no headway.

Interestingly, the traveled distance into anterograde and retrograde direction (Fig. 55) show very similar distributions and pattern. But with only a genotype effect in traveled distance into retrograde direction at day 5 post virus transduction and permanent stress application with decreasing retrograde traveled distance in PINK1-deficient neurons (traveled distance into anterograde direction at day 3: w/o control 1.17 ± 0.21 ; w/o Pink1sh 0.84 ± 0.16 ; 1 % Ca^{2+} control 0.24 ± 0.08 ; 1 % Ca^{2+} Pink1sh: 0.94 ± 0.36 ; 10 % Ca^{2+} control 0.524 ± 0.17 ; 10 % Ca^{2+} Pink1sh: 0.224 ± 0.07 ; Genotype x Treatment interaction: 0.0200; Bonferroni p-values of w/o, 1 % Ca^{2+} and 10 % Ca^{2+} : $p = \text{ns}$; at day 5: w/o control 1.57 ± 0.37 ; w/o Pink1sh 0.92 ± 0.29 ; 1 % Ca^{2+} control 1.14 ± 0.35 ; 1 % Ca^{2+} Pink1sh: 0.67 ± 0.15 ; Genotype x Treatment interaction: 0.7807; $p = 0.089$; traveled distance into retrograde direction at day 3: w/o control -1.58 ± 0.29 ; w/o Pink1sh -1.02 ± 0.21 ; 1 % Ca^{2+} control -0.32 ± 0.08 ; 1 % Ca^{2+} Pink1sh: -0.95 ± 0.36 ; 10 % Ca^{2+} control -0.46 ± 0.20 ; 10 % Ca^{2+} Pink1sh: -0.40 ± 0.16 ; Genotype x Treatment interaction: 0.0546; $p = 0.9991$; at day 5: w/o control -1.67 ± 0.39 ; w/o Pink1sh -1.11 ± 0.21 ; 1 % Ca^{2+} control -1.48 ± 0.27 ; 1 % Ca^{2+} Pink1sh: -0.77 ± 0.22 ; Genotype x Treatment interaction: 0.8030; $p = 0.0478$).

These observations apply only to the observed developmental state. More matured or aged neurons can have a different behavioral pattern and distribution.

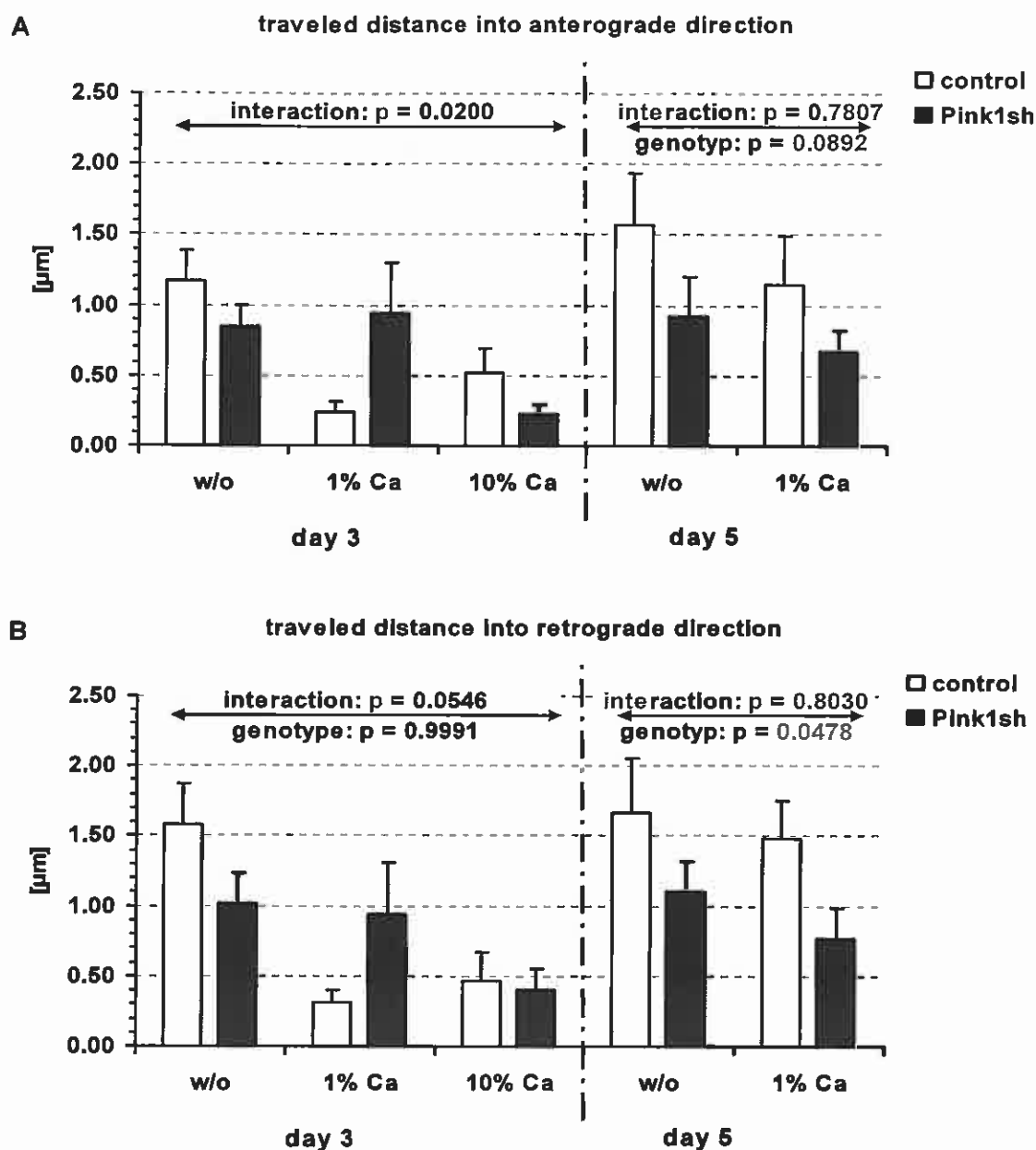


Fig. 55: Traveled distance of mitochondria into anterograde (A) and retrograde (B) direction with and without constant stress application via calcium increased environment. Both anterograde and retrograde traveled distance are very similar, but with a higher affinity into retrograde direction for each, control and Pink1sh neurons. The neurons on day 5 also seem to travel a longer distance than their counterparts on day 3.

Mitochondrial traveled distance in acute PINK1 knockdown primary cortex neurons during constant stress application										
young neurons	day 3						day 5			
	w/o		1 % Ca ²⁺		10 % Ca ²⁺		w/o		1 % Ca ²⁺	
	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh	control	Pink1sh
traveled distance from origin point	-0.41 ± 0.19	-0.18 ± 0.23	-0.08 ± 0.02	-0.01 ± 0.16	-0.03 ± 0.10	-0.17 ± 0.12	-0.10 ± 0.40	-0.19 ± 0.26	-0.34 ± 0.32	-0.09 ± 0.09
Interaction value	0.4967						0.6031			
p-value	0.6961						0.8132			
whole traveled distance	2.75 ± 0.47	1.86 ± 0.29	0.56 ± 0.15	1.86 ± 0.70	1.06 ± 0.34	0.62 ± 0.21	3.24 ± 0.64	2.02 ± 0.43	2.63 ± 0.53	1.44 ± 0.36
Interaction value	0.0220						0.9828			
p-value	ns		ns		ns		0.0342			
traveled distance into anterograde direction	1.17 ± 0.21	0.84 ± 0.16	0.24 ± 0.08	0.94 ± 0.36	0.524 ± 0.17	0.224 ± 0.07	1.57 ± 0.37	0.92 ± 0.29	1.14 ± 0.35	0.67 ± 0.15
Interaction value	0.0200						0.7807			
p-value	ns		ns		ns		0.0889			
traveled distance into retrograde direction	-1.58 ± 0.29	-1.02 ± 0.21	-0.32 ± 0.08	-0.95 ± 0.36	-0.46 ± 0.20	-0.40 ± 0.16	-1.67 ± 0.39	-1.11 ± 0.21	-1.48 ± 0.27	-0.77 ± 0.22
Interaction value	0.0546						0.8030			
p-value	0.9991						0.0478			

Tab. 23: Mitochondria traveled distance in acute PINK1 knockdown primary cortex neurons during constant stress application via calcium increased environment.

4.2.2.6 Summarized overview of mitochondrial trafficking in acute PINK1-deficient primary neurons with and without constant stress application

The table below shows a highly condensed, but very informative overview of all mitochondrial trafficking experiments, with and without constant stressors, in acute PINK1 knockdown neurons at young developmental state. Keep in mind that the speed data consist of three individual measuring points (average speed of mitochondria, average speed of mitochondria during anterograde and retrograde movement), whereas the mobility includes five different values (stationary phase during motion, anterograde and retrograde phase during motion, immobile mitochondria and mobile mitochondria). The data from direction changes and stop phases also consist of five different measuring points (direction changes: percentage of mitochondria with direction changes during motion, number of direction changes during motion / 10 frames, number of speed changes during mitochondrial motion, number of speed changes during anterograde and retrograde phase; stop phases: percentage of mitochondria with holding stops during motion, average duration of stop time during mitochondrial motion, number of mitochondrial stops during motion, number of stops after anterograde and retrograde motion). The last point, traveled distance, is also composed of several subunits (traveled distance from origin point, whole traveled distance,

traveled distance into anterograde and retrograde direction). The gray boxes show measuring points without any changes in PINK1 knockdown neurons versus neurons with normal PINK1 expression. Orange marked fields are those who had up to three significant changes in the individual measuring points, whereas red labeled fields represent those values which had more than three significant changes within this range.

It is apparent from the table (24) that on day 3 after acute knockdown of PINK1 just a few significant changes in the mitochondrial mobility occur with additional cell stress like calcium application. Compared with unstressed mitochondria at the same time point (see table 18 at page 97) a slight increase of a phenotype occur via stress application. It seems that with an additional stressor PINK1-deficient neurons can no longer compensate mitochondrial defects as well as healthy neurons. Interestingly, however, is the fact that this situation reinforced two days later. Not stressed PINK1-deficient neurons show changes in mobility and direction changes at day 5 after acute PINK1 down regulation, but with stress treatment a highly increase of significantly changes within mitochondrial transport occurs.

Almost all major values (mitochondrial speed changes, changes in mobility, direction changes and traveled distance) exhibit genetically based differences in mitochondrial trafficking under stress. Thus, PINK1-deficient mitochondria show much stronger deficits when they are stressed. These results fit perfectly to the multiple-hit theory and to the suggested major role of mitochondria in PD.

	young neurons				
	day 3			day 5	
	acute w/o	1 % Ca ²⁺	10 % Ca ²⁺	acute w/o	1 % Ca ²⁺
speed					≤ 3
mobility		≤ 3			> 3
direction changes					≤ 3
stop phases					
traveled distance					≤ 3

Tab. 24: Summarized results of mitochondria trafficking in acute PINK1-deficient primary cortex neurons with and without (w/o) constant stress application via high calcium concentration within the culture medium compared to neurons under normal condition.

4.3 organelle trafficking in B16 primary cortex neurons

I have performed several approaches to monitor the transport of other cell organelles such as LC3 or Rab5 (see Material and Methods, 2.2.7). Commercially available fluorescent target proteins as well as LC3 and Rab5 plasmid transfection have failed to provide meaningful data for their transport. While it was possible to label corresponding proteins, however, the number of transfected neurons was too low to obtain reasonable results. Since lentivirus is known to obtain high transfection rates *in vitro*, we have generated a Rab5 lentivirus to conduct future experiments with this.

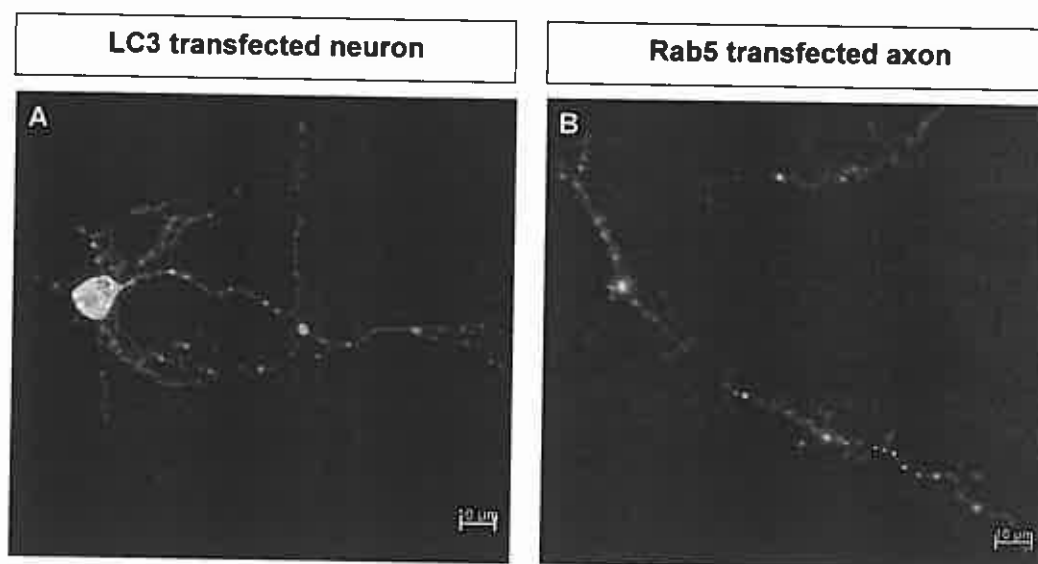


Fig. 56: LC3 (A) and Rab5 (B) plasmid transfected primary cortex neurons from C57/Bl6-J mice.

4.4 RNA expression of mitofission and –fusion genes and mitochondrial transport genes

4.4.1 Establishment of the standard controls

With growing and increasing cell size normal internal standard genes like β -actin, which is a structure protein and component of the cytoskeleton, may also vary. Therefore we tested five commercially available and widely used reference genes to their possible use in our experiments. In addition to β -actin, we tested glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase 1 (pgk1), hypoxanthine guanine phosphoribosyl transferase (HPRT) and ribosomal protein L27 (rpl27). GAPDH has multiple functions like a major involvement in glycolysis, an important pathway of energy and carbon molecule supply. It also has recently been implicated in transcription activation of vesicle trafficking in ER and Golgi and apoptosis. Pgk1 is a transferase enzyme also used in glycolysis where it catalyses

the formation of ATP to ADP and vice versa. HPRT is an enzyme involved in purine metabolism of eukaryotes and plays a central role in the generation of purine nucleotides through the purine salvage pathway. Rpl27 is located near the peptidyl-transferase centre at the interface of ribosomal subunits, and is important for ribosomal assembly and function. All RT-PCRs were performed by Annerose Kurz-Drexler.

