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DISSERTATION

“The role of *DJ-1* in neurodegeneration and aging in a *Drosophila* model of Parkinson’s Disease and its implications for novel neuroprotective therapies”

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This work is dedicated to my dear father,

Wolfgang Faust,

who enabled me to study medicine.

Gewidmet meinem lieben Vater, Wolfgang Faust,

der mir das Studium der Medizin ermöglichte.

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"*…That these friends to humanity and medical science, who have already unveiled to us many of the morbid processes by which health and life is abridged, might be excited to extend their researches to this malady, was much desired; and it was hoped, that this might be procured by the publication of these remarks….*

…..Particular circumstances might arise in different cases, in which the aid of medicine may be demanded ..."

James Parkinson, M.D. ***An essay on the shaking palsy*** **(1817)**

ABSTRACT

Background

Most current pharmacological treatments for Parkinson’s Disease (PD) alleviate symptoms without reversing disease progression.

Pathogenic factors for PD include oxidative stress, inflammation and excitotoxicity. Manipulation of these factors may allow the development of disease-modifying treatment strategies.

Identification of rare monogenetic forms of Parkinson`s (*PARK1-11*) has enabled the creation of genetic animal models. *PARK7* is caused by mutations in the *DJ-1* gene.

The aim was to describe the phenotype of a newly generated *Drosophila-DJ-1*-model and use it for testing potentially disease modifying drugs.

In addition to the *PARK7* studies, two new *Drosophila* clones (*PINK1* and *hMARK2*), relevant to *PARK6*, were to be engineered and transgenic lines established.

Methods

The *DJ-1* phenotypes were used in assays on neurodegeneration, lifespan and resistance to exogenous toxins.

The accelerated degeneration of dopaminergic neurons (DN) in *DJ-1*-deficient flies was assessed by histological staining of neurons immunopositive to anti-tyrosine-hydroxylase (TH) at different fly ages. Brain dopamine content was measured in brain homogenates by HPLC.

For life span studies *DJ-1* function was selectively over-expressed or inhibited in various body tissues by specific promoters. Survival curves were plotted.

Mutants were exposed to reactive oxygen species and metal compounds. Survival was plotted.

Drugs were fed in yeast paste. The Chinese herb celastrol, the antibiotic minocycline, the bioenergetic amine coenzyme Q10 (coQ10), and the glutamate antagonist 2,3-dihydroxy-6-nitro-7-sulphamoylbenzo[f]-quinoxaline (NBQX) were used. Numbers of

TH-positive cells were visualized immunohistochemically. Brain dopamine concentrations were measured by HPLC.

To establish new downregulated and overexpressing lines, the RNA interference technique and upstream activating sequences were introduced. Plasmids containing the gene-of-interest were amplified in bacteria and purified. Genes were isolated by sequential digests and cloned into the pUAST-vector. Chemically competent cells were transformed with the pUAST-clone. The pUAST-clone was injected into the pre-germ cells of wild-type fly embryos. The generated flies were crossed to balancer chromosome-bearing flies to establish stable lines.

Results

Loss of *DJ-1* function resulted in age-dependent loss of dopaminergic neurons as well as in dopamine depletion. Secondly, loss of *DJ-1* function resulted in shortened lifespans and hypersensitivity to exogenous toxins. Overexpression of *DJ-1* produced resistance to external toxins.

The drugs celastrol and minocycline, both having antioxidant and anti-inflammatory properties, conferred potent dopaminergic neuroprotection, while coQ10 showed no effect. NBQX exerted differential effects.

Conclusions:

The results support the hypothesis of *DJ-1* playing a role in cellular protection and repair mechanisms.

Drugs combining antioxidant and anti-inflammatory properties hold therapeutic potential for mechanism-based PD treatments.

ABSTRAKT

Einleitung

Die meisten gegenwärtigen Behandlungen des Morbus Parkinson (PD) führen zu einer zeitlich limitierten Symptomkontrolle, ohne Einfluss auf das Fortschreiten der Erkrankung zu nehmen.

Oxidativer Stress, Inflammation und Exzitotoxizität sind bekannte pathogenetische Faktoren für die Entstehung PD. Ihre Modifikation bildet einen krankheitsmodifizierenden Therapieansatz.

Die Identifizierung familiärer monogenetischer Parkinsonformen (*PARK1-11*) hat die Entwicklung genetischer Tiermodelle ermöglicht. *PARK7* wird durch Mutationen im sogenannten *DJ-1* Gen bedingt.

Ziel war es, den Phänotyp eines neu entwickelten *DJ-1*-Drosophila Modells zu beschreiben und dann zur pharmakologischen Testung potentiell krankheitsmodulierender Medikamente zu nutzen.

Zusätzlich sollten zwei weitere *Drosophila*-Klone (*PINK1 und hMARK2*) als neue Parkinsonmodelle konstruiert und stabile transgene Linien generiert werden.

Methodik

Der *Drosophila-DJ-1*-Phänotyp wurde in Bezug auf Neurodegeneration, Überleben und Resistenz gegenüber oxidativem Stress analysiert: Die dopaminerge Degeneration wurde immunhistochemisch an Hirnschnitten mit anti-Tyrosin-Hydroxylase-Antikörper-Färbungen und metabolisch mittels HPLC für Dopamin gemessen.

Überlebenskurven wurden ermittelt, nachdem die Expression von *DJ-1* mithilfe geeigneter Promotoren gewebespezifisch hoch- oder herunterreguliert wurde.

Ferner wurden Überlebenskurven aufgezeichnet, nachdem die Fliegen exogenen oxidativen Stressoren ausgesetzt wurden.

Die potentiell neuroprotektiven Substanzen wurden der Fliege in Hefepaste dargeboten. Das chinesische Kraut Celastrol, das Antibiotikum Minocyclin, das biogene Amin Coenzym Q10 und der Glutamatanatgonist 2,3-dihydroxy-6-nitro-7-sulphamoylbenzo[f]-quinoxaline (NBQX) wurden verwandt. TH-positive Neurone und Dopaminkonzentrationen wurden nach der Behandlung ermittelt.

Um neue transgene Linien zu schaffen, wurden die RNA-Interferenztechnik und Promotor-kontrollierte Transkriptionsfaktoren benützt. Die das jeweilige Gen enthaltenden Plasmide wurden in Bakterien vermehrt. Das Gen wurde mittels sequentieller Restriktionsverdaue isoliert und in den pUAST Vektor geklont. Die Klone wurden erneut in Bakterien vermehrt, gereinigt und in die Prä-Gonaden von

Fliegenembryonen injiziert. Die geschlüpften Fliegen wurden mehrmals mit Balancer-Chromosom-tragenden Fliegen gekreuzt, bis stabile transgene Linien entstanden.

Ergebnisse

Der Verlust der *DJ-1* Funktion führte in der Fliege zu einer Degeneration dopaminerger Neurone und zur Transmitterdepletion, sowie zu verkürzter Lebenszeit und Überempfindlichkeit gegenüber oxidativem Stress. Überexpression von *DJ-1* hingegen führte zu Stressresistenz.

Die beiden sowohl antioxidativ als auch antiinflammatorisch wirkenden Substanzen, Celastrol und Minocyclin, zeigten einen deutlichen neuroprotektiven Effekt in der Fliege. Coenzym Q10 zeigte keine Wirkung auf die Neurodegeneration. NBQX zeigte divergierende Effekte.

Schlussfolgerung

Die Ergebnisse unterstützen die Hypothese, dass *DJ-1* eine Rolle bei zellulären Schutz- und Abwehrmechanismen spielt.

Substanzen mit antioxidativer und antiinflammatorischer Wirkung tragen ein neuroprotektives/neurorestauratives Potential in der Behandlung des PD.

1. INTRODUCTION

1.1 Parkinson’s Disease

Clinical presentation

In 1817 a surgeon from London, England, named James Parkinson, first described a certain type of movement disorder, which from then on has borne his name.

Although there had been mention of movement disorders since antiquity, Parkinson’s achievement lay in distinguishing the disease’s unique constellation of cardinal symptoms: resting tremor, rigidity, slowness of movement (bradykinesia), paucity in spontaneous movement (akinesia), postural instability and gait disorder. During the course of the disease the classical motor disturbances are frequently accompanied by both psychiatric (e.g. dementia, depression, etc.) and autonomic (orthostatic hypotension, seborrhoeic dermatitis, etc.) symptoms, making it clear that Parkinson’s Disease (PD) is not merely a movement disorder, but a complex neurological and even systemic condition.

Pathology

Our understanding of Parkinson’s disease since James Parkinson’s “essay on the shaking palsy” of 1817 evolved parallel to the nascence of modern medical sciences, such as neuroanatomy, pathology and cell biology.

In the 19th century it was believed PD was a disease of the muscles; and some classified it as a form of psychosis. In 1894 the French neurologist Eduard Brissaud established the anatomical basis of PD in the substantia nigra (SN) of the midbrain when relating a tuberculoma found in that region to a patient's Parkinson’s symptoms. Subsequent pathological investigations revealed that the histological hallmark of PD is no tumorous or granulomatous lesion in the midbrain, but a progressive loss of the substantia nigra’s pigmented neurons.