The establishment of the internal standard control genes were performed at different time points of cell culture growth. Day 0 in this case means the start point of the time series, which was on day 4 of growth, to maintain comparability with the mitochondrial morphology study and also mitochondrial trafficking experiments. Day 3 e.g. means three days after the start point, which corresponds to the identical time day 3 after virustransfection in the other experiments. The results are listed in table 25 and are also graphically represented in Fig. 57. One can clearly see that rpl27 and β -actin have almost the same values at day 3 compared with day 5, which are the important time points (known from our mitochondrial morphology and trafficking studies). This implies that these two reference genes are most suitable for our study and were used as internal standard controls.

standard controls [CT mean]				
CT mean	day 0	day 1	day 3	day 5
GAPDH	18.14	18.60	19.27	18.96
pgk1	22.55	22.89	23.45	23.18
HPRT	21.16	23.39	23.55	23.04
rpl27	22.51	23.16	23.84	23.87
β -actin	20.27	20.43	20.90	20.87

Tab. 25: Testing of five internal standard controls at different cell culture time points. In particular day 3 and day 5 are the most important time points regarding the comparability of the morphology and mitochondrial trafficking tests. Especially rpl27 and β -actin do not change their CT value between these two time points and are thus well suited to serve as internal controls.

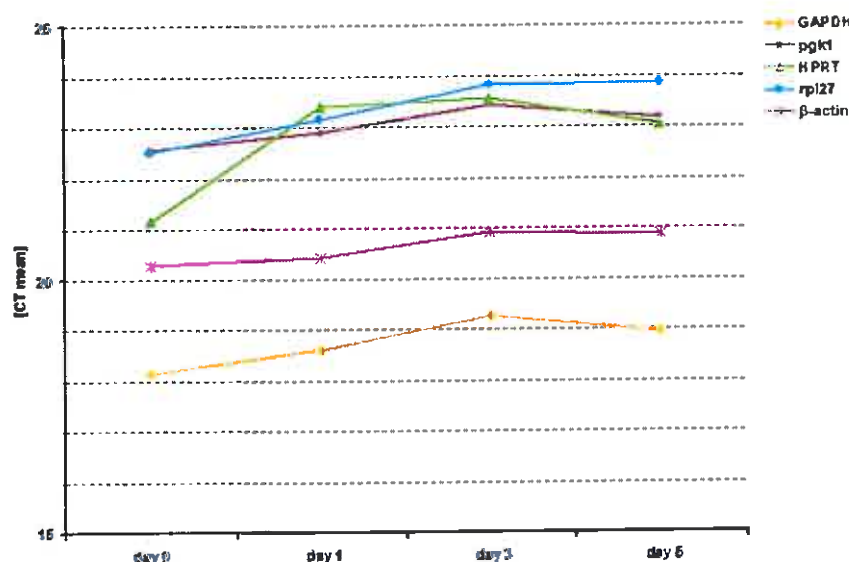


Fig. 57: CT values of different reference genes and at different cell culture time points.

The PCR cycle at which the sample reaches a fluorescent intensity above background is the Cycle Threshold (CT) and is inversely proportional to the amount of target nucleic acid in the sample. This value is used to calculate $2^{-\Delta\Delta CT}$ and thereby obtains the actual expression rate. Rpl27 and β -actin do not change their CT values at day 3 and day 5, which is a good sign for us to use them as reference genes.

4.4.2 RNA expression of mitofission and -fusion genes in primary cortex neurons after acute knockdown of PINK1

mRNA concentration of mitofission and -fusion genes in primary cortex neurons was measured at day 1, 3 and 5 after acute knockdown of PINK1 via virus transduction as well as with a control virus (without shRNA of PINK1). PINK1 knockdown efficiency of shRNA virus was checked in addition. Rpl27 served as reference gene. Expression values of the control neurons were averaged to 100 % and the corresponding PINK1-deficient cells were set in relation to this 100 %. An expression rate of 120 % for example means that this gene is expressed 1.2 fold higher in acute PINK1 knockdown neurons than corresponding neurons without shRNA for PINK1.

First, you can see clearly that the knockdown efficiency of PINK1 on day 3 and day 5 is very effective. At these time points we have a significant decreased expression rate of PINK1 with rpl-27 as internal control ($36.18 \% \pm 1.87$ at day 3; $26.21 \% \pm 1.36$ at day 5). All genes which are responsible for mitofission and -fusion processes (Fis1, Drp1, Opa1, Mfn-1 and Mfn-2) show a slight down regulation at day 3 and day 5 with rpl27 as reference gene, but this was not significant. The exact values are given in table 26.

mRNA expression of mitofission and -fusion genes in primary cortex neurons after acute knockdown of PINK1 in [%]			
rpl27	2- $\Delta\Delta$ CT in % $\pm$ S.E.M.		
	day 1	day 3	day 5
Pink1	124.88 $\pm$ 20.01	36.18 $\pm$ 1.87	26.21 $\pm$ 1.36
p-value	0.431	0.001	0.0003
Fis1	123.49 $\pm$ 18.91	89.79 $\pm$ 6.50	86.94 $\pm$ 10.05
p-value	0.431	0.257	0.323
Dnm1l = Drp1	107.69 $\pm$ 0.78	91.12 $\pm$ 9.37	83.67 $\pm$ 24.00
p-value	0.065	0.443	0.566
Opa1	110.45 $\pm$ 11.06	79.59 $\pm$ 12.97	79.60 $\pm$ 13.69
p-value	0.810	0.258	0.275
Mfn-1	94.73 $\pm$ 4.87	87.54 $\pm$ 4.63	77.64 $\pm$ 16.17
p-value	0.474	0.116	0.301
Mfn-2	97.69 $\pm$ 2.80	74.53 $\pm$ 9.76	66.83 $\pm$ 21.33
p-value	0.561	0.121	0.260

Tab. 26: mRNA expression of mitofission and -fusion genes in primary cortex neurons after acute knockdown of PINK1 in [%] with rpl27 as internal control. Knockdown efficiency of our Pink1sh virus is stable and quite high at day 3 and day 5.

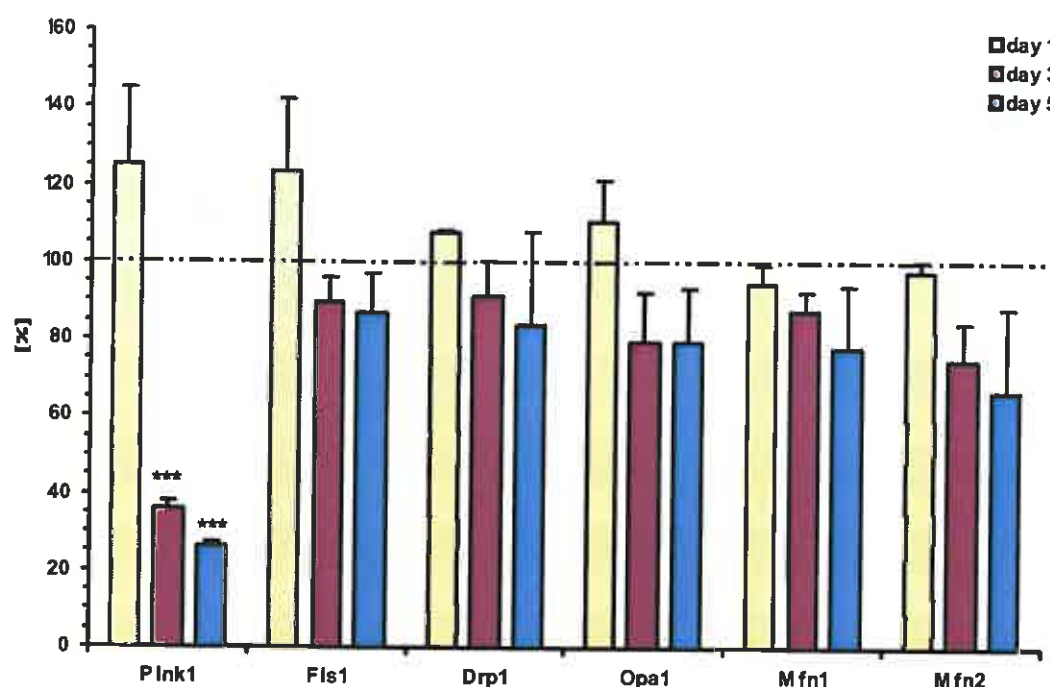


Fig. 58: mRNA expression of mitofission and -fusion genes in primary cortex neurons after acute knockdown of PINK1 with rpl27 as Internal control. Knockdown efficiency of our lentivirus at day 3 and day 5 is quite successful. The dashed line at 100 % represents the values of the control neurons which were transfected with a pLL3.7mitoEYFP lentivirus without any shRNA. $\Delta\Delta CT$ values of controls were set to 100 % and genes of interest were calculated relative to this 100 %. All genes calculated with the reference gene rpl27 show a slight down regulation at day 3 and day 5 but this was not significantly.

4.4.3 RNA expression of mitochondrial transport genes in primary cortex neurons after acute knockdown of PINK1

Expression rate of mitochondrial transport genes, as well as PINK1 knockdown efficiency in primary cortex neurons were measured at day 1, 3 and 5 after acute lentiviral knockdown of PINK1. Rpl27 served again as reference gene.

The knockdown efficiency of PINK1 is stable down regulated at day 3 and day 5 after acute knockdown ($37.68 \% \pm 3.20$ with rpl27 at day 3; $50.36 \% \pm 17.42$ with rpl27 at day 5), as expected. Furthermore a significantly down regulation of Dnhd1, a part of the dynein complex, appears already at day 3 with rpl27 control, but is not yet as strong as two days later at day 5 after acute PINK1 knockdown. This seemingly is the first indication that the mitochondrial transport, especially retrograde transport, is already disturbed in the molecular level of gene expression. All other transport factors are not significantly changed (see table 27).

mRNA expression of mitochondrial transport genes in primary cortex neurons after acute knockdown of PINK1 in [%]			
rpl27	2- $\Delta\Delta$ CT in % $\pm$ S.E.M.		
	day 1	day 3	day 5
PINK1	104.13 $\pm$ 4.10	37.68 $\pm$ 3.20	50.36 $\pm$ 17.42
p-value	0.371	0.00004	0.046
Miro-1	92.32 $\pm$ 12.96	78.59 $\pm$ 8.85	81.96 $\pm$ 16.32
p-value	0.594	0.094	0.350
Miro-2	120.53 $\pm$ 14.67	95.25 $\pm$ 12.69	97.29 $\pm$ 31.24
p-value	0.256	0.733	0.936
Milton / trak2	109.46 $\pm$ 15.39	80.82 $\pm$ 7.37	107.47 $\pm$ 36.57
p-value	0.582	0.080	0.851
Dctn 1	111.86 $\pm$ 14.45	83.95 $\pm$ 12.70	80.28 $\pm$ 28.08
p-value	0.472	0.205	0.533
Dnhd 1	113.49 $\pm$ 14.38	76.70 $\pm$ 6.73	61.47 $\pm$ 9.09
p-value	0.317	0.041	0.024
Oas1b	95.62 $\pm$ 37.73	108.88 $\pm$ 20.83	101.83 $\pm$ 26.68
p-value	0.818	0.698	0.950

Tab. 27: mRNA expression of mitofission and -fusion genes in primary cortex neurons after acute knockdown of PINK1 in [%] with rpl27 as reference gene. Again, the good efficiency of our lentivirus with siRNA against PINK1 is obvious. There are also no detectable differences in the interferon response (Oas1b), which is a good sign that no off-target effects are taking place. A decrease in the gene expression of Dnhd1 (Dynein heavy chain domain1), occurs at day 3 and day 5 after acute knockdown of PINK1. This may be a first hint of a defect in the retrograde transport. All other gene expression rates are not impaired.

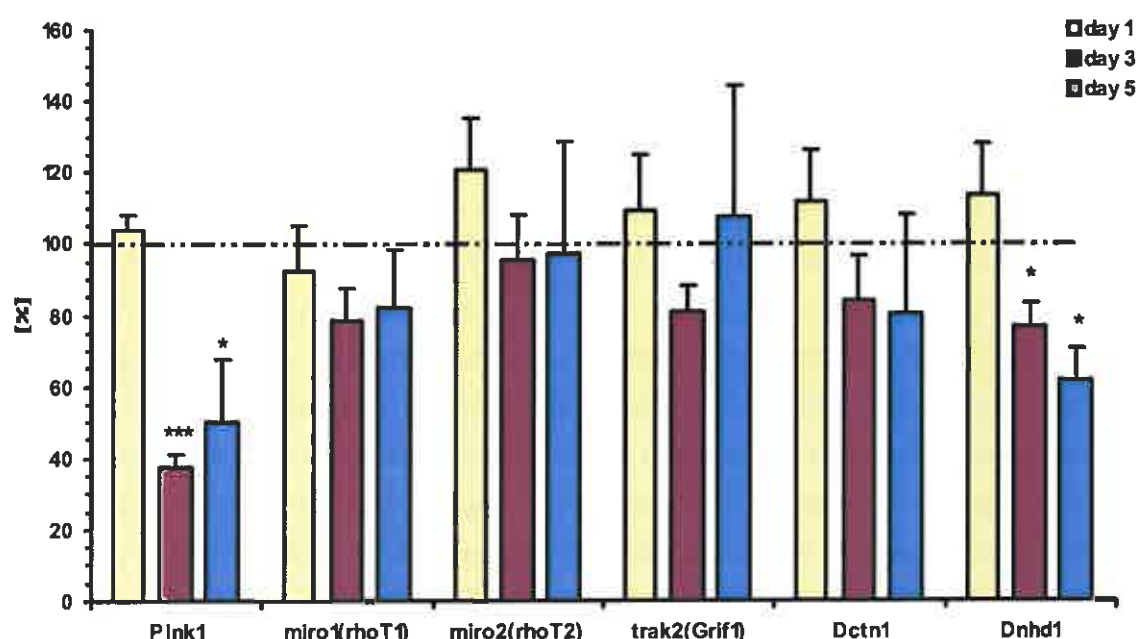


Fig. 59: mRNA expression rate of mitochondrial transport genes in primary cortex neurons after acute knockdown of PINK1 in [%] with rpl27 as reference gene. Our lentivirus for Pink1sh is working well at day 3 and day 5, the important time points of our study. The dashed line represents the relation to the neurons transfected with a control virus (pLL3.7mitoEYFP), which were scaled to 100 %. The expression rate of Dnhd1, is significantly decreased at day 3 ($p = 0.041$) as well as at day 5 after acute PINK1-deficiency ($p = 0.024$ for rpl27).

4.5 GMC screen

A PINK1 cohort was tested in the German Mouse Clinic for a broad phenotypic screen (for details see page 56ff or <http://www.mouseclinic.de/index.php?id=11096>). A summary of all results with displays of significances are given in the following table (Tab. 28). Arrow upwards ($\uparrow$) means a significant increase, arrow downwards ($\downarrow$) means a significant decrease, whereas an equivalent (=) refers to no changes between the genotypes. The strength of significant are expressed in the number of asterisks, whereas one asterisk (*) meaning significant with $p \leq 0.05$, two asterisks (**) meaning highly significant with $p \leq 0.01$ and three asterisks (***) meaning highest significant values with $p \leq 0.001$. Some data are additionally divided in genders. NA meaning no alternations and NAD meaning no alternations determined.

The behaviour screen shows an increase of forward locomotion in both genders and an anxiety-related behaviour in male mice of PINK1^{-/-}. In the neurology screen the GMC could found an increase in vocalization in both genders and a highest significant increase in lactate levels. Also a decrease of the first response time in a hot plate test was observed. This test is often used for subjective perception of pain, i.e. that a faster reaction leads to a decrease in response time. What allows the general accepted conclusion of an increased sensitivity to pain? The presumption of the GMC is obvious that our PINK1 mice have hyperalgesia. If we have a closer look into the energy metabolism, our PINK1^{-/-} mice exhibit a highly increased body temperature, a trend towards increased basal glucose levels of male mutant mice and a decrease in mean AUC 30-120 values. Moreover, both the glucose level of ad libitum fed mice as well as the urea concentration in blood is significantly increased in the constitutive PINK1 mice. However, the potassium values as well as the alkaline phosphatase (ALP) show a decline in these mice. Furthermore, the basic haematology in Pink1^{-/-} mice points out an increase in white blood cells and plated count, which is a measurement for thrombocytes. These results could hint towards an effect on glucose handling and / or kidney function. The Immunology screen could detect some significant changes in Pink1-deficient mice compared to wild type mice, like a lower proportion of CD44-expressing T cells (CD4+/CD44+), MHC class II expressing B cells and CD44 expressing T cells (CD19+/MHCII+B220+.). But these alternations are of unclear relevance. In addition, the cardiovascular screen exhibits an increase in the heart echo rate of female PINK1-deficient mice, whereas the heart echo rate of mutant male mice are decreased. The corticosterone level of male Pink1^{-/-} mice is also increased compared to the male control group. Interesting to note, also the pathology screen shows some elementary significant alternations in our constitutive knockout mice. In Prussian blue staining of the spleen an obvious hemosiderosis in six out of six tested male mice were found (see pic.60 A+B), which is normally characteristic in cases of hemolytic anemia or toxic insults.

Furthermore, the mutant male mice show a testicular degeneration in two out of six mice and in none of the wild type control animals (pic.60 C+D). One of these animals displayed an additional spermatocele in the epididymis.

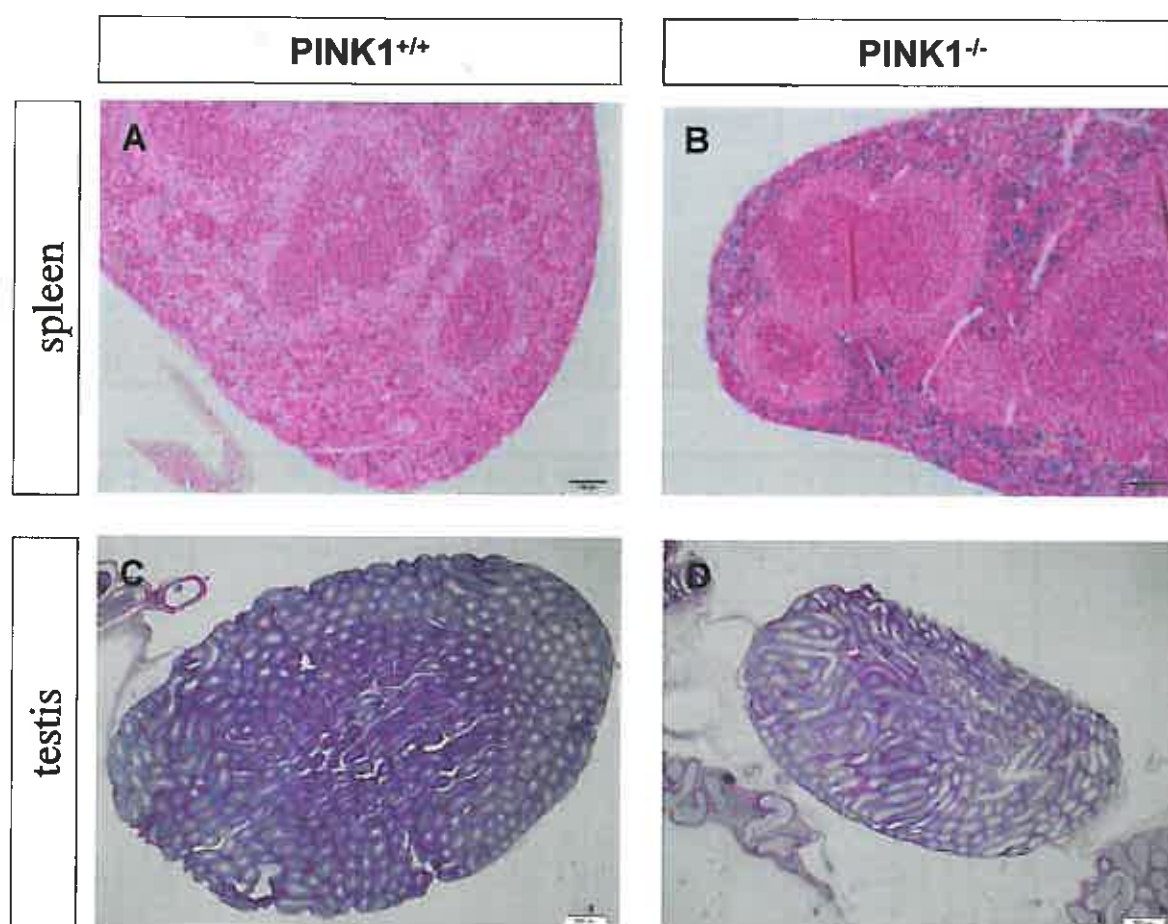


Fig. 60: Spleen samples stained with Prussian blue (A and B), adapted by GMC. Whereas in normal spleens no hemosiderosis is obvious (A), hemosiderosis in the spleen of a mutant male mouse (B) appears much more frequently. Testicular were stained with H&E (C and D). Mutant male mice show a clear degeneration of the testicular (D) compared to normal shaped testicular in wild type control mice (C). Scale bar of A and B: 100 μ m; of C and D: 500 μ m.

To get a quick overview of the different phenotypes of the individual experiments, the GMC has in addition performed Manhattan-Plots of each gender (Fig.61A for male mice and Fig. 61B for female mice). Manhattan-Plots are usually used to represent a large amount of data-points in which the negative logarithm of the associated p-values is displayed along the y-axis. The dashed line in the graph shows the p-value of 0.05. All values above this line are significantly altered compared to PINK1^{+/+} mice. As expected and generally occurring in most mouse models, there are major differences between male and female mice. Hits of the Manhattan-Plots reflect the results of the table 28 and all GMC results in detail.

tests	f	m
Body weight during screening time	=	=
Dysmorphology	=	=
Clickbox test	=	=
X-Ray analysis:	=	=
Behaviour	=	=
Forward locomotion	++	++
Vertical locomotion	=	=
Anxiety-related behaviour	=	++
Acoustic startle reactivity (ASR)	=	++
Prepulse inhibition (PPI)	=	=
Neurology	=	=
Modified SHIRPA-Protocol:	=	=
Vocalisation	++	++
Grip strength	++	++
Lactate	++	++
(AU400) [mmol/l]	++	++
Accelerating rotarod:	=	=
Noctepion	++	++
First response time	++	++
Second response time	=	=
Eye	=	=
Sit lamp biomicroscopy:	=	=
Funduscopy/Ophthalmoscopy:	=	=
Laser interference biometry:	=	=
Energy Metabolism	=	=
Body mass	=	=
Fat mass	=	=
Lean mass	=	=
Body mass	=	=
Body temperature	++	++
Food intake (g)	=	=
Mean VO ₂	=	=
Min VO ₂	=	=
Max VO ₂	=	=
Mean RER	=	=
Mean distance	=	=
Mean rearing	=	=

tests	f	m
Clinical chemistry	=	=
Fasting-induced body mass	=	=
Glucose tolerance test - glucose	=	=
Basal glucose level	=	=
AUC 0-30	=	=
AUC 30-120	++	++
Glucose (fasted)	=	=
Cholesterol (fasted)	=	=
HDL-cholesterol (fasted)	=	=
Non-HDL cholesterol (fasted)	=	=
Triglycerides (fasted)	=	=
NEFA (fasted)	=	=
Glycerol (fasted)	=	=
Sodium	=	=
Potassium	++	++
Chloride	=	=
Total protein	=	=
Albumin	=	=
Creatinine enz.	=	=
Urea	++	++
Cholesterol	=	=
Triglyceride	=	=
ALAT/GPT	=	=
ASAT/GOT	=	=
Alpha-Amylase	=	=
Glucose	++	++
LDH	=	=
Calcium	=	=
Inorganic phosphate	=	=
Iron	=	=
ALP	++	++
Animal blood counter (ABC):	=	=
RBC: Red blood cell count	=	=
HGB: Hemoglobin	=	=
HCT: Hematocrit	=	=
MCV: Mean corpuscular volume	=	=
MCH: Mean corpuscular hemoglobin	=	=

tests	f	m
RDW: Red blood cell distribution width	=	=
WBC: White blood cell count	++	++
PLT: Platelet count	++	++
MPV: Mean platelet volume	=	=
Immunology	=	=
Immunoglobulin levels in blood plasma	=	=
(IgG ₁ , IgG ₂ , IgG ₃ , IgM, IgA)	=	=
Leucocyte proportion:	=	=
CD3+T-cells	=	=
CD4+T-cells	=	=
CD8+T-cells	=	=
B cells	=	=
NK cells	=	=
Granulocytes	=	=
Monocytes	=	=
DC4+ / CD25+	=	=
Gamma-delta T c	=	=
CD4+ / CD82L+	=	=
CD4+ / CD44+	++	++
CD8+ / CD82L+	=	=
CD8+ / CD44+	=	=
NK+CD5+	=	=
EU_B cells	=	=
CD19+ / IgD+	=	=
CD19+ / CD5+	=	=
CD19+ / MHCII+ B220+	=	=
NK7 CD11b+	=	=
Cardiovascular	=	=
Weight	=	=
LVPW:d	=	=
LVID:d	=	=
LVID:s	=	=
Heart rate Echo	++	++
IVsd:	=	=
IVSs:	=	=
LVPW:s	=	=
Respiration rate echo	=	=

tests	f	m
Allergy	=	=
Total plasma IgE	=	=
Steroid Metabolism	=	↑*
Corticosterone	NA	NA
Androstendione	NA	NA
Testosterone	=	=
Lung function	=	=
Body mass (BM)	=	=
Volumetric parameters:		
Tidal Volume (TV)	=	=
Inspiratory Capacity (IC)	=	=
Expiratory Reserve Volume (ERV)	=	=
Vital Capacity (VC)	=	=
Functional Residual Capacity (FRC)	=	=
Residual Volume (RV)	=	=
Total Lung Capacity (TLC)	=	=
Forced Vital Capacity (FVC)	=	=
Flow parameters:		
Forced expiratory Volume (FEV100)	=	=
Peak Expiratory Flow (PEF)	=	=
Mechanical parameters:		
Chord (static Lung Compliance) (C)	=	=
Dynamic Lung Compliance (Cdyn)	=	=
Lung Resistance (R)	=	=
Pathology		
Heart weight	=	=
Tibia length	=	=
Liver weight	=	=
Spleen weight	=	=
Heart weight / tibia length	=	=
Heart weight / body weight	=	=
Liver weight / tibia length	=	=
Liver weight / body weight	=	=
Spleen weight / tibia length	=	=
Spleen weight / body weight	=	=
Results - Spleen	increased	↑*
Results - Testis	deposits of hemosiderin degeneration	↑*
Skin	NAD	NAD
Musculoskeletal system	NAD	NAD
Eyes	NAD	NAD
Brain	NAD	NAD
Cerebellum	NAD	NAD
Heart / Arteries	NAD	NAD

tests	f	m
Trachea	NAD	NAD
Lung	NAD	NAD
Teeth	NAD	NAD
Salivary gland	NAD	NAD
Oesophagus	NAD	NAD
Stomach	NAD	NAD
Small intestine	NAD	NAD
Large intestine	NAD	NAD
Liver	NAD	NAD
Mammary	NAD	NAD
Pancreas	NAD	NAD
Cervical lymph node	NAD	NAD
Thymus	NAD	NAD
Spleen	Yes	Yes
Thyroid	NAD	NAD
Parathyroid	NAD	NAD
Adrenal gland	NAD	NAD
Kidneys	NAD	NAD
Urinary bladder	NAD	NAD
Testes	Yes	Yes
Epididymis	NAD	NAD
Funiculus spermaticus	NAD	NAD
Ovaries	NAD	NAD
Uterus	NAD	NAD
Vagina	NAD	NAD
Body weight	NAD	NAD

Tab. 28: Table of content of the GMC-Screen of PINK1^{+/+} mice. Some screens are additionally divided into genders. Significances are expressed by asterisks (*), whereas one asterisk (*) mean significant, two asterisk (**) mean highly significant and three asterisk (***) mean highest significant. The direction of these significant changes is given as arrows. Therefore, "↑" are expressing a significant increase, whereas "↓" expresses a decrease of the values and "=" means no changes between PINK1^{+/+} and PINK1^{+/+} mice. Abbreviation: NA: no alternations; NAD: no alternations determined.