In 1912, Friedrich Lewy described a concentric inclusion body in the cytoplasm of neurons in patients who had died of PD. These inclusions, hence called the Lewy bodies, are considered the signature of idiopathic PD (see fig.6). In 1919, C.Tretiakoff, a young French physician and scholar of Lewy, first identified the loss of pigmented neurons within the SN to be etiological for PD (see fig 2).

In the second half of the 20th century, pathology was joined by newer tools for understanding the brain: biochemistry, pharmacology and, more recently, molecular genetics. The concept of catecholamines as transmitters in the brain emerged, and in 1957 Kathleen Montagu identified dopamine (DA) as a neurotransmitter in the brain. In 1960, Oleh Hornykiewicz, pharmacologist from Vienna, demonstrated an 80% loss of dopamine in the striatum of Parkinson patients and suggested the administration of levo dopa, a precursor of dopamine, to PD patients. In 1972 Dahlstrom and Fuxe demonstrated a dopaminergic pathway between the SN in the midbrain and the striatum (putamen and caudate nucleus).

Today, several decades of intense research on the pathogenesis of PD later- the cause of the disease remains unknown.

Epidemiology

Sporadic PD is the second most common neurodegenerative disease in the western world after Alzheimer's, and the single most common movement disorder. Over one million people in the United States are affected [1]. Prevalence increases with age and age remains the single most important risk factor, with a rise of prevalence from 1.4% at the age of 55, 2% at the age of 65, 3.5% at 75 and up to 5% at 85 years [2]. Mean age of onset is 55 years. Lifetime risk is 1 in 40.

In contrast to sporadic PD rare familial PD forms exist. In 5-10% of PD cases PD is caused by inherited gene alterations in the so called PD genes (*PARK 1-11,* see ch.1.4). In familial PD, the onset of symptoms is generally much earlier, with juvenile-onsets occurring before age 20 and early-onsets between age 20 and 50.

The incidence of PD has risen dramatically after the Industrial Revolution 200 years ago, due to extension of average lifespan. The relevance of PD in clinical practice, as

well as its impact on society and economy, is expected to become even more significant with the continuing increase of life expectancy in the decades to come.

Therapy

Current therapy for PD is mainly pharmacological. All first line drugs employed aim at increasing the concentration of functional dopamine in the striatum by one of a number of mechanisms: Either by replacing dopamine itself (e.g. L-Dopa), or by inhibiting dopamine degrading enzymes, such as COMT and MAO (see fig. 1), and thus prolonging its half-life (e.g. entacapone, selegeline), or by mimicking the effect of dopamine on its receptors with so called dopamine agonists (e.g. bromocriptine, pergolide, pramipexole etc.).

Interestingly, L-Dopa has remained the single most effective Parkinson drug since its discovery 40 years ago. L-Dopa is a precursor of dopamine. L-Dopa, but not dopamine, can cross the blood brain barrier (BBB). Therefore L-Dopa is administered in combination with another drug that peripherally inhibits the conversion of L-Dopa by the enzyme Dopa-Decarboxylase (DDC) (see fig. 1).

Fig. 1:
Overview of dopamine metabolism
L-DOPA: L-Dihydroxyphenylalanine; DOPAC: 3,4 Dihydroxyphenylacetoacid; 3-MT: 3-Methoxytyramine;
HVA: Homovanillinic acid
MAO: Monoamine oxidase
COMT: Catechol-O-methyltransferase

All of the drugs mentioned above serve to mask symptoms for a very limited amount of time. None of them retards or reverses disease progression. Many display serious psychiatric and motor side effects; L- Dopa produces a wearing off phenomenon; and tolerance is developed inevitably to dopaminergic agonists.

Since there is a steady-state interplay between dopamine and all the other cerebral neurotransmitters, its loss results in a dysregulation. Within the striatum, acetylcholine is the most relevant one. Thus second line treatment of PD involves a class of drugs that counterweigh acetylcholine predominance. Anticholinergics are used in combination with L-dopa to treat tremor-dominant PD-forms. Clearly, they target a subset of symptoms only, namely tremor and (less efficiently) rigor (-these are the so called plus symptoms), while leaving bradykinesia, postural instability, psychiatric symptoms etc, uninfluenced.

An interesting second-line drug is Amantadine, - a virostatic that has coincidentally been discovered and empirically used in the treatment of PD, as it successfully alleviates symptoms, until it was discovered years after its introduction that one of its mechanisms of action is glutamate inhibition. The hypothesized benefit of glutamate inhibitors involves not merely the buffering of excess glutamate within the globus pallidus, but also a recently discovered mechanism called excitotoxicity.

Excitotoxicity is a form of apoptotic cell death that is caused by glutaminergic overstimulation and mediated by calcium influx (see also glossary). The pathogenetic role of excitotoxicity will be examined in the following drug study and a novel glutamate inhibitor (NBQX) will be put to the test in an animal model of PD.

Physical exercise, including physiotherapy, ergotherapy and speech therapy, has been proven to alleviate PD symptoms and plays an increasing role as sustentative therapy [3].

Alternative treatments

In patients with tremor dominant forms of PD and a very poor response to pharmacological treatment, surgical procedures like deep brain stimulation of the subthalamic nucleus and other targets have been employed in recent years. They are

applicable in a small subset of patients only. Risks and cost of the operation have to be carefully weighed up against its benefits.

Recent experimental efforts of transplanting fetal SN stem cells into diseased brains have produced conflicting results. Clearly, the unpredictable behavior of these fetal cells, their oncogenic potential, as well as the currently unresolved technical difficulties, make this a highly improbable treatment option for the near future.

The vital need for causal therapeutic venues, applicable to the vast collective of PD patients, is undeniable; and there is a broad consent that they will be pharmacological.

At present, new pharmacological strategies are emerging that are designed to attempt to slow neuronal death. The development of neuroprotection may be the second breakthrough in PD after Hornykewicz's 1960 findings.

In the present study a genetic animal model of PD (see ch. 1.4 and 1.7) is used to test potential neuroprotective drugs. The drugs applied are the Chinese herb *Celastrol*, the antibiotic *Minocycline*, the bioenergetic amine *ubiquinone (Coenzyme Q 10)* and the glutamate antagonist *NBQX.* All of them target main pathogenetic mechanisms of neurodegeneration, as described below under 1.3, and thus consititute a causative approach to PD.

1.2 The Etiology of PD

Although the cause and the mechanisms underlying nigrostriatal dopaminergic cell loss in idiopathic PD remain obscure, various etiologies have been proposed over the years. Apart from genetic forms, which will be discussed in more detail below, these include: *toxins, trauma, infection*, and *drugs*:

Trauma

Patients who had suffered from serious head injury, e.g. chronic head trauma of boxers, have a significantly increased risk of developing PD in later life due to traumatic loss of dopaminergic neurons [4, 5].

Drugs

Antipsychotics, as used in the treatment of schizophrenia, provoke a “secondary” form of parkinsonism due to their dopamine-antagonistic effect. In fact, L-dopa and dopamine agonists, as used in the treatment PD, contribute to a worsening of PD symptoms in later stages of the disease, because they reduce the number and sensitivity of dopamine receptors.

Infection

An infection hypothesis emerged after a 1915-1926 epidemic of encephalitis lethargica. There was approximately a 100-fold rise in the incidence of PD in the generation affected by the epidemic during childhood compared to previous generations. The virus causing the epidemic disappeared “mysteriously” in 1931 followed by a drastic fall of the incidence in individuals born after that year [6, 7]. From that time on various infection- or post infection-based theories on PD were proposed [8], e.g. that it is a slow virus disease [9, 10, 11] or a consequence of intrauterine influenza [12, 13].

The role of inflammation in the disease's pathogenesis is interesting in the context of an infection-based theory of PD. In the following drug study anti-inflammatory drugs will be administered to a fruit fly model of PD and their effects will be evaluated.

Toxins

But the most popular supposition on the etiology of PD for much of the late 20th century was the so called *exogenous toxin hypothesis*:

This hypothesis postulates that the selective and progressive neurodegeneration in PD is due to an environmental dopaminergic neurotoxin. This acts either via chronic or temporally limited exposure, which then initiates a prolonged, self-propagating cascade of degenerative events.

MPTP

The toxin-based hypothesis became very popular after an outbreak of PD in a cohort of young drug addicts in California in the early 80s, who consumed a synthetic “street heroin” contaminated with MPTP.

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) itself is not toxic, but easily crosses the BBB where it is oxidized into the toxic MPP+ by MAO-B (see fig.1) [14]. MPP+ enters dopaminergic neurons due to its affinity to the dopamine transporter (DAT) [15]. This readily explains the selectivity of its toxicity. MPP+ inhibits complex I in the mitochondrial respiratory chain, which leads to the production of superoxide radicals [16, 17] (see fig. 3). Thus, the mechanism of MPTP toxicity is the generation of oxidative stress (see below, see glossary).

Pesticides

Epidemiological studies have implicated a geographic heterogeneity of PD, with a higher prevalence in rural areas. This has been traced back to the chronic exposure to pesticides: Herbicides like paraquat, insecticides like rotenone, which is also used to kill unwanted fish, and fungicides like maneb [18, 19] all have been mentioned in relation with PD. Interestingly, paraquat bears a striking molecular resemblance to MPTP, although its precise mechanism of toxicity is not clearly understood. (In the toxicology study below, various effects of a pesticide are examined in a genetically designed animal model of PD.)