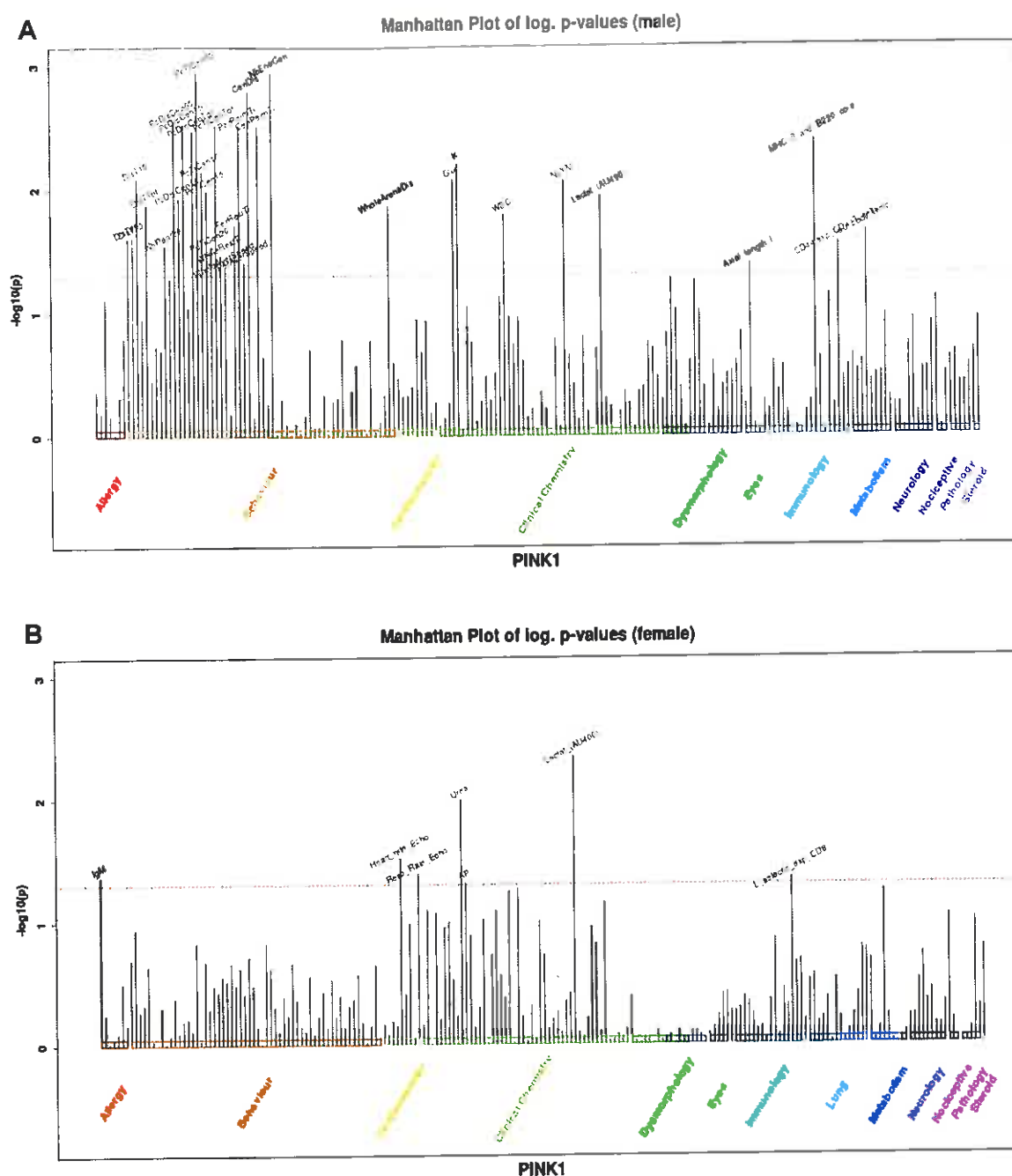


Fig. 61: Manhattan-Plots of PINK1^{-/-} male (A) and female (B) mice exhibit phenotypes of the GMC experiments. The different screens are shown in different colors and the red dashed line indicates a p-value of 0.05. Every hit of PINK1^{-/-} above this line points a significant difference to PINK1^{+/+} mice.

4.6 Walking phenotype of PINK1 mice

4.6.1 Gait analysis of PINK1 mice

The main results of the previously described and published automated gait analysis system CatWalk® [Glasl et al.; 2012] in male PINK1^{-/-} mice and littermate controls at an age of 24-27 months is presented here briefly:

The base of support (BOS) for the hind paws, i.e. the average width between the hind paws, was significantly decreased in PINK1^{-/-} mice ($p=0.0076$). In addition, the print length of the hind paws ($p=0.0435$) as well as the stand duration ($p=0.0350$) was decreased. Whereas the maximum contact at (%) in the hind paws of PINK1^{-/-} mice showed a tendency towards increasing ($p=0.0653$). "Maximum contact at (%)" reflects the time of maximum contact, at which the largest part of a paw contacts the glass plate during the total time of the contact, relative to the duration of stand phase of an individual paw placement and can be seen as the point where the breaking phase turns into the propulsion phase" [Glasl et al.; 2012]. To check the interpaw coordination Lisa Glasl from our behaviour team calculated the temporal relationship of placements of the two paws in phase dispersion and couplings. Ipsilateral phase dispersion from the left front and left hind paws as well as the right front and right hind paws show an obvious increase (L-F and L-H: $p=0.0279$; R-F and R-H: $p=0.0279$) in PINK1-deficient mice. Furthermore, phase dispersion values for both diagonal paw pairs, left front and right hind paw ($p=0.0220$), right front and left hind paw ($p=0.0350$) are also significantly increased, as well as coupling of left hind and right front paw ($p=0.0279$) and a markedly change in right front and left hind paw (0.0535).

In summary, the gait of old PINK1^{-/-} mice measured via CatWalk® system differs from PINK1^{+/+} mice at same age. This different response patterns is mainly affected by the hind paws. Note, that the hind paws are more important for stabilization of the gait than the front paws. In addition, there are changes in phase dispersions and couplings, but in both directions, increased and reduced. For further details of this data read Glasl et al., 2012.

4.6.2 Stepping test of PINK1 mice

In addition to the automated gait analysis system CatWalk®, Lisa Glasl performed a stepping test, which is commonly used to assess akinesia and gait deficits in Parkinson rodent models. Akinesia itself is one of the major motor deficits in PD. With the results of the stepping test we got additional information about the spatial and temporal parameters of the adjusting steps. PINK1 mutants made the same number of adjusting steps with their front paws as the corresponding control mice (PINK1^{+/+}: 27.78; PINK1^{-/-}: 27.07; $p=0.6934$; Tab.29; Fig. 62A), but the PINK1-deficient animals needed more time for it (PINK1^{+/+}: 4.24 sec.; PINK1^{-/-}: 6.35 sec.; $p=0.0074$; Tab. 29; Fig. 62B). Furthermore, the swing phase duration of

PINK1^{-/-} exhibit no significant increase (PINK1^{+/+}: 0.14 sec.; PINK1^{-/-}: 0.24 sec.; $p=0.0605$; Tab. 29; Fig.62C). The maximal contact time (max cont. at (%)) of the paws occurs earlier in PINK1^{-/-} animals than in PINK1^{+/+} mice (PINK1^{+/+}: 39.54%; PINK1^{-/-}: 31.96%; $p=0.02898$; Tab. 29; Fig.62D), which reflects a shorter breaking phase.

Stepping test of PINK1 mice		
	PINK1 ^{+/+}	PINK1 ^{-/-}
stepping adjustments [number]	27.78	27.07
p-value	0.6934	
crossing duration [sec]	4.24	6.35
p-value	0.0074	
swing duration [sec]	0.14	0.24
p-value	0.0605	
max contact [%]	39.54	31.96
p-value	0.02898	

Tab. 29: Stepping test of PINK1 mice. The mean values of the stepping test of each genotype are summarized of three trials per mouse. The crossing duration of mutant PINK1 mice is increased, whereas the max contact of the hind paws is decreased.

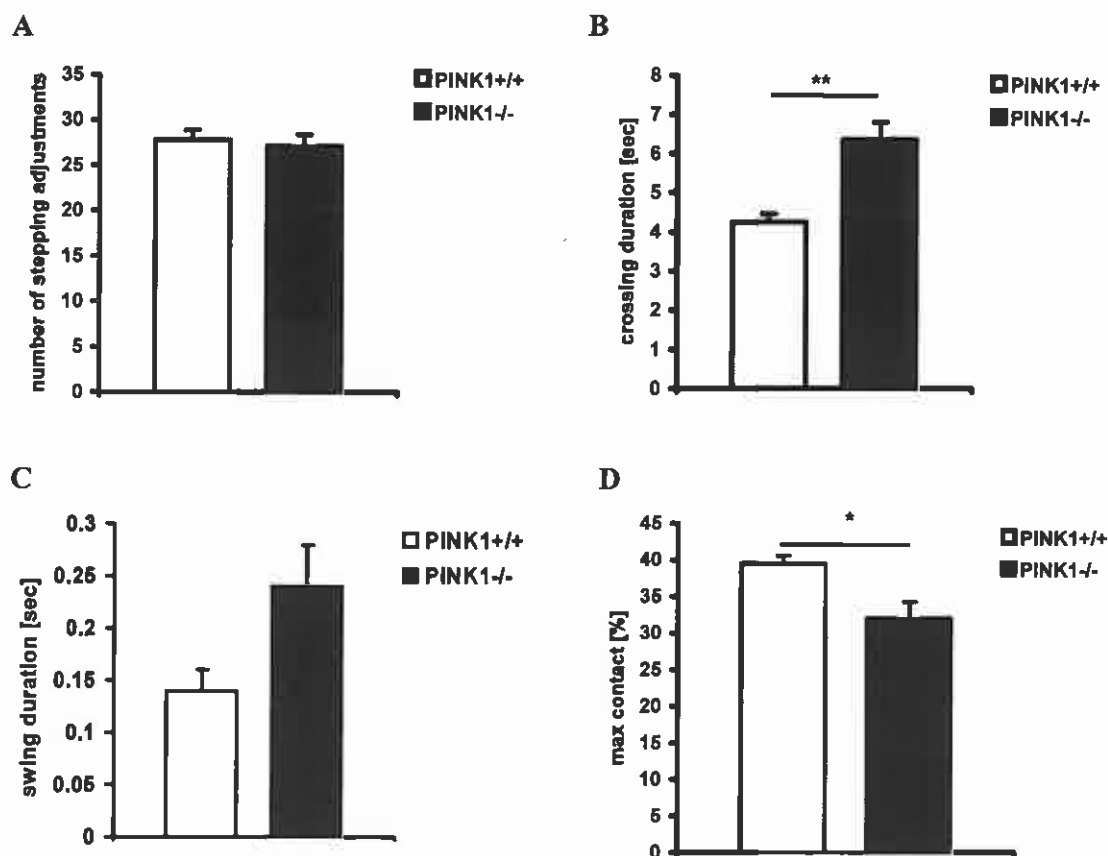


Fig. 62: Stepping test results. PINK1^{-/-} mice need more time (B) for the same number of adjusting steps (A) than PINK1^{+/+} mice. The swing duration (C) is unchanged, although the maximal contact time (D) is significantly reduced.

5 Discussion

In my thesis, the role of PINK1 in the pathoethiology of Parkinson's disease was investigated. A major part of the study focused on mitochondrial integrity and trafficking in PINK1-deficient neurons *in vitro* and *in vivo*. Our mitochondrial morphology study reveal an important new finding regarding the compensation of mitochondrial dysfunction over time and *in vivo*. This may be the reason that the disease do not attained the full extent in our mice, also like in all other mouse models of PD. Furthermore, the mitochondrial trafficking study could show obvious transport dysfunctions in PINK1-deficient neurons relating to impairment in the mobility and direction changes. With increased aging process of the neurons, this transport dysfunction exacerbated and additionally impairs mitochondrial speed and stop phases. By treatment with an additional cell stressor we were able to enhance the transport defect significant. Our results clearly show that under loss of PINK1 function the mitochondrial transport is considerable restricted and with an additional stressor the transport is even more disturbed. Thus, our results fit perfectly to the multiple-hit theory in Parkinson's disease. Differences in the gene regulation, particularly in a gene that regulates the retrograde transport, was also found in our study. Furthermore, in our systemic analysis we could found a slight gait phenotype in aged constitutive PINK1 knock-out mice, similar to the altered gait pattern which is found in PD patients. In addition, our mice seem to reveal a systemic inflammation response syndrome and impaired glucose tolerance. This was quite interesting, because PD patients with mutations in PINK1 show a high increased risk to get diabetes type 2. Our PINK1^{-/-} mice which exhibit early onset symptoms of PD are perfect models to investigate further molecular and compensatory mechanisms in Parkinson's disease.

5.1 Mitochondrial morphology studies *in vitro* and *in vivo*

Mitochondrial integrity is mainly influenced by a process called mitofission and -fusion. Unbalanced fusion leads to mitochondrial elongation (tubular mitochondria), whereas an unbalanced and higher rate of mitofission leads to smaller, fragmented mitochondria [Chan, 2006]. Under normal conditions, these opposing processes are balanced. Therefore, due to changes in the categorization of fragmented, intermediate and tubular mitochondria, conclusions to an unbalance within mitofission and -fusion processes can be taken. PINK1 is known to play a role within this process together with parkin [Weihofer et al., 2009; Sandebring et al., 2009; Winklhofer and Haass, 2010]. Striking different results of PINK1 influencing mitochondrial morphology occur within literature, depending on different animal models or used cell lines. Studies from *Drosophila melanogaster* models e.g. show that

PINK1-deficiency induces more elongated mitochondria. This effect could be rescued by increasing mitofission or decreasing mitochondrial fusion, as well as by an increase in parkin [Deng et al., 2008; Poole et al., 2008; Yang et al., 2008; Park et al., 2009]. However, PINK1-deficiency in HeLa and SH-SY5Y cells increases the percentage of fragmented mitochondria *in vitro*, which could be rescued by enhanced expression of Mfn2, Opa1 (mitochondrial fusion proteins) or dominant negative Drp1 (mitochondrial fission protein) [Lutz et al., 2009]. In these mammalian cells, PINK1 down regulation promotes mitochondrial fission. The few existing *in vivo* studies of mitochondrial morphology in mammals also represent contradictory results [Gautier et al., 2008; Gispert et al., 2009] and it is still controversial whether Pink1 modulates mitochondrial fission or fusion [Glasl et al., 2012]. Therefore, further *in vitro* and *in vivo* analyses were needed to study the influence and role of PINK1. In addition, the lack of a profound Parkinson phenotype in PINK1^{-/-} mice led us to the idea whether compensatory mechanisms are taking place.

For the *in vivo* study (partially published in Glasl et al., 2012) mitochondrial morphology study in PINK1^{-/-} and PINK1^{+/+} mice was performed to investigate endogenous PINK1 knockout. In striking contrast to the hitherto reported *in vitro* phenotypes, the results obtained in constitutive knock-out of PINK1 *in vivo* show no changes in overall mitochondrial length. In contrast, PINK1^{-/-} show a slight decrease in fragmented mitochondria compared to PINK1^{+/+}. Interestingly, Gautier and group 2008 showed an increase of larger mitochondria in PINK1^{-/-} mice, a phenotype resembling our results. Thus, the mitochondrial phenotype observed *in vitro* seems to be compensated *in vivo*. This compensation could contribute to the missing profound PD phenotype of these constitutive knock-out mice. Therefore, stereotactic injections of the Pink1sh and control lentivirus into the cortex of adult C57/Bl6-J mice were performed to investigate the effect of acute PINK1-deficiency. No significant differences in mitochondrial length or signs of an imbalance of mitochondrial fission and fusion processes occur at day 3 of acute PINK1 knockdown *in vivo*. However, mitochondria of PINK1-deficient neurons are slightly shorter at day 5 compared to controls, different to what we observed in the constitutive knock-out. Thus, these results show that acute PINK1-deficiency *in vivo* influences mitochondrial morphology comparable to the hitherto reported knock-down approaches in mammalian cell culture, an effect which seems to be compensated *in vivo*. To further substantiate our hypothesis of compensation taking place within cells, we investigated mitochondrial morphology *in vitro* at different time points post Pink1sh transduction.

In our *in vitro* study (published in Glasl et al., 2012), a highly increase of fragmented mitochondria of primary hippocampal neurons can be observed three days after Pink1sh transduction. In contrast to the higher amount of fragmented mitochondria, the intermediate and tubular mitochondria decrease inversely proportional. Consequently, the mitochondrial length in soma and processes decreases in PINK1-deficient neurons compared to control

virus transduced neurons. Just one day later, at day 4 of acute PINK1-deficiency, only a trend towards decreased mitochondrial length occurs, except shorter mitochondria in the soma. No changes in the categorization of fragmentation, intermediate and tubular mitochondria, to detect misbalances of mitofission and –fusion, are obvious anymore. At day 5 post transduction, all phenotypes and tendencies are vanished. Thus, we could demonstrate for the first time a highly significant shift from fragmented and shorter mitochondria to normal shaped mitochondria in acute PINK1-deficient neurons over time. To exclude a possible ineffective action or decline of our Pink1sh lentivirus, quantitative Real-Time-PCR was performed. At all critical time points in our experiment series the Pink1sh virus shows an effective down regulation (see also chapter 4.3). Additionally, possible side effects of the usage of the lentivirus could be excluded by detection of interferon response (see 4.3). Changes in mitochondrial morphology stir solely to the lack of PINK1.

An explanation for the contradictory results of drosophila and mammalian systems as well as between *in vitro* and *in vivo* studies could be a different regulatory mechanism of mitochondrial fission and fusion in arthropods and mammals, as well as different or at least different effective compensation mechanisms and time dependent factors (also highlighted in Lutz et al., 2009 and Glasl et al., 2012). For example in *Drosophila melanogaster* a fuzzy onions (Fzo) mitofusion gene exist which does not occur in mammals. Two closely related mitochondrial fusion homologs Mfn1 and Mfn2 existing in mammals, whereas flies only have dmnf/Marf [Chan, 2006; Lutz et al., 2009]. Although Mfn1 and Mfn2 have largely overlapping functions, there, nevertheless, exist differences. Mfn1 function, i.e. depends on Opa1 activity whereas Mfn2 function not [Chan, 2006]. In addition, the functional regulation of Opa1 seems to be different in flies, e.g. regarding the presenilin-associated rhomboidlike (PARL) protease, which is involved in Opa1 processing [Jeyaraju et al., 2006; Lutz et al., 2009]. Furthermore, the “age” of the investigated cell, especially in acute PINK1-deficient cell lines, as well as acute PINK1 knockdown *in vivo*, seems to play a crucial role. Moreover, several other molecules are discussed to be involved in mitochondrial dynamics, like endophilin B1, mitochondrial protein 18 kDa (MTP18), ganglioside-induced differentiation associated protein (GDAP), Miro-1 and Miro-2 [Chan, 2006]. Interestingly, Miro-1 and -2 are also known to play a role in mitochondrial trafficking. Mitochondrial quality control mechanisms like mitophagy may also be eligible to be involved in a compensatory mechanism.

Taken together, our *in vitro* results provide information that rapid compensatory mechanisms might prevent impaired mitofission and –fusion processes and therefore cellular dysfunctions. We describe for the first time “a clear difference between the consequences of acute Pink1 knockdown in mammalian cell culture and the constitutive knock out of Pink1 in mice (acute versus chronic loss of protein function)” [Glasl et al., 2012]. However, further

analyses are needed to unravel the molecular mechanisms underlying the observed compensation.

However, the existence of such compensatory mechanisms has an implication on the etiology of PD. It implicates that the loss of a single gene function is not sufficient to elicit the full blown disease phenotype, because compensatory mechanisms keep the system in check. However, with increasing stress factors or toxic insults these compensatory and regulatory pathways may not be able to restore mitochondrial function effectively, especially in neurons with low bioenergetic threshold and a high oxidative burden, like dopaminergic neurons in the substantia nigra [Lutz et al., 2009]. Therefore, challenging experiments like environmental stressors or toxins could deliver promising results and trigger a stronger phenotype *in vitro* and *in vivo* [Glasl et al., 2012]. This would be consistent with the prevalent multiple-hit theory, in which additionally to the genetic background, several environmental factors play an important role in the outbreak of the disease.

5.2 Mitochondrial trafficking

As already mentioned, PINK1 together with Miro and Milton build a multiprotein complex and bind mitochondria to kinesin, thus linking PINK1 to anterograde transport [Stowers et al., 2002; Glater et al., 2006; Rice and Gelfand, 2006; Cai and Sheng, 2009; Weihofen et al., 2009; Brickley and Stephenson, 2011; Wang et al., 2011]. If this transport is disturbed, e.g. due to PINK1 misregulation, it may in turn lead to a chain reaction in cells, because energy demands at different locations in the cells are no longer be covered accurately in time with fatal consequences for the neurons. Therefore, the technique of live cell imaging was successfully established to detect cytoskeleton transport of mitochondria and other organelles. Mitochondrial trafficking in cortical neurons with PINK1-deficiency were analyzed 3 and 5 days after virus transfection at different developmental cell stages (young, matured and aged neurons). In order to quantify mitochondrial speed, mobility, direction changes, stop phases and covered distance, the software "kymograph" from ImageJ was successfully brought to application. Interestingly, the mitochondria in our cell culture are generally slower than the so far known speed of mitochondria within explants or *in vivo* models [see Misgeld et al., 2006], which might be due to technical differences (e.g. culturing conditions), to different cell geometry in cell cultures or measured developmental stages ("age of the neurons"). There are also natural occurring differences between different subtypes of neurons [Molyneaux et al., 2007]. In addition, like for the mitochondrial morphology, the "age of the neuron" = "time spent in culture" seems to play a significant role. During neuronal growth and network formation, as well as in matured and aged neurons there are always phases with higher or lower energy demand. To cover an increased energy requirement of

the cell, e.g. at the synapses during neural development, it is known that mitochondria, as energy suppliers of the cell, are transported to the appropriate location [Saxena and Caroni, 2007; Yanwei et al., 2010]. Thereby also the transport of mitochondria increases. Thus, during a normal life cycle of a cell also naturally occurring fluctuations in mitochondrial transport appear. These natural variations can be observed in our control neurons. The average speed (general speed, anterograde and retrograde speed) of mitochondria at day 3 in young and matured neurons e.g. is always higher than at day 5. Especially among young neurons which are still growing and connecting with each other, the energy demand is very high. Our control neurons show here a high value in the speed of mitochondria, as well as a high rate of mobile phases. Interestingly, this higher speed can not be found in the acute PINK1-deficient neurons, whereas the mobile phase is also very high. Mitochondria of aged neurons are generally not as mobile as young ones, also clearly seen with the increasing stop time during movement at increasing cell stage. Here again, aged neurons of PINK1-deficient cells show a longer stop time than normal PINK1 expressing neurons.

In general, we could show that acute PINK1-deficiency influence the mitochondrial transport, especially the mobility and direction changes of mitochondria. Significant changes in mitochondrial transport take place almost exclusively on day 5 post virus transduction. Except aged neurons, which show an additional increase in mitochondrial speed already at day 3 of acute PINK1 knockdown. In acute PINK1 down regulated neurons the increased energy requirement of young neurons seems not to be regulated by the speed of mitochondria, because we could not see any changes in mitochondrial speed at this time point. This naturally occurring energy demand is also obvious in the mobility values of mitochondria. More mitochondria are transported in young neurons, whereas in matured and aged neurons half of the mitochondria are immobile in healthy neurons. In acute PINK1-deficient neurons more mitochondria are immobile even in early time points. With age, the rate of immobile mitochondria increases up to 74 %. Nearly 20 % more mitochondria are not moving in PINK1-deficient neurons at a given time period. A misregulation in neuronal mitochondria mobility as well as mitochondrial distribution is predicted to cause neuronal dysfunctions and degenerations, and are implicated in many neurodegenerative and psychiatric disease models [Lovas and Wang, 2012], like Amyotrophic Lateral Sclerosis [De Vos et al., 2007], Alzheimer's Disease [Lovas and Wang, 2012], Charcot-Marie-Tooth [Baloh et al., 2007], Huntington [Trushina et al., 2004] and Schizophrenia [Atkin et al., 2011]. Our results clearly show that the mobility and thus the mitochondrial trafficking in PINK1-deficient neurons, particularly in aged neurons, are disturbed.

A general striking observation was that PINK1-deficient neurons normally do not show a high natural variability than their controls. What might indicate that they can not respond to external circumstances as well as healthy neurons and appropriate changes can no longer

be well buffered. Additional factors that could affect mitochondrial functions, such as environmental factors, toxins and also age may increase this effect, which in turn would again be consistent with the multiple hit theory. For that matter, we have done another set of experiments in which mitochondria transport under additional stress conditions (elevated calcium levels) was investigated. This additional factor may emphasize mitochondria to a potentially stronger phenotype. In fact, a stronger mitochondrial transport phenotype in young acute PINK1-deficient neurons under stress application is obvious 5 days after acute PINK1 down regulation. Almost all major mitochondrial transport factors like speed, mobility, direction changes and traveled distance are significantly changed already in young stressed Pink1sh neurons compared to their stressed counterparts with normal PINK1 function. Under permanent stress application already the young neurons at day 3 post virustransduction show first changes in mitochondrial trafficking, namely in the mobility. We could therefore prove that with an additional stressor acute PINK1-deficient neurons react even more sensitive with severe changes in mitochondrial transport than unstressed neurons. So far, we only have observed mitochondrial transport changes in stressed neurons of young neuronal cortical culture. It would be interesting to see also the affect of stress application in aged PINK1-deficient neurons. Since differences in mitochondrial transport of acute PINK1 down regulated neurons already occur at day 3 in aged neurons.

The question remains open why with acute PINK1-deficiency mitochondrial transport impairment occurs at later time points compared to mitochondrial morphology impairments. Is it an independent process, or is it even triggered by the imbalance of mitofission and – fusion, changes in mitochondrial membrane potential and / or mitochondrial respiratory changes? These processes can have an impact on the energy balance and thus on the mitochondrial transport. To solve this question at least partially, other transport processes, such as endosomal transport of Rab5 need to be examined. This allows us to determine whether there is a general disturbance in the transport, or this is only related to mitochondria. If it is “only” mitochondria related, it is likely that the impairment is a disorder of energy balance, or even as a result of not optimally functional connection to Miro and Milton, two primary motor-adaptor complexes that bind to mitochondria. In this context, Wang and group uncovered some very interesting new insights of the role of PINK1 in transport mechanisms. They found out, that damaged and depolarized mitochondria recruited PINK1 on the surface of mitochondria where PINK1 phosphorylates Miro. Phosphorylated Miro, in turn, activates proteasomal degradation of Miro in a Parkin-dependent manner. Furthermore, removal of Miro from mitochondria also detaches kinesin from the surface. Therefore, “PINK1/Parkin pathway may quarantine damaged mitochondria prior to their clearance” [Wang et al., 2011]. With a lack of PINK1, the abolishment of defect mitochondria does no longer take place. Possible expression differences in gene regulation and protein regulation of Miro and Milton

are being investigated. If, however, PINK1-deficiency leads to a general transport problem, we would have to find out where exactly this kind of impairment is defined. In some other neurodegenerative diseases [reviewed by De Vos et al., 2008], axonal transport disruption occurs via damage to kinesin and cytoplasmic dynein (e.g. p38, GSD3 β or c-Jun N-terminal kinases (JNK) inhibits kinesin transport), damage to microtubules (MT) (e.g. phosphorylated tau destabilize MT), damage via inhibiting their attachment to motor cargos (e.g. via phosphorylation) or damage to ATP fuel supply [De Vos et al., 2008]. It is also known that mitochondria and also other axonally transported organelles, can move along actin filaments in axon [Morris and Hollenbeck, 1995; Hollenbeck and Saxton, 2005]. Also a mitochondria transition from the transport along microtubules to actin filament, via Myosin binding especially in the synapse region, is known [Reynolds and Rintoul, 2004; Sheng and Cai, 2012] and could also be disturbed.

Another important question that needs to be investigated in the near future is, whether the implementation of so-called rescue experiments *in vitro*, in which PINK1 deficiency should be compensated by an overexpression of human PINK1 and / or parkin, can rescue mitochondrial transport impairment. Furthermore, mitochondrial trafficking of constitutive and conditional mice would be interesting to investigate. In constitutive mice maybe there is again a compensatory mechanism taking place. Other genes involved in Parkinson and showing interaction with PINK1 should be examined in addition, like Parkin, LRRK2, DJ-1, PACAG and Omi/HtrA2 [Deng et al., 2008; Poole et al., 2008; Yang et al., 2008; Dagda, 2009; Lutz et al., 2009; Venderova, 2009; Weihofen et al., 2009; Whitworth and Pallanck, 2009; Thomas, 2010; Wilson, 2010].

Due to the fact that PINK1 is ubiquitously expressed and that with PINK1-deficiency mitochondria show strong impairments, the question remains open, why especially the dopaminergic neurons in the substantia nigra of humans are so sensitive in PD? First of all, in contrast to most other neurons, dopaminergic neurons of the substantia nigra have a spontaneous pacemaking activity with a high metabolic rate [Bezprozvanny, 2009; Elstner et al., 2011]. The pacemaking activity is driven by L-type voltage-gated calcium channels (VGCCs), the CaV1.3 type [Bezprozvanny, 2009; Elstner et al., 2011]. Neuronal calcium signalling itself undergoes significant age-dependent changes, e.g. an increase in calcium release from the endoplasmatic reticulum (ER) to the cytosol, increased calcium influx into neurons via VGCCs and reduced cytosolic calcium buffering capacity. Neurons are known to be extremely sensitive to intracellular calcium changes [Bezprozvanny, 2009]. Furthermore, an increased calcium level in the cytosol activates calcineurin and calpains, which in turn may trigger apoptotic pathways [Bezprozvanny, 2009]. In addition, calcium can activate pro-apoptotic proteins like Bax, Par-4 and p53. This leads to mitochondrial membrane permeability changes, release of cytochrome c and caspase activation [Bezprozvanny,

2009]. Moreover, mitochondria from aged neurons have accumulated oxidative damage and are less efficient in handling calcium [Toescu et al., 2007; De Vos et al., 2008; Bezprozvanny, 2009]. Continuous calcium influx in dopaminergic neurons from the substantia nigra makes the neurons more vulnerable to secondary insults on mitochondrial function [Chan et al., 2007; Bezprozvanny, 2009], e.g. like the above mentioned membrane permeability changes. Age-related mitochondria damage may amplify additional defects in mitochondrial integrity, trafficking, quality control (via mitophagy) etc.. Besides these functional changes, dopaminergic neurons have a low antioxidative capacity but high content of toxins and reactive metals bound to neuromelanin [Zecca et al., 2008]. In addition, they suffer from oxidative stress of dopamine metabolism [Hunter et al., 2009] and during brain inflammations, due to the high density of microglia around this region [Gao et al., 2011]. Taken together, aged neurons and especially dopaminergic neurons from the substantia nigra are getting more and more in a situation where all these defects can not be balanced anymore. The threshold of getting PD is exceeded.