Metals

Other toxins implicated in the pathogenesis of PD involve carbon monoxide (CO) and so called “transition metals”, such as manganese (Mn) and copper (Cu). Both will be included in the toxicology experiments below.

Wilson's syndrome, for example, a metabolic condition that leads to abnormal storage of copper in various body tissues including the basal ganglia, is frequently accompanied by Parkinson's syndrome [20]. Likewise the prevalence of PD is higher in certain occupational groups, such as welders and miners, that are exposed to manganese containing fumes, as assessed by public health-based epidemiological studies [21, 22].

1.3 Hypotheses on the pathogenesis of Parkinson's disease

In the course of our growing knowledge about several players in the biochemical events that eventually lead to PD, several hypotheses on the disease pathogenesis have emerged. Four important contributory mechanisms have been identified: *oxidative stress, inflammation, excitotoxicity* and *protein aggregation*.

At the current stage of our understanding, it is assumed that these four players form a complex network of events that eventually leads to dopaminergic neuronal death. Consequently, effective disease modifying therapy might require addressing a combination of neurodegenerative mechanisms within the specific setting of the substantia nigra (SN) and related structures (e.g. the locus coeruleus).

1.3.1 Oxidative stress

Oxygen is essential for cellular respiration in all aerobic organisms, and due to its high electronegative potential it is indispensable for adenosine triphosphate (ATP) production in the mitochondrial electron transport chain. Yet, it possesses the propensity to transform into more reactive forms, so called reactive oxygen species (ROS) that are toxic to cells, as they produce damaging oxidizing events. At times, the cell uses the toxic properties of oxygen to its advantage, e.g when defending itself against bacterial invasion.

ROS include oxygen-free radicals, such as the highly reactive hydroxyl radical, the superoxide anion and peroxy-radicals as well as oxygen non-free radicals, such as hydrogen peroxide and singlet oxygen.

ROS react with proteins at proline, histidine, cystein or methionine residues, leading to damage of essential enzymes and structural proteins. They can nick DNA, lead to breaks in carbohydrate chains and promote lipid peroxidation, often resulting in uncontrolled autooxidative chain reactions.

In a healthy cell, antioxidant forces maintain a homeostasis with oxidative events: The major antioxidants in a cell are reducing molecules (such as glutathione, ascorbate and

tocopherol) and radical scavenging enzymes (such as catalase, superoxide dismutase and glutathione peroxidase). Oxidative stress is the result of an imbalance between pro-oxidant and antioxidant forces, be it either by increased production of oxidizing species or by loss of scavaging enzymes or reducing agents.

The brain has a higher susceptibility to oxidative damage than any other body tissue. This is due to its high oxygen consumption, relatively low antioxidant properties and restricted regenerative capacity. Neurons are considered post-mitotic cells. Even though the brain accounts only for approximately 2% of the total body weight, it consumes about 20% of the total oxygen intake. During normal metabolism up to 2% of the consumed oxygen is converted into reactive oxygen species.

Post-mortem biochemical analysis

There is overwhelming evidence for the involvement of oxidative stress in the pathogenesis of PD in human brain histology. Post mortem analyses of tissue taken from the SN of PD patients demonstrate increased levels of lipid hydroperoxides, malondialdehyde, basic protein oxidation and reactive carbonyls. An increase in 8-hydroxyguanine and 8-hydroxy-2'deoxyguanosine indicates oxy-radical mediated damage to DNA [23].

In addition, glutathione (GSH) is reduced in the substantia nigra of parkinsonian brains, but not in other brain regions [24, 25]. Interestingly, there is a correlation between the amount of reduction and severity of PD symptoms [25]. The enzyme glutathion peroxidase, which is the major pathway for decomposing hydrogen peroxide, has been reported to be reduced in the SN, putamen and globus pallidus in parkinsonian brains [26]. There is also some evidence for a reduction of catalase levels [27].

The substantia nigra, pars compacta, (SNpc) has certain characteristics, which lead to increased basal levels of oxidative stress under physiological conditions. These include mitochondrial dysfunction and a high content of dopamine, neuromelanin and iron.

1.3.1.1 The role of dopamine

One of the most conspicuous characteristics of PD is that it primarily affects a relatively restricted neuronal population in the brain: the SNpc. The most overt difference between this cell population and other brain regions is the high level of dopamine, which is suspected to put cells at risk. In fact, oxidative stress in nigral neurons may result from the metabolism of dopamine itself. Dopamine is known to react with oxygen enzymatically and non-enzymatically through auto-oxidation:

Monoamine oxidases (MAO) catalyze the oxidative deamination of dopamine to DOPAC and homovanilllinic acid (see fig.1). In the brain, there are two MAOs, A and B. MAO-B prevails in the SN with approx. 80% [28]. In the deamination process, hydrogen peroxide (H_2O_2) is formed. Hence, dopamine turnover is linked stochiometrically to H_2O_2 production.

The autooxidation of dopamine produces toxic quinones and semi-quinone species. H_2O_2 and oxy-radicals are also generated in the process. For this reason, dopamine and its metabolites can, in a way, be considered endogenous toxins.

1.3.1.2 The role of neuromelanin

The age-dependent accumulation of neuromelanin within nerve cells is a characteristic feature of the SN and leads to its dark-brown pigmentation. In PD, the more heavily pigmented cells of the SN are preferentially lost [29].

Neuromelanin is mainly composed of DA redox products, which accumulate during dopamine's metabolism and auto-oxidation [29, 30, 31, 32].

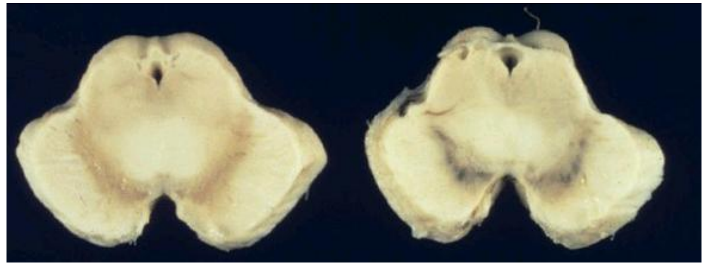


Fig. 2:
Loss of pigmented neurons in the SN of PD
Substantia nigra of a healthy aged human, right; substantia nigra of an age-matched PD patient, left. Taken from: www. pathology.mc.duke.edu

Importantly, neuromelanin binds metal ions, particularly iron. As a catechol, dopamine is a good metal chelator. It has been postulated that neuromelanin might serve as an iron-storage molecule, possibly protecting the cell from the oxidation-catalyzing properties of iron (Fe^{3+}) [33]. But the iron bound to neuromelanin is redox-active. Dopamine is a potential electron donor and can reduce the oxidation state of metals, thus supporting the production of hydroxyl radicals via the Fenton reaction (see glossary). The function of neuromelanin is being debated controversially and might be iron-dependent: At low iron concentrations, melanin exhibits antioxidant properties, possibly protecting against dopamine induced redox-associated toxicity, while at high metal concentrations it acts pro-oxidantly [34].

In summary, an upset in the delicate regulation of dopamine-neuromelanin-iron biochemistry in the SN could result in oxidative stress to the neurons.

1.3.1.3 The role of metals

The role of iron and other metals in the etiology of PD is of special interest due to their ability to generate free radicals. In particular, they catalyze the formation of hydroxyl radicals from hydrogen peroxide. (see glossary-> Fenton reaction)

In comparison with other body tissues, the brain is very rich in iron (and also copper); it concentrates metals [35]. Studies in mice have shown a progressive rise in cerebral iron and copper content with normal aging [36, 37, 38]. Certain areas within the brain have a

particularly high iron content: the substantia nigra, the globus pallidus and the nucleus dentatus.

In patients affected by PD, there is a further fundamental increase of iron content in the pars reticulata of the substantia nigra (SNpr), where the dendrites of the pars compacta are situated [39], but not in other brain regions. In addition, the pathological hallmark of PD, the Lewy bodies (see fig. 6), show a strikingly high iron content.

1.3.1.4 The role of mitochondrial function

Many of the toxins discussed earlier (MPTP, rotenone, 6-hydroxydopamine) exert their effect by inhibiting complex I of the mitochondrial respiratory chain (see fig. 3). It is compelling that the identical mitochondrial complex I abnormality seen with MPTP toxicity has been found to be present in patients with the sporadic from of the disease [34]. Complex I deficiency seems to be specific for PD, since it has not been described in any other neurodegenerative disease, and it is not present in any other brain area but the SN [40]. Yet, interestingly, complex I deficiency is also observed in platelets of PD patients (- suggesting blood tests as a possible pre-clinical diagnostic tool).

PINK1, a recently discovered familial Parkinson gene, is a mitochondrial protein kinase.

It remains unclear whether the complex I defect is primary or secondary in PD. Free radicals can lead to respiratory chain defects, yet they should affect complexes II, III and IV in a similar manner as complex I. Thus, the selective vulnerability of complex I makes it unlikely to be a by-product of oxidative damage.

At the same time, no mutation in mitochondrial DNA (mDNA) has ever been reported in the context of PD and symptoms of PD are not associated with any of the mitochondriopathies (e.g. Kearns-Sayre syndrom, MELAS-syndrome, Leber's hereditary optic neuropathy etc.) Nor there is no clue as to a maternal mode of inheritance.

After all, the role of mitochondrial function in PD is still unclear [41].

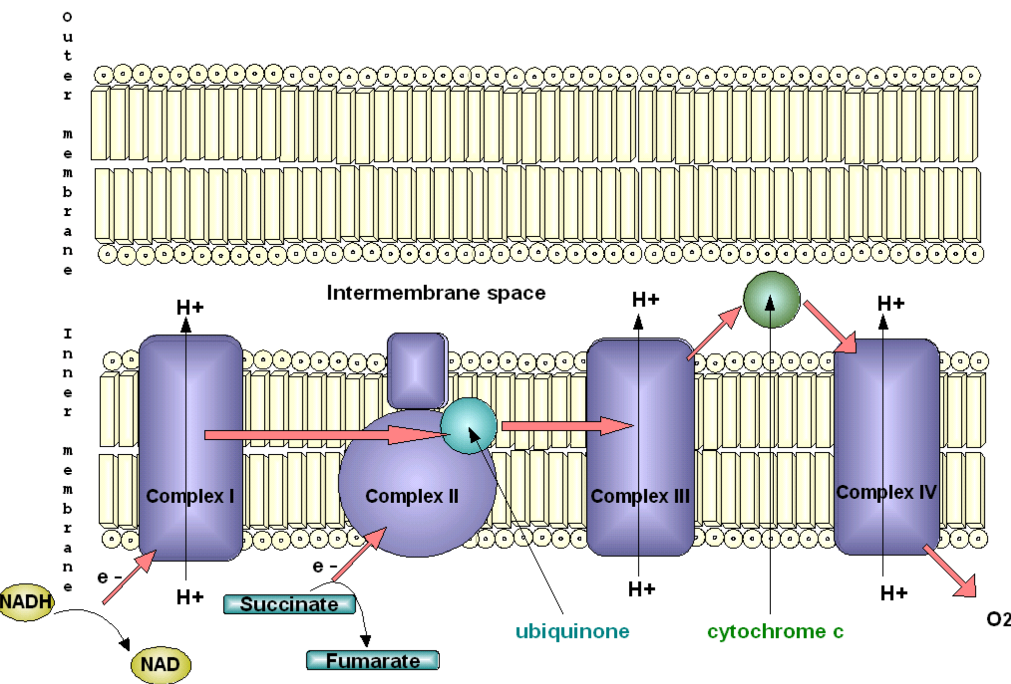


Fig. 3:
Mitochondrial respiratory chain

Complex I = NADH-ubiquinone oxydoreductase
Complex II = Succinate- ubiquinone oxoreductase
Complex III= Ubiquinon- cytochrome-c oxidoreductase
Complex IV= Cytochrome-c- O2-oxidoreductase

1.3.1.5 The role of aging

As individuals age there is a physiological decline in sensory, motor and cognitive function. Cells in all regions of the nervous system are affected by the natural aging process.

Since the incidence of PD also increases with age, it is important to mention that there is a natural loss of monoaminergic neurons with age: At birth, the human SN contains approximately 400,000 cells, at the age of 60, there are approximately 250,000 left [42]. A similar continuous loss takes place in the locus coeruleus, which contains also catecholamine. In the cerebral cortex neuronal numbers stay constant with aging. The loss of dopaminergic neurons in the SN is paralleled by an age-dependent decrease of dopamine levels in the striatum [43, 44]. Consistent with these findings, there is an age-dependent decline of dopamine synthesizing enzymes [42] and an increase in the dopamine-degrading enzymes MAO-A and MAO-B [45], leading to a naturally increased rate of H_2O_2 formation with age (see above).

When the dopamine concentration within the striatum is reduced to 20% of its original level, Parkinson symptoms begin to appear [46]. At this stage, loss of at least 50% of the dopaminergic neurons in the substancia nigra pars compacta (SNpc) has occurred [1].

Several studies in mice have shown that one of the consequences of normal aging is a rise in the levels of iron and copper in brain tissue [36-38].

The finding, that normal aging leads to an increased vulnerability to oxidative events in the SN, could lead to the interesting assumption that PD is a physiological result of aging and that all human beings would eventually suffer from PD symptoms if they lived long enough for them to appear. The majority of us might die of other reasons before the critical level of less than 20% striatal dopamine content could be reached. In line with this thought the aging process of striatal neurons in PD might be accelerated due to a genetic predisposition, or to endogenous or external influences. Thus, selective manipulation of the aging process could be a potential therapeutic target of PD (see ch. 1.6).

Taken together, oxidative events, linked to abnormal catecholamine metabolism or mitochondrial dysfunction, promote altered protein conformations and can damage the main protein degrading machinery in the cell itself, the proteasome (see also ch. 1.3.4.:"The UPS"). In turn, accumulation of damaged proteins, caused by proteasomal inhibition, can induce oxidative stress. This interdependency between the two main hypothesised causative players may lead to a vicious circle of degenerative events.

In the following drug study, potent anti-oxidants, such as coenzyme Q10 and Minocycline are considered as potential neuroprotective therapeutics in a genetic animal model of PD: the *DJ-1* deficient fly.

1.3.2 Inflammation

In addition, there is increasing recognition of the possible role of neuroinflammation as a contributing factor in the pathogenesis of PD [47-56]. Both, human Parkinsonian brains and animal models of PD, exhibit a multitude of features of inflammation within the SN,

including activated microglia, phagocytosis, complement activation, increased synthesis and release of pro-inflammatory cytokines.

Microglia are resident immunocompetent cells in the central nervous system (CNS). They constitute a morphologically separate cell type from other glial cells, such as astrocytes and oligodendrocytes, since they derive from blood monocytes in the bone marrow and migrated into the brain at early embryogenic stages when the blood brain barrier (BBB) is not yet fully developed [57]. Microglia are close relatives of macrophages and considered to be the innate immune system of the brain, exhibiting the same cell surface antigens as those found on the latter. Microglia react highly sensitively to disturbances within the microenvironment of the brain and act as scavenger cells in the event of infection, inflammation, trauma, ischemia, intoxication and neurodegeneration [58].

Microglia are antigen presenting cells, which display the major histocompatibility complex class II (MHC class II) on their cell membrane. When activated upon neuronal injury or inflammatory stimulation, they are capable of phagocytosis and release inflammatory cytokines, of which e.g. interleukin-1β (IL-1β), tumor necrosis factor -α (TNF-α) and interleukin-6 (IL-6) are relevant ones, that amplify the inflammatory response by recruiting other cells to the respective lesion, as well as inflammation-associated molecules, such as prostaglandins.

In addition, microglia release a number of further substances, some of which are potent neurotoxins, like nitric oxide (NO), superoxide (O_2^-) and other ROS [59, 60]. The SN contains four to five times more microglia than any other area in the brain [61]. In brains of patients with PD, a further extensive proliferation and focal accumulation of activated, phagocytosing microglia in the substantia nigra (SN) has been reported [62, 63, 50, 53] and is accompanied by progressive dopaminergic neuronal loss.

Moreover, an upregulation of MHC class II molecules [53] and β2-microglobulin, the light chain of MHC class I [64], as well as highly elevated levels of IL-1, IL-6 and TNF-α are found both in the SN [65], the striatum [64] and the cerebrospinal fluid [52] of patients with PD.

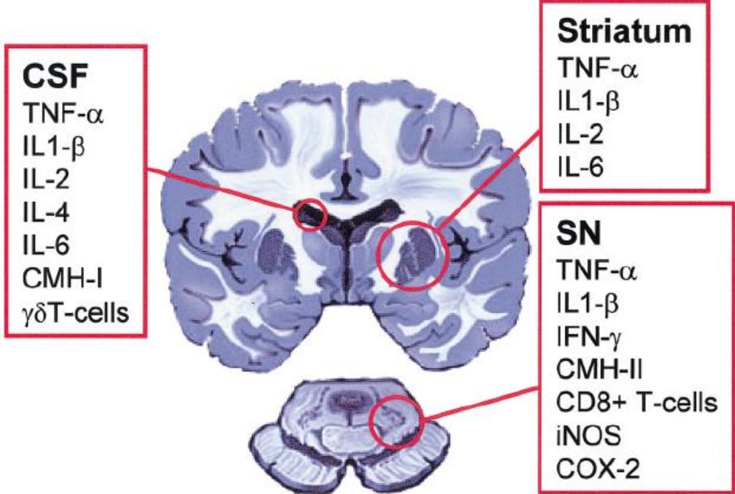


Fig. 4:
Inflammation related changes in PD brains. CSF: cerebrospinal fluid; SN: substantia nigra. *taken from: Hunot S (2003); Neuroinflammatory Processes in Parkinson's Disease; Annals of Neurology 53 (suppl 3) 49-60* [65].

These findings were reproduced in experimental animal models of the disease, such as the MPTP model [66], the rotenone model [59] and the 6-hydroxydopamine (6-OHDA, see fig. 1) model [47]. All of them show increased numbers of activated microglia within the SN as well as a focal increase of inflammmatory cytokines [64, 51, 52].

Interestingly, in post mortem examination of human brains exposed to MPTP, activated microglia were present up to 16 years after exposure [67], indicating a chronically sustained neuroinflammation after the initial toxic insult. The sustained inflammatory response is accompanied by ongoing dopaminergic neuron loss that continues to occur even decades after MPTP exposure. In neuronal cell cultures exposed to MPTP, DA toxicity is increased significantly after addition of microglia [59].