With the help of our PINK1^{-/-} mice, with PINK1 exhibiting severe imbalance of mitochondrial functions at certain time points after acute PINK1-deficiency *in vitro* and not as severe defects *in vivo*, it is possible to analyse individual or several aspects of these pathways. Although the calcium homeostasis seems to play a role in aged neurons and therefore maybe also in PD, not much is known about the role of calcium in this disease and needs to be investigated. A promising hypothesis could be an increase of neuronal calcium due to an inflammation via environmental factors and thus activation of microglia, which in turn leads to a glutamate release and opens glutamate dependent calcium channels in neurons. Previous mitochondrial dysfunctions (like changes in morphology, membrane potential or trafficking), e.g. by PINK1 mutations – which could be compensated so far – together with increased oxidative stress and ROS production, resulting from the increased calcium influx, lead to an additional impairment of mitochondrial function. The accumulation of all of these mitochondrial malfunctions can not be handled anymore from the cell and neurons start to die. To proof this assumption, it would be useful to initially measure basal calcium concentrations in healthy and PINK1-deficient neurons, challenge them then with oxidative stressor and look for differences in calcium homeostasis in the cytosol and mitochondria. If differences in calcium buffering are obvious, further experiments are needed, like rescuing this phenotype via blocking appropriate calcium channels (e.g. inhibition of CaV1.3 L-type calcium channels with dihydropyridine isradipine or inhibiting glutamate activated NMDAR receptors). This hypothesis is partly supported by findings of Chan et al., 2007. They could show that subcutaneous delivery of isradipine, a pharmacological inhibitor of CaV1.3 L-type calcium channel, significantly protects substantia nigra neurons in animal models of PD [Chan et al., 2007].

Taken together, all of our findings suggest that PINK1 knockout mice are very promising candidates to study early non-motoric phases of Parkinson's disease, as well as compensatory mechanisms, and maybe a starting point to find early diagnostic procedures of PD or neuroprotective therapies.

5.3 RNA expression of mitofission and –fusion genes and mitochondrial transport genes

To find out whether the previous shown effects in mitochondrial morphology and trafficking are due to a change in mRNA expression, like changes of Fis1, Drp1, Opa1, Mfn-1 and -2 for mitochondrial morphology, or Miro-1 and -2, Milton, Dynein Cytoplasmic 1 heavy chain 1 (Dctn1) and Dynein heavy chain (Dnhd1) for transport mechanisms, we performed RT-PCRs. The relative quantification of our RT-PCR is based on internal reference genes to determine fold-differences in the target gene expression. A biologically meaningful experiment series of target mRNA copy numbers requires accurate normalization to a standard gene. Any unwanted variation in the standard gene will obscure real changes and produce artificial changes [Bustin, 2000]. Only very few genes show equal levels of expression across a range of different conditions [Nolan et al., 2006; Dhanasekaran et al., 2010], like our different timeframes in which the neurons grow and form network connections. For that reason, we searched for the best reference gene and tested the following five commercially available and widely used reference genes: β -actin, GAPDH (play a major role in glycolysis, energy and carbon molecule supply), pgk1 (catalyses ATP to ADP in glycolysis), HPRT (involved in purine salvage pathway) and rpl27 (important for ribosomal assembly and function). The CT curves, the number of cycles at which the fluorescence exceeds the threshold, of all of these standard genes (Fig. 57, page121) show clearly that there are almost no changes in β -actin and rpl27 at day 3 and day 5. Therefore, they are the most suitable internal standard genes for our experimental trial series and were used as reference genes.

5.3.1 RNA expression of mitofission and –fusion genes in primary cortex neurons after acute knockdown of PINK1 show a slight reduced tendency at day 3 and day 5

To see the mitochondrial morphology changes, which I could detect in primary neuronal culture at different time points after acute knock down of PINK1, as well as *in vivo*, mRNA concentration of mitofission and mitofusion genes were measured at day 1, 3 and 5 after virus transduction for acute PINK1-deficiency, identical to the morphology experiments. First of all we had to determine the knockdown efficiency of our virus. On day 3 and day 5 after

PINK1sh treatment, the interesting time points in our experiment series, the virus displays a high and significant efficiency to down regulate the gene PINK1.

Looking for the mitofission genes Fis1 and Drp1, no significant changes in gene regulation are obviously at day 1, 3 and day 5 after acute PINK1 down regulation. The same applies to the mitofusion genes Mfn1, Mfn2 and Opa1. No significant changes could be found. But all expression rates of the mitofission and -fusion genes seems to be slightly decreased at day 3 and 5 after PINK1-deficiency compared to the internal control. No changes in gene expression of mitofission and -fusion genes are taking place in primary neuronal culture at day 1, 3 and 5 after acute knockdown of PINK1. In order to detect possible changes in the protein expression, especially on day 3, western blots with antibodies against the mitofission and -fusion proteins have to be done (ongoing process). Furthermore, other genes or proteins may play a role here, e.g. endophilin B1 and the mitochondrial membrane protein MTP18, GDAP etc. (see also chapter 5.1) [Chan, 2006]. Direct interacting genes of PINK1 and / or Parkin, like DJ-1, LRRK2, Omi/HtrA2, Parkin co-regulated gene (PACRG), may influence mitochondrial morphology and are interesting candidates, which should be checked.

5.3.2 RNA expression of mitochondrial transport genes in primary cortex neurons after acute knockdown of PINK1 provide indications of a disturbed retrograde transport

To detect changes in genes associated with mitochondrial transport in cortical primary neurons after acute knockdown of PINK1, we analysed the RNA expression of mitochondrial transport cargos. Among them are some of the components of the dynein transport like Dctn1 and Dnhd1 and components of the kinesin transport like Miro-1, Miro-2 and Milton. A significant decrease in Dnhd1 expression (decline of 24 %), a part of the dynein complex, is taking place already at day 3, but is not yet as strong as two days later at day 5 after acute PINK1 knockdown (decline of 39 %). This may be a first hint for an impairment of mitochondrial retrograde transport also on molecular level. A detailed analysis of Dnhd1 protein expression is necessary to proof also protein impairment of mitochondrial transport processes in our acute PINK1-deficient neurons. In addition, the other transport cargos have to be checked. There may be possible protein changes in endogenous PINK1-deficient neurons of our PINK1^{-/-} mice, which also have to be examined. So far, in the literature is nothing known about the involvement of PINK1 in mitochondrial transport via the dynein complex. But it is supposed that many functions of PINK1 are yet not completely understood or decoded.

Miro1, which has a tendency of reduced mRNA expression, may also be an interesting candidate in a protein expression study. Miro is known to form a multiprotein complex with

Milton and PINK1 [John et al., 2005; Fransson et al., 2006; Guo et al., 2006; Glater et al., 2006; Rice et al., 2006; Weihofen et al., 2009; Wang et al., 2011], and phosphorylated Miro (activated via PINK1 recruitment to mitochondria membrane of damaged mitochondria) activates proteasomal degradation of Miro. Therefore, Miro is removed from binding to kinesin [Wang et al., 2011]. Thus, there is strong evidence that PINK1-deficiency may influence also the Miro (and / or Milton) protein expression.

Furthermore, we checked a possible interferon response (Oas1b) of the virus, which is sometimes described in literature [Bridge et al., 2003; Fish et al., 2004; Pebernard et al., 2004; Bauer et al., 2008]. No change of Oas1b expression could be found on any experimental time point. Therefore, possible occurring side effects of lentivirus usage can be excluded in our experiments.

5.4 GMC screen

PINK1 is known to be ubiquitously expressed and seem to have a pleiotropic role within PD. Therefore a systemic analysis of the PINK1 knockout mice would shed more light on the function of PINK1 and possible defects in PD. Hence, a large PINK1 cohort was prepared and screened in the German Mouse Clinic. An overview of all significantly changed results and their connection with each other is shown in Fig. 64. In the following article these striking phenotypes and some hypothesis to be tested are described.

An increased forward locomotion of our PINK1 mutant mice could be found in the open field test of behavior screen. Interestingly, also in human PD patients a phase of hyperactivity occurs very often [Walitza et al., 2007]. In addition, the behavior screen showed an increase in anxiety-related behavior in our male PINK1 knockout mouse line within the first 5 minutes of the test, which is measured by the total time mice spent in the center of the open field. This means that our male PINK1^{-/-} mice show less anxiety, which was somehow surprising and stands in contrast to previous done behaviour tests of our group, but under different conditions, and in contrast to published data in PD patients. Anxiety is a major non-motor symptom in Parkinson's disease patients with lifetime prevalence about 49 %. But it is still not well characterized [Walitza et al., 2007; Protone et al., 2010]. However, it is widely believed that hyperactivity may influence the anxiety-related behaviour [Tohda et al., 2009; Yen et al., 2011]. It is conceivable that if the hyperactivity increases, also the anxiety-related behavior increases. Furthermore, stress is also known to influence anxiety-related behavior. With increasing stress, corticotropin-releasing hormone (CRH) is secreted by the hypothalamus and paraventricular nucleus (PVN) and stimulates the synthesis of adrenocorticotrophic hormone (ACTH) in the pituitary, which is then released into the blood. The adrenal gland binds ACTH and thus activates corticosterone, which is also released into

the blood and regulates a feedback loop on hypothalamus and pituitary [literature for corticotrophin-releasing factor pathways: Elliott-Hunt et al., 2002; Taché et al., 2005; Laryea et al., 2012]. Corticosterone itself can be used as a marker for stress [Laryea et al., 2012]. Our PINK1-deficient male mice have a significantly higher corticosterone level. Under normal conditions an increase in corticosterone leads to a decrease in anxiety-related behavior. Our results contrast to that fact, which is maybe a first indication that seemingly at least in our male PINK1 knockout mouse model a disorder in the stress pathway and thus in the CNS is taking place. A first approach to check this hypothesis is to proof the density and amount of glucocorticoid receptors (GR) and mineralocorticoid receptors (MR). GR are less sensitive to corticosterone concentrations, which means they are only active at high levels of corticosterone. MR in contrast, which is mostly located in the hippocampus, is more sensitive and is already activated at very low corticosterone levels [Wu et al., 2012]. It would be interesting to see if these values are also changed in our PINK1^{-/-} mice. Furthermore, we could calculate the weight of the adrenal gland and thymus with respect to the body mass. Normally, with high corticosterone levels, the adrenal gland enlarges whereas the thymus reduces. Additionally, we could perform an acute restrain test that triggers a real strong stress in mice (see publication of Annemarie Wolff-Muscate from the group of Sabine Hölter, in preparation) and so draw conclusions about a possible disturbed CRH-stress system in PINK1-deficient mice. But why do this corticosterone and anxiety-related behavior remains gender specific? A possible explanation could be the neuroprotective effect of estrogens, which of course is present in female mice at elevated levels. Furthermore, higher corticosterone values normally occur in response to an acute stress situation, but our mutant mice already have a higher corticosterone concentration under basal conditions. Perhaps there is an inflammatory process taking place in our PINK1 knockout mice, which in turn increases the corticosterone due to cell stress – perhaps triggered by mitochondrial impairment.

An interesting discovery that we could see from the data of the GMC, was the realization that our PINK1^{-/-} mice probably have a so-called systemic inflammation response syndrome (SIRS). SIRS is defined, at least in humans, when two or more of the following factors occur simultaneously: an elevated heart rate, high respiratory rate, abnormal white blood count and elevated or lower body temperature. In fact, the GMC screen shows two of these factors changed in our male PINK1 mutants (higher body temperature and higher rate of white blood cells) and even three factors are changed in female PINK1^{-/-} mice (higher body temperature, higher rate of white blood cells and elevated heart rate) [reviewed by Robertson and Coopersmith, 2006]. Also, the fact that the increased corticosterone value, which was found in the steroid metabolism of male PINK1-deficient mice, can be triggered by a systemic inflammation response and reaffirm this presumption. Furthermore, the pathology found

deficits in the spleen of PINK1 knockout mice. The ratio spleen to body weight was significantly reduced in female PINK1^{-/-} mice, as well as increased deposits of hemosiderin in male PINK1^{-/-}. Hemosiderin is an iron-storage complex of ferritin, denatured ferritin and other materials [Harrison and Arosio; 1996; Chasteen and Harrison, 1999]. But the iron within deposits of hemosiderin is very poorly available. Iron itself is essential in almost all cells, e.g. for oxidase reductase reactions and some catalysis, but is also toxic in certain amounts because of its propensity to form oxygen radicals. Hemosiderin can be found e.g. in macrophages and is suggested to be related to phagocytosis of red blood cells and hemoglobin [Harrison and Arosio; 1996; Chasteen and Harrison, 1999]. It is normally characteristic in cases of hemolytic anemia or toxic insults. What in turn is again a hint to a possible inflammatory process by yet unknown environmental factors. But for the pathology just 6 animals of each genotype were analyzed. Nevertheless, the fact that 6 out of 6 animals have deposits of hemosiderin showed the high penetrance of this phenotype. With deposits of hemosiderin usually an increase of platelet count (PCT) is expected, which indeed was found in PINK1-deficient mice. Moreover, in the substantia nigra of Parkinson's disease patients a highly increased amount of iron can be detected [Hunter et al., 2009; Zecca et al., 2006].

With SIRS the body needs more insulin which in turn leads to a reduction of insulin within the blood [Van den Berghe et al., 2001; Jeschke et al., 2002; Jeschke et al., 2004; Albacker et al., 2008]. Our PINK1 mice were not tested for insulin, but basal glucose levels were measured. With a reduction of insulin in the blood the concentration of glucose have to increase, because almost all cells need insulin to accommodate glucose into the cell. Indeed, PINK1^{-/-} mice show an increase of basal glucose in the blood. When SIRS would be active, it would lead in addition to an increase in blood urea and an increased requirement of potassium and as its consequence to a reduction of potassium in the blood. Also these values, the reduction of potassium as well as the increase of urea, could be shown from the clinical chemistry screen. A reduction of potassium in addition influences the heart rate. Changes in heart echo rate were also found in our PINK1 knockout mouse model.

In the nociception screen a decrease in first response time to the hot plate test was found. This means that PINK1-deficient mice are more sensitive to pain. An increased sensitivity to pain is called hyperalgesia and may be caused by damaged nociceptors or peripheral nerves and can be increased due to evolved response in inflammation (a new clue to SIRS). In patients with PD pain is a common symptom playing a major role and influencing quality of life enormously [Djaldette et al., 2004; Schestatsky et al., 2007; Mylius et al., 2011; Perrotta et al., 2011]. Pain is reported by 40-75 % of patients with PD [Gerdelat-Mas et al., 2007]. Various types of pain in PD are known and can be an early symptom in PD. Pain can precede the diagnosis of the disease [Djaldetti et al., 2004]. Increased experimental pain

sensitivity was observed in patients exhibiting Parkinson's disease-related pain [Mylius et al., 2011]. Furthermore, Tinazzi and group could show a significantly reduced heat pain threshold in PD patients [Tinazzi et al., 2009]. Furthermore, a measurement of the nociceptive withdrawal reflex at both sides of the body would give a deeper insight into hyperalgesia in Parkinson's disease mouse models. Symptoms in PD also usually occur first in one side of the body and spreads out with proceeding disease. Furthermore, it probably exist a direct correlation between basal ganglia deficits and deficits in nociception (see Fig.63) [Gerdelat-Mas et al, 2007; Tinazzi et al., 2009]. Under increased stress e.g. by fear or pain mice produce more sounds, which leads to an increase in vocalization. In fact, in the neurology screen an increase in vocalization was found.