Activation of microglia upon exposure to pathogens or toxins is a vital and protective process for normal CNS function. Usually, it is self-regulated and transient. However there is mounting evidence, that the inflammatory response in PD is both sustained and progressive. Inflammation can lead to neuronal death and degeneration. At the same time neurons, injured e.g. through oxidative damage or inflammation, release cytokines which in turn activate microglia. Hence, a vicious circle of inflammatory insult may develop.

Several studies have shown that microglia can enhance both neuromelanin and α-synuclein mediated toxicity in PD. Neuromelanin, which is released by dying dopaminergic neurons in PD, has chemotactic effects and activates microglia [68]. Extracellular α-*synuclein* aggregates and Lewy bodies are often surrounded by microglia and complement [53, 69]. In microglia-depleted cell culture studies, α-*synuclein* failed to induce dopaminergic neurotoxicity at low concentrations [70].

Additionally, Parkinson patients exhibit a specific IgG response to dopamine-o-quinone (see above) modified proteins [71], which may indicate involvement of autoimmune processes in PD, possibly similar to the ones already described in multiple sclerosis (MS), and AD [72, 73]. Yet, although the theory of PD being an autoimmune disease is tempting, there is no proof of any relevant involvement of B and T cells in PD.

An important microglial enzyme, markedly increased in the SN of PD patients compared to healthy age matched controls [65, 74] and in experimental PD models [16, 48], is the inducible nitric oxide synthase (iNOS). INOS mediates the synthesis of nitric oxide (NO), which has been shown to be highly neurotoxic. NO reacts with superoxide to form the highly reactive peroxynitrite. It can nitrosylate and thus disrupt the function of many neuronal proteins, including tyrosine hydroxylase [75]. Lewy bodies in PD stain positive for 3-nitrotyrosine [76], and nitrite levels have been shown to be elevated in the CSF of PD patients [77]. In mice, deficiency of iNOS protects against MPTP induced toxicity (Dehmer 2000), while upregulation enhances MPTP toxicity [16].

Secondly, NO can release iron from its intracellular binding protein, ferritin, thus leading to the build-up of free iron [78], which participates in the generation of H_2O_2 (see above).

In summary, neuroinflammation is an indisputable neuropathological feature in PD that probably propagates the degenerative process. Even though much remains to be clarified, as to how, when and where inflammation is involved in the cascade of events leading to nerve cell death, there may be a therapeutic implication to consider the use of anti-inflammatory drugs in PD, as they may prove effective in slowing down disease progression.

In the MPTP and 6-OHDA mouse model a variety of immunosuppressive and anti-inflammatory drugs have been applied, including aspirin, thalidomide, dexamethasone and cyclosporine A, all of which have shown to confer some protection against drug-induced dopaminergic depletion [79-82].

In the drug study below, the potential benefit of two anti-inflammatory drugs are investigated in a genetic animal model of PD: the *DJ-1* deficient fly. These drugs are the Chinese herb Celastrol and the antibiotic Minocycline.

1.3.3 Excitotoxicity

After nigrostriatal dopaminergic denervation in PD, there are functional modifications in the circuitry of the basal ganglia [83-85]. The resulting altered neurotransmission underlies many of the clinical manifestations in PD. Glutaminergic pathways from the subthalamic nucleus (STN) become overactive, leading to an overexcitation of the substantia nigra pars reticulata (SNr) and the globus pallidus internus (GPi). Altered glutaminergic neurotransmission also leads to regulatory changes in the different glutamate receptors (GluR) in various loci of the basal ganglia circuit [85, 85].

Glutamate is the major excitatory neurotransmitter both in the vertebrate and invertebrate central nervous system (CNS). It acts through two types of receptors, ionotropic and metabotropic. The ionotropic receptors have been subdivided based on agonist specificity into N-methyl-D aspartate (NMDA), 2-amino-3-(3-hyxdroxy-5-methylisoxazol-4-yl)-propionic acid (AMPA) and kainic acid (KA) receptors [87].

In addition to being the main excitatory neurotransmitter in the CNS, paradoxically glutamate is also a neurotoxin. Increased concentrations of glutamate within the synaptic cleft can lead to apoptotic nerve cell death mediated through high levels of calcium (Ca^{2+}). Overexcited ionotropic glutamate receptors (GluR) allow abnormal Ca^{2+}-influx into the cell. The Ca^{2+} influx activates a number of enzymes, e.g. phospholipases, proteases and endonucleases, which damage key components of the cell. In addition, excess Ca^{2+} in the cytosol leads to opening of mitochondrial Ca^{2+}-permeable pores, causing disturbances in energy metabolism, altered electrochemical gradients, and the release of mitochondrial apoptotic proteins.

This phenomenon, called excitotoxicity has been well established to occur in a number of both acute (stroke, trauma, epilepsy) and chronic neurodegenerative and neuroinflammatory (PD, ALS) diseases [86, 88-90], triggering serious secondary damage after a primary insult.

Parkinsonian nigral neurons, already oxidatively damaged and with an impaired mitochondrial function (see ch. 1.3.1), are especially vulnerable to the toxic effects of glutamate [90]. Thus, the secondary glutaminergic overstimulation of the SN after dopaminergic denervation of the striatum sustains and amplifies the progressive dopamine neuronal death, contributing to a vicious circle of degenerative events. For this reason, glutamate receptor antagonists have been developed as research tools and potential therapeutic agents [91].

In *Drosophila*, one metabotropic (DmGlu-RA) and 8 ionotropic (DGlu-RI, DGlu-RIB, DGlu-RIIA, DGlu-RIIB, DGlu-RIIC, DGlu-RIIE, DNMDA-RI and DNMDA-RII) glutamate receptors have been identified. Of those, DGlu-RI and DGlu-RIB are AMPA selective, and DGlu-RIIB shows affinity for both AMPA and KA. (www.flybase.bio.indiana.edu).

In the human SNr, AMPA receptors have a higher relative density, compared to NMDA receptors. Microinjections of AMPA, but not NMDA, into the SNR of rats induced parkinsonian rigidity [85] and lesions of the STN in rats produced selective upregulation of AMPA, but not NMDA, receptors. In the MPP+ mouse model of PD, AMPA/kainate receptor antagonists protected against MPP+ toxicity, while selective NMDA receptor agonists failed to protect [92].

Therefore, in the drug study below, a selective antagonist of AMPA receptors, NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione), was chosen to investigate its neuroprotective potential in the *DJ-1A* RNAi fly model of PD.

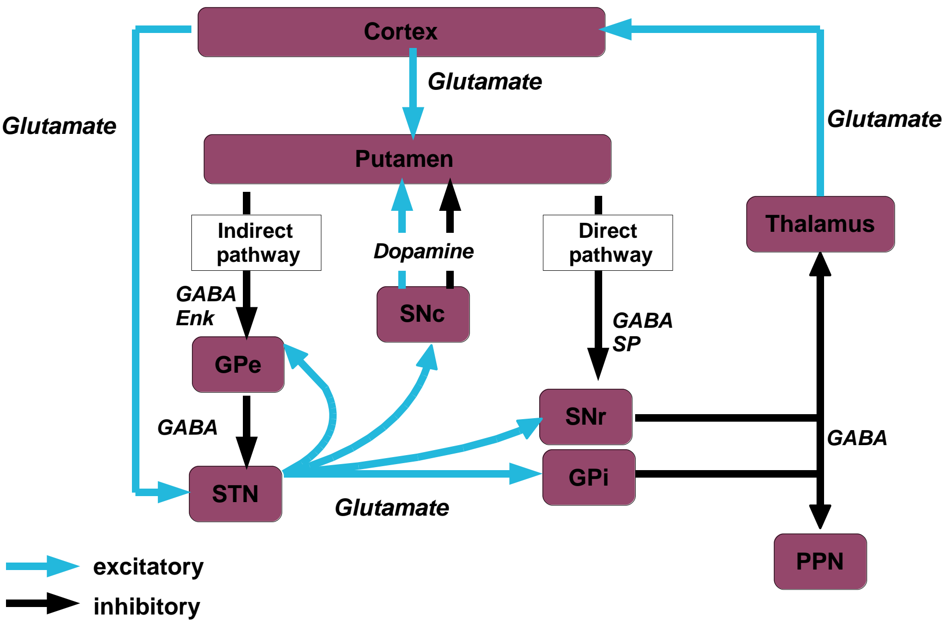


Fig.5:
Basal ganglia circuitry, schematic
Enk: enkephalin, GABA: gamma-aminobutyric acid, GPe: globus pallidus external segment, GPi: globus pallidus internal segment, PPN: pedunculopontine nucleus, SNc: substantia nigra pars compacta, SNr: substantia nigra pars reticulata, STN: subthalamic nucleus.After dopamine depletion in the striatum, the natural balance in the circuit is lost.