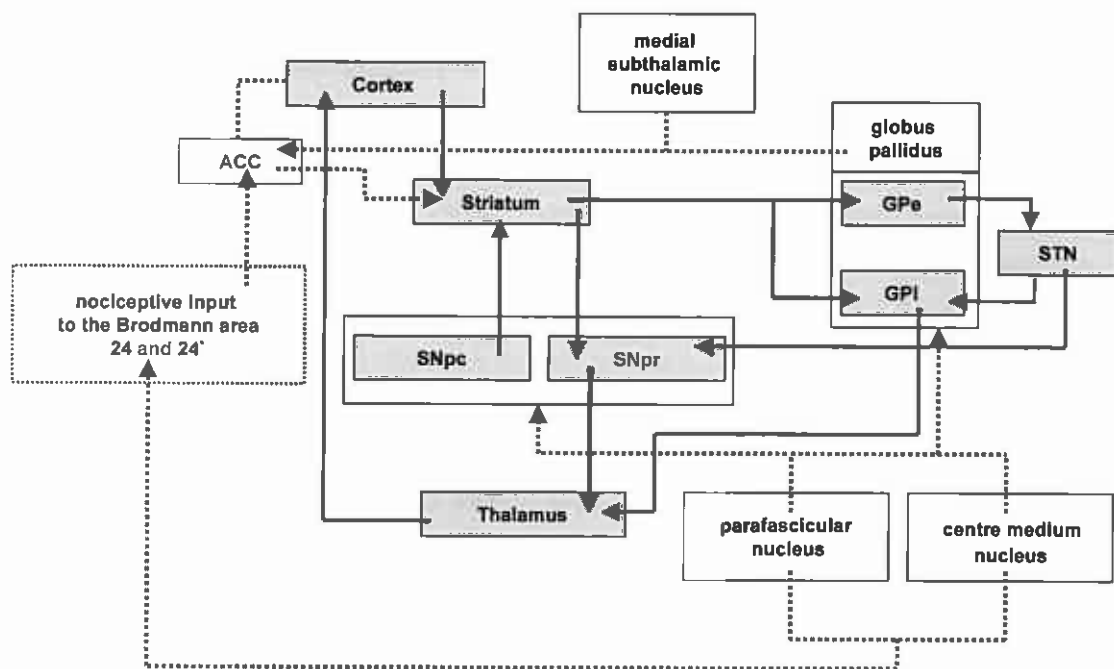


Fig. 63: Involvement of nociception in the basal circuit. The gray areas and arrows are part of the basal ganglia circuit, whereas the dotted arrows indicate the brain regions involved in nociception. Nociception inputs to the Brodmann areas 24 and 24' have their origin in the parafascicular nucleus and centre medianum nucleus. Both of these nuclei send projections to the globus pallidus and substantia nigra [Valeriani et al., 2000]. Almost 30 years ago it was shown that neurons in the globus pallidus and medial subthalamic nucleus project to the anterior cingulate cortex (ACC) [Jurgens, 1983]. And neurons from the ACC in addition projects to the striatum [Chudler et al., 1995]. **Abbreviations:** ACC: anterior cingulate cortex; GPe: external part of the globus pallidus; GPi: internal part of the globus pallidus; SNpc: substantia nigra pars compacta; SNpr: substantia nigra pars reticulata; STN: subthalamic nucleus.

Recent studies from Scheele et al. (2007) and Franks et al. (2008) linking PINK1 deficiency to type 2 diabetes. Misregulated PINK1 in human neuronal cell lines impaired basal glucose uptake [Scheele et al., 2007]. With impaired glucose tolerance (insulin resistance) the risk of type 2 diabetes rises. Impaired glucose tolerance would lead to a decrease of potassium, an

increase in urea and an increase in glucose which we can find in our PINK^{-/-} mice. But to make a statement for an impaired glucose tolerance or type 2 diabetes within our mice it is too early. Additional tests are needed to confirm this assumption, like glucose tolerance test under different conditions, most important insulin measurements, as well as analyzing pancreas and more detailed the beta and alpha cells in the pancreatic glands. In a previous study of our group we could show that PINK1 is strongly expressed in pancreas. Therefore, an immunofluorescence staining against insulin for the beta cells and against glucagon for alpha cells could be performed. Alpha cells can also be indentified morphological by their characteristic granules. Furthermore, polyuria with increased drinking and eating should be observed in addition in our PINK1^{-/-} mice.

One final point of interest is found in the pathology screen. Here it was clearly shown that 1/3 of our PINK^{-/-} mice have testicular degeneration and one of these animals displayed an additional spermatocele in the epididymis. These are very interesting results, because in the animal model of *Drosophila melanogaster* a very striking phenotype of reduced male sterility (defective spermatogenesis) was shown. Furthermore, evidences were given that immature testis are vulnerable to oxidative stress and mitochondrial dysfunction [Chandrashekar and Muralidhara, 2009]. Mitochondria are very frequently in testis and with a loss of PINK1 function also mitochondrial dysfunction appear and can influence testis function. But for this screen only 6 mutant mice were tested and to corroborate this phenotype more mice have to be tested.

From our mitochondrial analysis we already know that a lack of PINK1 impairs mitochondrial integrity and trafficking. Also respiratory chain efficiency, energy production and thus, changes in membrane potential were found [reviewed by Büeler, 2010; Chu, 2010; Xi et al., Zhu and Chu 2010; Exner et al., 2012; Pils and Winklhofer, 2012]. This means, that the oxidative phosphorylation is disturbed, which consequently affects the glycolysis as a precursor step of oxidative phosphorylation. Therefore, pyruvate can not be approved into mitochondria very well. Pyruvate inclusion may somehow be disturbed and is converted into lactate via LDH and an increase of lactate occurs. Indeed, our PINK1-deficient mice show an increased lactate concentration. Increased lactate concentration may function as an indicator for mitochondrial dysfunction. A more detailed study of mitochondrial metabolism, especially glycolytic and oxidative phosphorylation pathways, are planed via a "Seahorse bioenergetic profile" study. Finally the GMC data also support the hypothesis of a strong influence of mitochondrial dysfunction in Parkinson's disease.

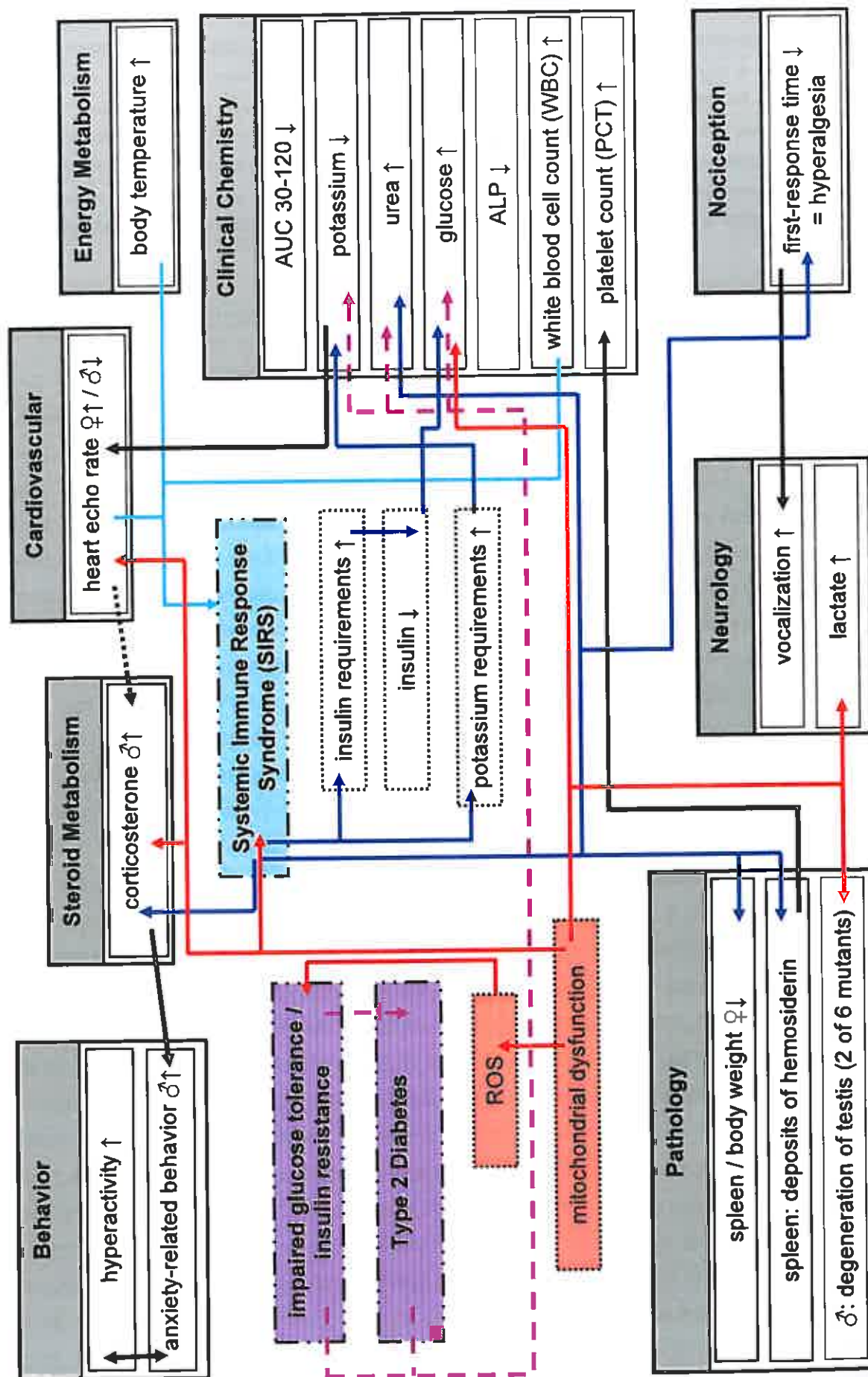


Fig. 64 on the previous page: Overview of the discovered significant changes in the GMC screen and their connection with and to each other. In addition the influence of mitochondrial dysfunction is also shown. A detailed description of the graphic is found in the text above. Arrows are shown in different color to better recognize the course of the arrows. The recently suspected connection of PINK1 with impaired glucose tolerance and diabetes is represented in pink color, the influence of mitochondrial dysfunction is represented in red, while the influence of SIRS on our determined values is shown in blue color. Light blue arrows represent the indication for correct diagnosis of SIRS, whereas dark blue arrows represent the direct consequences of SIRS.

5.5 Gait impairment of PINK1 mice

Our PINK1-deficient mouse model exhibits clear behavioral symptoms of early non-motor phases of PD, like olfactory dysfunctions. In order to detect other early Parkinson syndromes in our mice a number of different motoric tests were performed and analyzed in collaboration with the behaviour group containing Open Field, gait analysis via CatWalk, Vertical Pole Test, Rotarod and Grip Strength. In particular, interesting anomalies were observed in gait tests in two years old PINK1^{-/-} mice [Glasl et al., 2012]. The PINK1^{-/-} mice showed different response patterns especially in the hind paws with reduced base of support, print length and stand duration, and a tendency of increased maximum contact. In addition, stepping test is commonly used to assess akinesia, one of the major motor deficits in Parkinson's patients, and gait deficits in Parkinson rodent models. With this test we would get additional information about the spatial and temporal parameters of the adjusting steps. Consequently, we performed a stepping test which revealed that our PINK1 mutants need more time for the same number of adjusting steps and also the maximal contact time of the paws occurs earlier than in PINK1^{+/+} mice, resulting in a shorter braking phase. The swing phase duration remained unchanged.

Although mice are walking around on four paws and therefore have a different center of gravity, the changes in gait found in our PINK1 mice are partly very similar to the gait phenotype in PD patients. In humans, gait disorder in PD consist of reduced walking speed and step length [Gantchev et al., 2000; Kemoun and Defebvre, 2001a,b,c; Chastan et al., 2009], reduced base of support [Morris et al., 2001a,b; Glasl et al., 2012], start hesitation, freezing and festination [Giladi, 2001; Chastan et al., 2009]. Furthermore, poor braking just prior to foot-contact, a reduced maximum activity in gastrocnemius and controversially discussed, also a decreased tibialis anterior muscle activity occur [Chastan et al., 2009]. Gastrocnemius and tibialis anterior muscle are antagonists in the leg of mammals. In PD patients, it is known that monosynaptic and polysynaptic reflexes are diminished in the leg extensor muscles and pre-programmed response of the flexor muscles are impaired [Mitoma et al., 2009]. But not all of the found gait differences in PD patients have been studied in mice. So far, no electrophysiological studies on muscles involved in gait were performed in

PD mouse models or studies of the reflex arc of leg nerves. Therefore, we have started a collaboration to analyse different muscles responsible for the gait in mice, the tibialis anterior muscle and the gastrocnemius muscle. Tibialis anterior muscle is also called musculus tibialis cranialis in domesticated mammals. Fluorescent tracers and electrophysiological studies will be performed to detect changes in the reflex arc as well as to analyze the electrochemical signal transmission. The major goal of the retrograde tracing study of hind limb nerve fibers is the investigation of possible impaired neural pathways of the hind limb which regulates walking in mice. Mitoma et al. assume that different modulation of monosynaptic reflexes, and / or different compensatory polysynaptic responses in the leg muscles are playing a role in PD [Mitoma et al., 2000].

Preliminary results of the tracing and electrophysiology studies have shown promising evidence with respect to an altered gait phenotype found in human Parkinson patients. Detailed studies in humans showed that gait is a dynamic and periodic process during which loss and recovery of balance alternate [Chastan et al., 2009]. In the gait initiation, a falling phase in the centre of gravity is followed by a phase of breaking of the centre of gravity fall. This centre of gravity fall can be arrested by activation of the ankle plantar flexor, prior to foot-contact of the swing limb, or by the swing limb hitting the ground [Chastan et al., 2009]. If the ankle plantar flexor does no longer function correctly, it may result in falls within PD patients. In fact, almost 50 % of all falls occur while walking, in particular during the initiation and termination of gait [Masud and Morris, 2001; Chastan et al., 2009]. A dysfunctional reflex arc caused by incorrect or inadequate nerve networking or stimulus transmission in the spinal cord of our PINK1-deficient mice may also exist. Furthermore, despite the absence of dopaminergic neurodegeneration in our PINK1^{-/-} mice, it is still possible that functional changes in dopamine turnover could be responsible for the observed gait alterations [Glasl et al., 2012]. In addition the group of Chastan suggests that the gait initiation process is not directly controlled by the dopaminergic system but by the activity of basal ganglia, in particular that of the subthalamic nucleus (STN). In line with these hypothesis, high frequency stimulation of the STN or the internal part of the globus pallidus (GPi), a main output of the basal ganglia, was shown to alleviate the gait dysfunction very well [Chastan et al., 2009; reviewed by Alam et al., 2011]. Also low frequency stimulation of the pedunculopontine nucleus (PPN) region has been shown to improve impaired gait in PD [Stefani et al., 2007]. Mesencephalic structures, which project efferent pathways to the lower brainstem and spinal cord and receive afferent inputs from the basal ganglia structures like substantia nigra pars reticulata (SNr), STN and GPi [Takakusaki et al., 2005a,b], seem to play at least in part a role in gait controlling [Chastan et al., 2009].