1.3.4 Protein aggregation

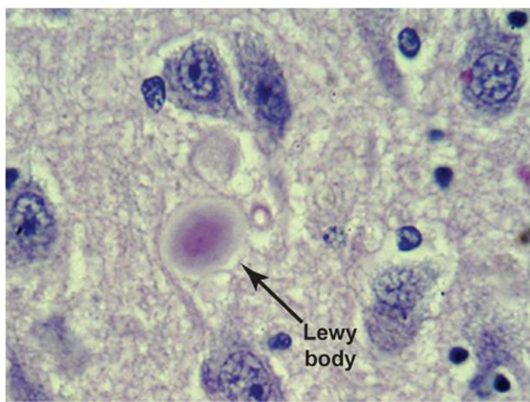


Fig. 6:
Neuron with a Lewy body, human brain tissue
taken from www.genome.gov, National Human Genome Research Institute

PD involves deposits of intracytoplasmic inclusion bodies (the Lewy bodies, LB), which contain abnormally aggregated protein, and it has been suggested that these might be

pathogenic. The major component of LBs in idiopathic Parkinson's syndrome (IPS) is α-*synuclein* (*SNCA*) and it has been shown that a mutation in *SNCA*, which leads to its misfolding, causes a familial form of PD [93]. SNCA is a synaptic protein, whose folding, post-translational modification and recycling is compromised in PD. Misfolded SNCA forms complex oligomers and finally aggregates (the Lewy bodies), which are considered toxic to the cell [61, 94, 95].

The UPS

The main protein degrading machinery in the cell is the ubiquitin proteasome system, UPS. The notion that defects in the UPS may contribute to neurodegeneration in PD arose with the identification of two familial PD genes that are key players in the ubiquitin proteasome pathway, *Parkin* (=*PARK2*) [96] and *UCH-L1* (=*PARK5*) [97, 98].

Until 2003, when *DJ-1* was discovered as new PD gene and drew attention to the oxidative stress theory of PD pathogenesis, the theory on protein aggregation of PD was strongly favored by experts for many years and is still today [99, 100].

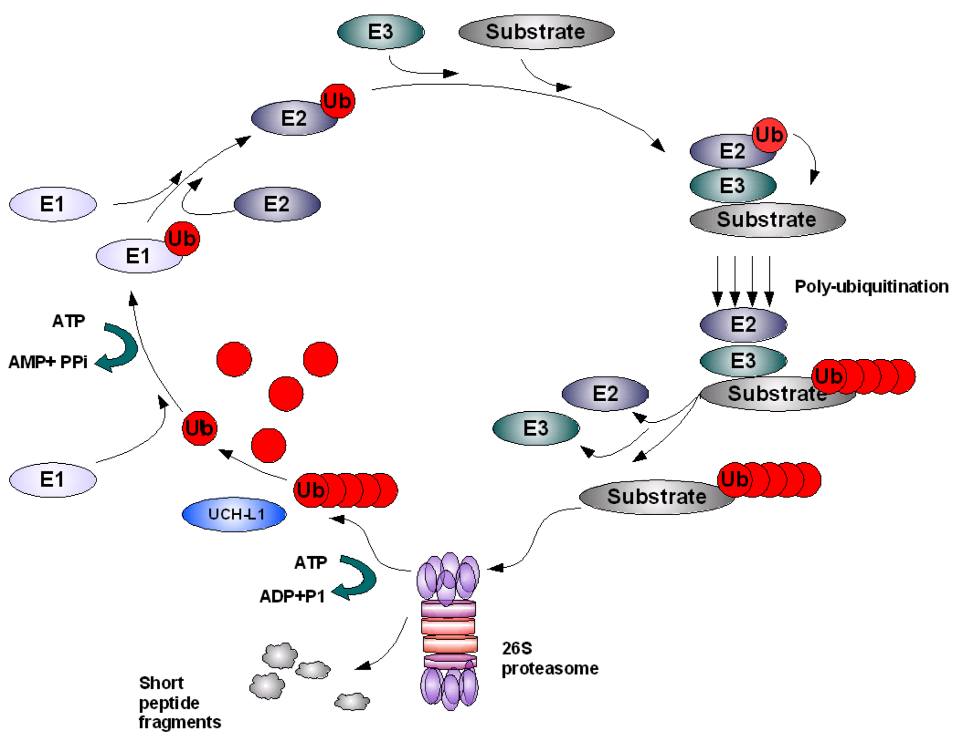


Fig. 7:
The ubiquitin-proteasome system

However, as discussed above, protein aggregation and oxidative stress are not mutually exclusive pathogenetic phenomena. They might even be interrelated. After all, oxy-radicals lead to protein oxidation, and thus misfolding and aggregation; while protein toxicity promotes and sustains the intracellular production of free oxy-radicals [101, 102].

1.4 Genetics of Parkinson's disease

Even though the vast majority of PD cases are sporadic idiopathic forms, recent identification of a number of genes responsible for rare familial forms has provided tremendous insight into the pathogenesis of the disease. The reasoning behind studying rare genetic forms of a common sporadic illness is the assumption that both share a key biochemical pathway.

Further studies on the physiological role of these gene products, as well as identification of additional novel PD genes, will continue to provide further pieces of a puzzle that will eventually come together and lead to understanding the pathogenesis of both familial and sporadic disease, thus paving the way to developing new treatment strategies that will actually modify the progression of neuronal degeneration and therefore, for the first time, be causal in their approach. Genetic information may also prove useful in identifying the pre-clinical phase of PD, allowing treatment to be preventative.

The following work is based on the PD gene *DJ-1*, which is presented below. But since various PD genes relate to or even interact with each other, and since models of other Parkinson genes (such as *SNCA*, *Parkin* and *PINK1*) have already shed tremendous insight into the pathogenesis of PD, a short overview over all Parkinson genes known so far is provided below in tabular form.

1.4.1 Overview of Genetic Forms of Parkinson's Disease

Designation	Gene product	Chromosomal region	Mode of inheritance	Onset	Reference
PARK 1	*α-Synuclein*	4q21	AD	40s	Polymeropoulus et al. 1997
PARK 2	*Parkin*	6q25	AR	20s	Kitada et al. 1998
PARK3	unknown	2p13	AD	60s	Gasser et al. 1998
PARK 4	*α- Synuclein* duplication and triplication	4q21	AD	30s	Singleton et al. 2003
PARK5	*UCH-L1*	4p14	AD	~50	Leroy et al. 1998
PARK6	*PINK 1*	1p35-37	AR	~40	Valente et al. 2004
PARK7	***DJ-1***	**1p38**	**AR**	**~30**	**Bonifati et al. 2003**
PARK 8	*LRRK2*	12cen	AD	~50	Zimprich et al., 2004 Paisán- Ruiz et al. 2004
PARK 9	*ATP13A2*	1p36	AR	~10	Hampshire et al. 2001
PARK10	unknown	1p32	AD	50-60	Hicks et al. 2002
PARK 11	*GIGYF2*	2q36	AD	>60	Pankratz et al. 2003

Tbl. 1: overview of monogenetic forms of Parkinson's disease

AD: autosomal- dominant, AR: autosomal recessive

Of the ten gene-loci that have been identified to be associated with inherited PD, six gene products have been characterized so far: *α- synuclein, Parkin, UCH-L1, PINK 1, LRRK 2* and *DJ-1*.

1.4.2 *PARK 7: DJ-1*

1.4.2.1 Characterization and general description

DJ-1 has been shown to segregate in families with PD [103]. *DJ-1* maps to chromosome 1p36 of the human genome and contains 9 exons, encoding a relatively small 189 amino acid protein of approximately 20kDa.

It is unclear how mutations in *DJ-1*, an ubiquitously abundant protein of unknown function, produces neurodegeneration in familial cases of Parkinson's disease. However, its role in oxidative stress defense, as well as in the regulation of cell survival, indicates a neuroprotective role. In creating a *DJ-1*-loss-of-function phenotype in *Drosophila* we expected to create a model of Parkinson's disease.

The following work focuses on elucidating possible roles of *DJ-1* within the cell, its relevance within the healthy organism and its role in the pathogenesis of Parkinson's disease.

Clinics

The clinical presentation of *DJ-1* associated PD is similar to *parkin*- and *PINK*-associated PD, with an early age of onset (17 to 42 years, -average age of onset is in the early 30s-), slow disease progression, and good response to L-dopa [103-106]. Psychiatric and behavioral disturbances, as well as focal dystonias (e.g. blepharospasm) seem to be more frequent and to emerge at earlier stages than in other PD forms.

Due to the rare incidence, relatively recent discovery and slow disease progression, no mortem brain material of *DJ-1* has been evaluated as of yet. However, post mortem studies in sporadic PD have demonstrated region-specific decreases of *DJ-1* protein, and also a preponderance of the acidic pI isoforms of *DJ-1* monomer (see below), suggesting a putative role of *DJ-1* in the pathogenesis of sporadic PD [107]. Brain single positron emission computed tomography (PET) with a tracer for DA transporters (6-fluorodihydroxyphenylalanine) was consistent with severe abnormalities in the pre-synaptic function of DA-transporters [108].

Genetics

A number of autosomal recessive mutations have been identified in the *DJ-1* gene, including exonic deletions, truncations, homozygous and heterozygous missense mutations [103, 105, 106]. One of the known point mutations (L166P-lysine is replaced by proline at aminoacid 116) has clearly been associated with a loss-of-function phenotype, due to structural breaks in the helix. Therefore, this mutation is well suited for disease modeling in human cell culture studies.

DJ-1 is ubiquitously expressed in the brain, - without being confined to a preferential anatomical region or functional system-, as well as in all extracerebral body tissues [109, 110]. Both neurons and glia (see glossary) are immunopositive for the protein, yet it seems to localize predominantly in astrocytes (see glossary) [111, 112]. *DJ-1* protein is detected both in the cytoplasm and the nucleus [105, 110] - yet literature on the subcellular localization is not unequivocal. There also appears to be an endogenous proportion of *DJ-1* which localizes to mitochondria. Interestingly, this mitochondrial pool enlarges under oxidative stress [113].

Structure

The crystal structure of human *DJ-1* has been resolved and shows that it exists in solution as a homodimer (see fig. 6) and dimerisation appears to be a prerequisite for proper functioning.

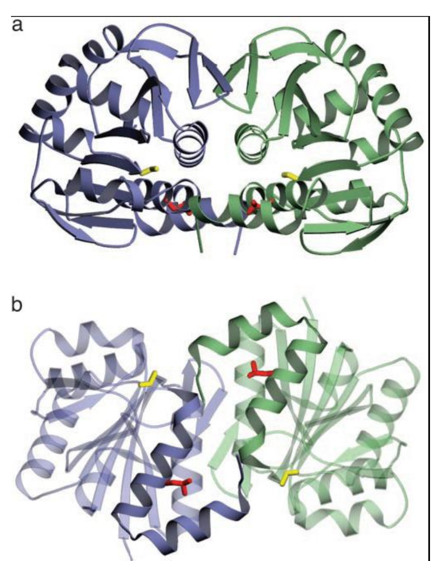


Fig. 8:
Crystal structure of human *DJ-1* protein, dimer.
(b) is rotated by 90° from (a). Monomer A is blue and monomer B green.
The readily oxidizable Cys 106 is yellow and Lysine166, which is mutated to Proline in PD, is red.

Taken from: Wilson A (2003), The 1.1.A° resolution crystal structure of *DJ-1*, the protein mutated in autosomal recessive early onset Parkinson's disease. PNAS 100:9258

The L166P mutation disrupts dimer formation [114]. Interestingly, when mutated, *DJ-1* also loses its uniform cytosolic distribution in order to colocalize with mitochondria [70].

Neuropathology

DJ-1, like *Parkin*, is not an essential component of Lewy bodies, however *DJ-1* immunoreactive inclusions are found in other α-synucleinopathies and also tauopathies (see glossary: tau). Immunohistochemical staining for *DJ-1* labels tau-positive lesions in Alzheimer's disease (AD) (see glossary), Picks disease (see glossary), progressive supranuclear palsy, and frontotempotal dementia with parkinsonism linked to chromosome 17 [115]. In multisytem atrophy (MSA, see glossary) *DJ-1* co-localizes with *α-synuclein* in cytoplasmic inclusions. Compellingly, *DJ-1* staining is positive within reactive astrocytes in most diseased tissue, independent of the respective type of disease [115, 116].

Taken together, these data suggest that different neurodegenerative diseases share a common pathogenic mechanism, in which *DJ-1* might play a key role that might reach far beyond its role in Parkinson's disease.

The DJ-1 superfamily

DJ-1 belongs to a superfamily of proteins called *ThiJ/PfpI/DJ-1* family that has been highly conserved throughout evolution. (See fig. 9)

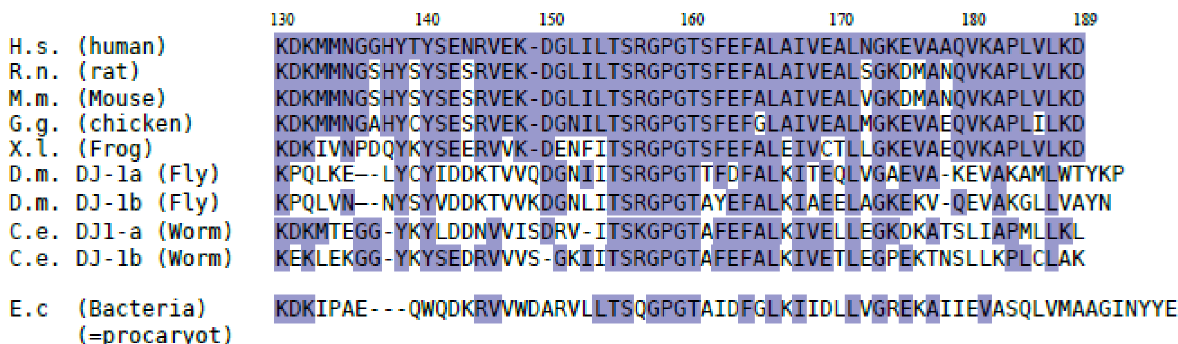


Fig. 9: Highly conserved amino acid sequence of *DJ-1* and its ThiJ, PfpI homologues in different species, C- terminus.
(H.s. = Homo sapiens, R.n. = Rattus norvegicus, M.m. = Mus musculus, G.g = Gallus gallus, X.l = Xenopus laevis, D.m. = *Drosophila* melanogaster, C.e. = Caenorhabditis elegans, E.c. Escherichia coli)

The other members of the *ThiJ/PfpI/DJ-1* family (-*ThiJ* and *PfpI* are E.coli proteins) include chaperones (see glossary), proteases and transcriptional regulators [111], yet *DJ-1* function remains to be elucidated. Protease activity has been suggested [117], but crystallography studies of *DJ-1* make this very unlikely [118], since the catalytic triad, that is present in other members of the *PfpI* family, proves absent in the corresponding region of *DJ-1*.

1.4.2.2 *DJ-1* function

DJ-1 has been implicated in a number of diverse cellular processes involving cellular transformation and tumorigenesis [112, 119], transcriptional regulation and RNA binding [120], androgen receptor signalling [137, 121], spermatogenesis [122, 123], and, probably most interesting in the context of PD: oxidative stress [124-127].

Cellular transformation

DJ-1 was first discovered in 1997 as a novel oncogene, which is able to transform cells in cooperation with activated Ras [112]. In a number of cancer types, including lung cancer, prostate cancer and breast cancer [128-130], *DJ-1* is significantly overexpressed in the tumor cells. In breast cancer patients, antibodies against circulating *DJ-1* antigen have been described [131]. *DJ-1* overexpressing tumor cells demonstrate an increased resistance to apoptotic signals [119, 132]. Recent studies have revealed *DJ-1* to participate in a number of cell survival pathways, via interference with apoptosis-mediating effector kinases: By inhibiting the function of tumor suppressor protein PTEN, *DJ-1* is a positive regulator of the anti-apoptotic Akt signalling pathway (see glossary), [128, 133]. Furthermore, *DJ-1* has been implicated in binding tumor suppressor protein p53 (see glossary) and "death associated" protein Daxx, thus hindering Daxx to translocate and bind its partner Ask to initiate apoptosis (see glossary) [134, 129].

Other studies have shown that *DJ-1* may be involved in androgen receptor signalling [37, 121], spermatogenesis and male fertility [122, 123]. Moreover, it has been cloned as part of an RNA-binding multiprotein complex [120] and several studies have associated it with transcriptional regulation [132, 135]. *DJ-1* has also been suggested as a regulator of protein SUMOylation (see glossary: "SUMO") [135, 136].

Oxidative stress

In the context of PD, *DJ-1*'s most alluring putative function concerns its involvement in oxidative events: Accumulating evidence from cell culture studies suggests a cytoprotective role of *DJ-1* in the defense against oxidative stress. In vitro studies employing 2-dimensional gel electrophoresis showed that *DJ-1* responds to oxidative stress, induced by paraquat exposure, with a shift of its iso-electric point towards a more acidic isoform (from pI 6.2 to pI 5.8) [137]. Post-mortem analysis of PD brains detected higher concentrations of the acidic *DJ-1* isoforms, as compared to healthy controls [111].

In a 2001 screen looking for hydrogen peroxide (H_2O_2) responsive proteins, *DJ-1* was identified amongst others. H_2O_2 exposure oxidizes all its cysteine residues (cys 46, 53, 106) to cysteine sulfonic acid [138], with cysteine 106 being the most stress sensitive residue amongst them. These studies demonstrate a direct modification of the *DJ-1* protein by ROS, nourishing the notion that *DJ-1* might act as a free radical scavenger.

Cell culture studies of *DJ-1* deficient neuronal cells exhibit an increased susceptibility to H_2O_2, MPP+, 6-hydroxydopamine, and rotenone toxicity [121, 128, 139], whereas *DJ-1* overexpression could dramatically reduce H_2O_2 induced cell death in neurons [139, 140]. Oxidative stress results in a mitochondrial relocalization of *DJ-1* that is mediated by oxidation of the cysteine 106 residue [141].

Taken together, cell culture studies have provided many valuable clues as to the potential radical scavenging properties of *DJ-1* protein. However, in order to describe loss of function phenotypes, the expression in an integral, live organism is indispensable. We therefore created animal models of *DJ-1* loss of function and *DJ-1* overexpression genotypes, employing the fruit fly *Drosophila melanogaster*.

1.5 The *Drosophila* model of *DJ-1* associated Parkinson's disease

A model organism is an extensively studied and understood species used to investigate causes and new treatments for human diseases, when experimentation on humans would be unethical or not feasible. Due to the common descent of all living organisms, genetic material has been conserved throughout the species, resulting in shared biochemical, metabolic and developmental pathways. Thus, knowledge gained in a model organism provides useful insight into the workings of humans and other species.

Since the very beginning of the twentieth century, the fruit fly *Drosophila* melanogaster has emerged as one of the most powerful genetic model organisms. For over 100 years of use it has led the way in elucidating many complex biological problems, including neurological and neurodegenerative disease.