An additional interesting but complex test would be the possible rescue of our obtained gait phenotype by activation of the STN via high frequency stimulation similar to that of human

application. Also the high frequency stimulation of the GPi or the low frequency stimulation of the PPN region would be an alternative. Furthermore, the muscle activation sequence of the tibialis anterior in human PD patients was normalized by facilitatory insole, at the time of initial ground contact and may lead to an overall improvement in gait pattern and stability [Jenkins et al., 2009]. But the use of facilitatory insole is not practicable in mice. Rather more promising is the treatment of our mice with L-DOPA. Although a full recovery of gait disturbances were not achieved in people with Parkinson's disease [reviewed by Alam et al., 2001], which in turn is an additional indication that also other neurotransmitter systems are involved. In animal models of monkeys and cats the locomotion and postural control was modulated with SNr activity via injection of GABAergic agents, lesions or electrical stimulation [Burbaud et al., 1998; Takakusakt et al., 2003; Henderson et al., 2005; Chastan et al., 2009]. Impaired motor performance may be controlled by a combination of the different mechanisms discussed above. Additional experiments are necessary in order to bring full light into the gait phenotype of PD mice, which in turn allows conclusions to PD patients and help to unravel the complexity of this disease.

5.6 Conclusions remarks

The aim of the present study was the systemic analysis of PINK1 for further investigation of the PD etiology and to get a closer insight into the physiological role of PINK1 in mitochondrial integrity and trafficking in health and disease. Previous studies of our PINK1^{-/-} mouse model exhibits early pre-motor phases of PD, like olfactory impairment and subtle gait alternations. A characteristic symptomatic phase with degeneration of dopaminergic neurons in the substantia nigra could not be observed. The so far recorded non-motor phenotypes in this ubiquitous expressed knock-out model indicate that beyond the dopaminergic system other neurotransmitter systems may be involved in the pathoetiology, like serotonergic or noradrenergic pathways [Glasl et al., 2012]. In addition, compensatory mechanisms in the living mouse may impede the appearance of major motor-symptoms. Thus, inducible conditional knock-outs of PINK1 are needed to investigate the contribution of the different neurotransmitter systems and to investigate whether compensatory mechanisms maybe overcome in inducible cell type specific knock-outs. Therefore, morphological, behavioral and neuronal functions should be investigated in PINK1-floxed mice crossed with Dat-Cre mice to analyse the dopaminergic system, with Pet1-Cre mice to analyse the serotonergic system and with DBH-Cre mice to analyse the noradrenergic system.

Furthermore, our mitochondrial integrity studies *in vivo* and *in vitro*, based on the widespread assumption that mitochondrial dysfunction may be a major cause of PD, revealed some important new findings. The detailed analysis of mitochondrial alternations *in vivo* in our

constitutive PINK1 loss-of-function mice and in acute PINK1-deficient mice reinforces the presumption of compensatory mechanism which may take place. In addition, we performed an analysis of mitochondrial morphology *in vitro* of acute PINK1-deficient primary cortical neurons and could show that mitochondrial fragmentation can be compensated with time. Thus, these results exhibit for the first time, that distinct differences exist between acute PINK1-deficient neurons in primary cell culture and acute and chronic PINK1-deficiency *in vivo* and that cellular dysfunction induced by Pink1sh can be compensated with time.

PINK1 is also involved in mitochondrial transport processes, although the dysfunction of mitochondrial trafficking in PD is largely unknown. For the first time, we have carried out a wide-ranging study of the role of PINK1 within mitochondrial transport processes and could show transport impairments due to acute PINK1-deficiency at different developmental stages of neurons *in vitro*. Further experiments in constitutive PINK1 knockout are needed to make a statement about the degree of a possible compensatory mechanism. Although the present study gave new interesting insight into the role of PINK1 in mitochondrial transport, we wanted to meet the prevalent multiple-hit-theory and therefore investigated mitochondrial trafficking in acute PINK1-deficient neurons under challenged conditions. Our initial assumption that the calcium challenge may exacerbate the phenotype could be clearly shown. But whether a lack of PINK1 affects only mitochondrial transport processes or general organelle transport remains open and needs to be explored next.

In addition, a detailed analysis of mRNA expression levels of mitochondrial fission and fusion genes, as well as transport cargos exhibit a decrease in dynein heavy chain, which is a part of the dynein complex and is mostly responsible for retrograde transport. A corresponding protein expression study is also currently executed.

The obtained results of mitochondrial integrity and trafficking lead to the hypothesis (Fig. 65), that neurons with acute PINK1-deficiency impair mitochondrial energy production and membrane potential. This in turn leads to impaired mitochondrial fission and fusion processes, as well as defects in the kinesin transport via an impairment of the complex binding with miro and milton, and defects in dynein transport, due to a decrease on dynein heavy chain gene expression. Consequently, mitochondrial transport is disturbed. Taken together, impaired mitofission and -fusion processes, as well as impaired mitochondrial trafficking may lead to mitophagy, a process which separates out dysfunctional mitochondria. With increasing cell stress, via aging, environmental factors, toxic insults, inflammation, etc. neurons are not able to compensate these defects anymore and thus, activating apoptosis.

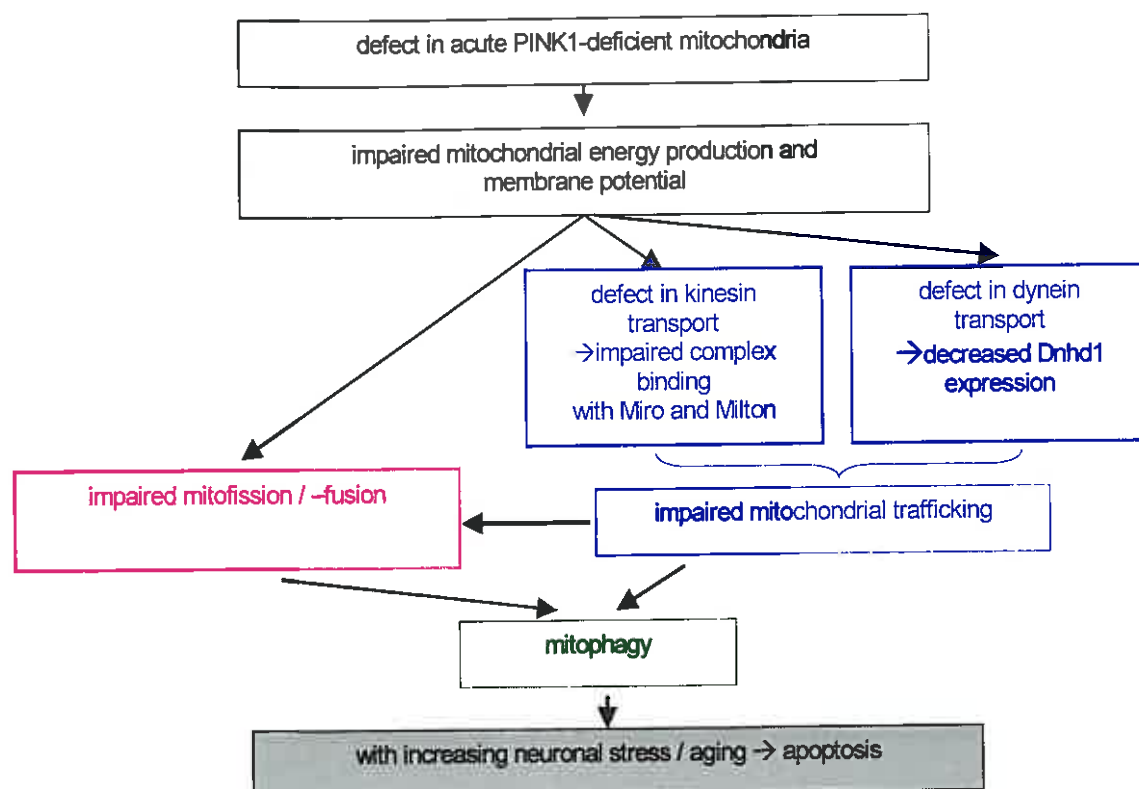


Fig. 65: Hypothesis about the Influence of acute PINK1-deficiency. Neurons lacking PINK1 influencing mitochondrial energy production and impair membrane potential. As a consequence, imbalanced mitochondrial fission and fusion processes occur, as well as defects in the transport of mitochondria. These dysfunctional mitochondrial mechanisms activate mitophagy, which can compensate malfunctioning working mitochondria. But with increased stress, e.g. due to aging or environmental factors, the clarifying process of mitophagy can not compensate all these defects anymore and neurons start to die.

In my thesis, I could show that mitochondrial dysfunction via PINK1-deficiency, including mitochondrial integrity and trafficking, may play a crucial role in the disease and can be compensated to some extent *in vitro* and *in vivo*. Not only that PINK1 influences mitochondrial functions, the malfunction of mitochondria due to a lack of PINK1 will get worse with time and additional stress factors. Our PINK1^{-/-} mice with early onset symptoms of PD seem to be a perfect model for analyzing further molecular and compensatory mechanisms involved in PD as well as to find biomarkers to allow an early diagnosis and treatment of PD.

6 Appendix

6.1 Abbreviations

6-OHDA	6-hydroxydopamine
ABC	Animal Blood Counter
ACC	anterior cingulate cortex
ACTH	adrenocorticotrophic hormone
ASAT/GOT	alpha-amylase, aspartat-aminotransferase
ALAT/GPT	alanine-aminotransferase
AP	alkaline phosphatase
ATP	Adenosine Tri-Phosphate
AUC	area under the curve
bp	basepair
BSA	bovine serum albumin
BOS	base of support
°C	degree Celsius
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
Cchord	chord compliance
Cdyn	dynamic lung compliance
cko	conditional knockout
CMV	cytomegalovirus promotor
CRH	corticotropin-releasing hormone
cpm	counts per minute
Cre	cyclization recombination enzyme (Cre recombinase)
dATP	desoxy adenosine triphosphate
dCTP	desoxy cytidine triphosphate
dGTP	desoxy guanosine triphosphate
dNTP	desoxy nucleoside triphosphate
DA	dopamine
DAB	3,3'-diaminobenzidine
DAT	dopamine transporter
DBS	deep brain stimulation
DMEM	Dubecco's modified Eagle's medium
Dctn 1	Dynactin 1
Dnhd1	Dynein heavy chain
Drp1	Dynammin-related protein 1
Dync1h1	Dynein cytoplasmic 1 heavy chain 1

E15.5	embryonic day 15.5
ECG	Echocardiography
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia (Latin), for example (English)
ER	endoplasmatic reticulum
ERV	expiratory reverse volume
et al.	et al. et alii (Latin) / others (English)
EtBr	ethidium bromide
ETC	electron transport chain
EtOH	ethanole
FCS	fetal calf serum
FEV100	forced expiratory volume
Fig.	Figure
Fis1	Anti-Mitochondrial fission 1 protein
flp	flp flipase recombination enzyme
FRC	functional residual capacity
FRT	flipase recognition target
FV	fast flow volume
FVC	forced vital capacity
Fzo	fuzzy onions mitofusion gene
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDAP	ganglioside-induced differentiation associated protein
GFP	green fluorescent protein
GMC	German Mouse Clinic
GPe	external part of the globus pallidus
GPI	internal part of the globus pallidus
GR	glucocorticoid receptors
H&E	haematoxylin-eosin stain
HEK 293T	Human Embryonic Kidney 293 cells expressing large T antigen
HCT	hematocrit
HGB	haemoglobin
HPLC	high performance liquid chromatography
HPRT	hypoxanthine guanine phosphoribosyl transferase
IC	inspiratory capacity
IDG	the Institute of Developmental Genetics
i.p.	intraperitoneally

IpGTT	Intraperitoneal Glucose Tolerance Test
IRES	internal ribosomal entry site
IVSd	interventricular septum diastole
IVSs	interventricular septum systole
JNK	c-Jun N-terminal kinases
kb	kilobasepairs
Kif5a	kinesin family member 5A
LC3	Microtubule associated protein light chain 3
LDH	lactate-dehydrogenase
L-F	left front paws
L-H	left hind paws
LIB	Laser Interference Biometry
LVIDd	left ventricular internal diameter diastole
LIVDs	left ventricular internal diameter systole
LVPWd	left ventricular posterior wall diastole
LVPWs	left ventricular posterior wall systole
Maneb	manganethylen-1,2-bis-dithiocarbamat
MAO-B	monoamine oxidase B
MCV	mean corpuscular volume
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
mDsRed	mDsRed mitochondrially targeted DsRed
MEF	mouse embryonic fibroblast
Mfn1	mitofusion 1
Mfn2	mitofusion 2
min	minute
MPTP	1-methyl 4-phenyl 1,2,3,6 tetrahydropyridin
MPV	mean platelet volume
MR	mineralocorticoid receptors
mRNA	messenger ribonucleic acid
MT	microtubules
mtDNA	mitochondrial DNA
MTP18	mitochondrial protein 18 kDa
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
NA	no alternations
NAD	no alternations determined

NEFA	non-esterified fatty acids
NP-40	Nonidet P40
n.s.	not significant
NTP	nucleoside triphosphate
Oas1b	2'-5' oligoadenylate synthetase 1
OF	open field test
Opa1	optic atrophy
PARL	presenilin-associated rhomboidlike
PBS	phosphate buffered saline
PCR	polymerase Chain Reaction
PCT	platelet count
PD	Parkinson's disease
PEF	peak expiratory flow
PFA	paraformaldehyde
pgk1	phosphoglycerate kinase 1
PINK1	PTEN (phosphatase and tensin homologue-) induced kinase 1
Pink1sh	PINK1 short hairpin
PLT	platelet count
PPN	pedunculopontine nucleus
PTP	mitochondrial permeability transition pore
PV	quasistatic pressure volume
PVN	paraventricular nucleus
R	lung resistance
Rab5a	Ras-related protein Rab-5A
RBC	red blood cell count
RDW	red cell distribution width
R-F	right front paws
R-H	right hind paws
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rpl27	ribosomal protein L27
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
RV	indirect residual volume
SB	Southern blotting
SDS	sodium dodecyle sulfate

sec	seconds
S.E.M.	standard error of the mean
SHIRPA	Smithkline Beecham, MRC Harwell, Imperial College, Royal London hospital, phenotype assessment
siRNA	siRNA small interfering RNA
SIRS	systemic inflammation response syndrome
SN	substantia nigra
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
SSC	sodium saline citrate
STN	subthalamic nucleus
Tab.	Table
TAE	tris acetate with EDTA
TBE	tris borate with EDTA
TBS(T)	tris buffered salind (with Tween)
TE	tris-EDTA
TH	tyrosine hydroxylase
TLC	total lung capacity
TV	tidal volume
VC	vital capacity
VGCCs	voltage-gated calcium cahnnels
WBC	white blood cell count
w/o	without

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Declaration of independency / Selbständigkeitserklärung

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Sehr geehrte Damen und Herren,

hiermit versichere ich, Karina Kloos, dass die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt worden ist, sowie entsprechende Zitate kenntlich gemacht worden sind. Die Dissertation wird vollständig eingereicht und wurde nicht einer anderen Prüfungskommission vorgelegt.

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Karina Kloos