Flies have several experimental advantages including:

- their relatively short life span (~ 3 months),
- their short life cycle of only ~10days,
- their very high reproduction rate, thus large numbers of individuals can be generated,
- their ease of maintenance, which is little space-, time- and cost-consuming,
- the fully sequenced genome, amendable to genetic manipulation,
- well understood modes of inheritance and fly husbandry
- well established, powerful molecular genetic tools and techniques,
- availability of stocks containing altered genes at the Bloomington *Drosophila* Center at Indiana University, USA.

Especially in neurodegenerative disease, *Drosophila* is emerging as a valuable model: Its highly complex brain and nervous system are composed of numerous specialized cell types that utilize all the major classes of neurotransmitters, receptors, ion channels and signalling pathways found in humans. *Drosophila*'s nervous system possesses approximately 300,000 neurons (as opposed to several hundred in the nematode) and millions of synaptic connections. The grade of complexity in *Drosophila* 's brain makes the fly capable of memory and learning. Moreover, *Drosophila* exhibits numerous

sophisticated behaviors [142-144], such as flight, courtship, climbing etc., which can be assessed to describe disease phenotypes.

Furthermore, more than 70% of disease-related loci in humans have a clear orthologue in the fly, as revealed by proteomic analysis.

The limitation of the fly model mainly consists in the absence of a comprehensible neurological disease phenotype. Obviously, the extrapyramidal symptoms of PD, such as tremor, rigor and bradykinesia cannot be reproduced in the fly. For such purposes, mammalian models are indispensable.

1.5.1 Dopamine-containing neurons in *Drosophila* melanogaster

Most importantly for Parkinson's research, insects possess dopamine-containing and -metabolizing neurons [145-148], which employ the same metabolic steps as those described in mammals (see fig. 1), i.e. the two main dopamine synthesizing enzymes, tyrosine hydroxylase (TH) and DOPA- decarboxylase (Ddc), are present also in the fly, and can be used methodically.

TH immunostaining is an excellent way of visualizing dopaminergic neurons specifically. Although the conversion of tyrosine to L-DOPA by TH is the primary step in all monoamine syntheses, including norepinephrine, norepinephrine has never been detected in the *Drosophila* CNS. It is thus most likely that all monoaminergic neurons contain dopamine (DA). (Staining of the enzyme DOPA- decarboxylase (Ddc) (see fig. 1) is less specific, since Ddc is also involved in serotonin synthesis.)

In the drug study below, TH-staining will therefore be employed to identify DA neurons. TH enzyme is a highly conserved protein, and cross reaction with TH from diverse vertebrate species is observed [149, 150].

The distribution and development of dopamine containing neurons in the *Drosophila* nervous system have been studied extensively [151-153]: In the adult fly, each monoamine, although widespread through the nervous system, has a specific location. Monoamine-containing cells present in confined cellular clusters. TH staining shows a prominent cluster of dopaminergic neurons that is always represented dorsomedially

behind the so called giant interneuron commissure, where axons cross to the other brain hemisphere (see fig. 10): The dorsomedial cluster, DM (see fig. 11).

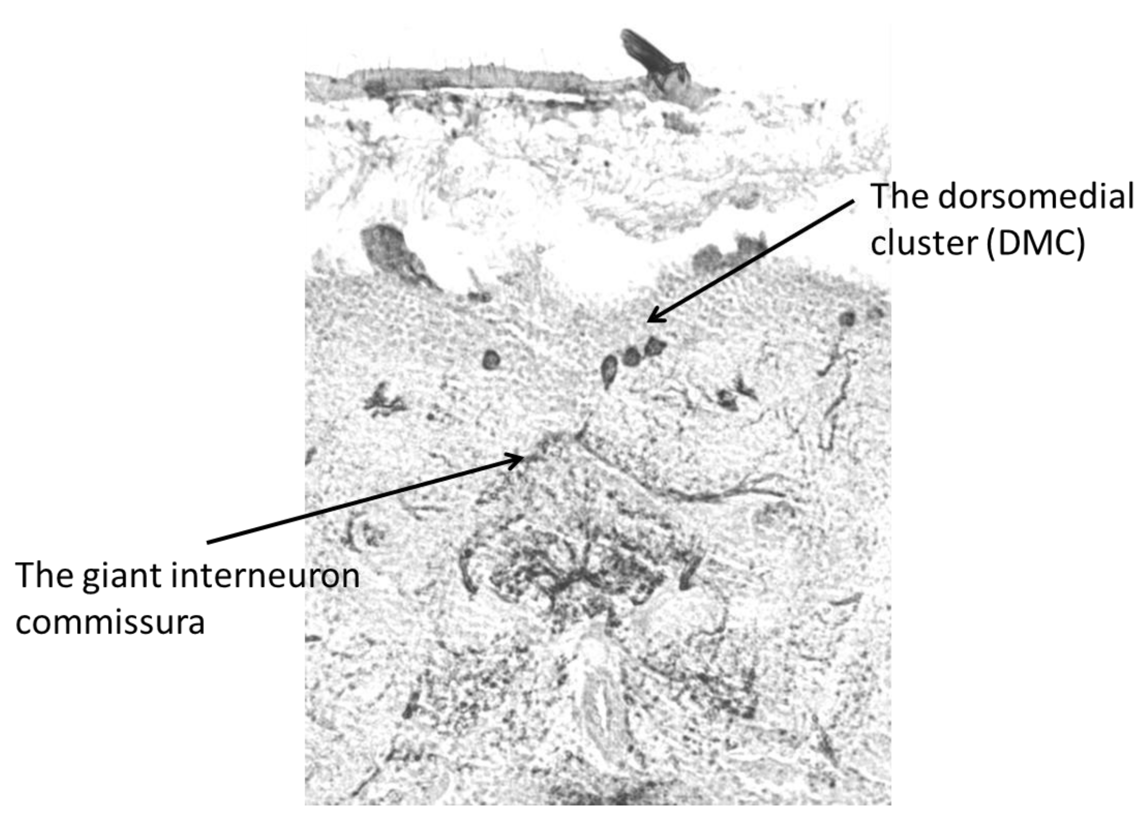


Fig.10:

The giant interneuron commissura,
light microscopy from the neurodegeneration assay (see below)

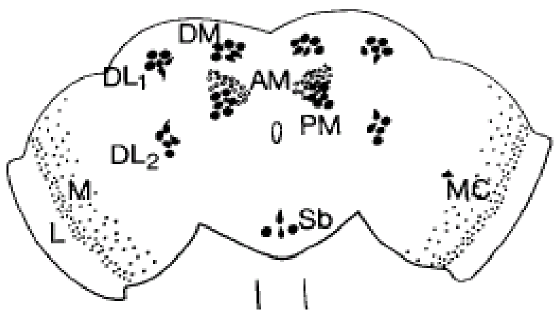


Fig. 11:
Schematic representation of CA containing neuronal clusters in the adult *Drosophila* midbrain.
Axial section. DM: dorsomedial, DL1: dorsolateral1, DL2: dorsolateral 2, AM: anteromedial, PM: posteromedial, L: lamina, M: medulla, MC: medulla cells, Sb: subesophageal. Open cells indicate anteriorly orientated clusters, filled cells posterior clusters. L+M mark the optical lobes. Numerous small cells in the medulla (MC) are indicated by dots. (The thoracic CNS of the fly, which also contains CA cells, is not depicted here.)
Modified from: Budnic V & White K (1988): Catecholamine-containing neurons in *Drosophila melanogaster:* Distribution and development. The Journal of Comparative Neurology 268:400-413 [152]

In another genetic fly model of Parkinson's, the *α-synuclein* model, it has been demonstrated that the DM cluster disappears preferentially and selectively [150]. In our evaluation, we will therefore also focus on the DM cluster, while the other clusters (see fig. 11) will serve as internal controls.

Even though a pharmacological animal model of Parkinson' disease, the MPTP mouse model, has already existed since the late eighties, its value is limited. One of the big caveats with the pharmacological model is its impracticality for drug studies, since it is uncertain, whether administered therapy is truly neuroprotective for PD pathogenesis, or if protection is achieved chemically by attenuating the toxin's pathogenicity.

Genetic studies in *Drosophila* have already implicated numerous key neuronal functions as critical to the maintenance of function during aging. At the time of the start of the present study, a handful of different genetic Alzheimer's model flies [154, 155], several Huntington flies [156] and two genetic Parkinson's model flies (*α -synuclein, Parkin*) [150, 157] existed, all of which recapitulated essential features of the human disorder and have generated tremendous novel insights into the respective disease.

1.5.1.2. *Drosophila* genetics

Fly genetics is facilitated by several unique characteristics of the *Drosophila* genome. Firstly, in the fly there are only 4 chromosomes. Recombination only happens in females and never in males. Furthermore, there are balancer chromosomes, which additionally prevent recombination. Balancer chromosomes are chromosomes which are no longer capable of pairing with their normal homologue during the meiotic phase. Thus, heterogametic mutations can be completely suppressed. Furthermore, marker mutations exist. They are used to tag the chromosome arm one is to follow on the respective balancer chromosome. Phenotypic expression of marker chromosomes involves eye colour, wing shape, bristle shape and cuticle colour. For the *DJ-1* mutants, we always used “dark cuticle”, which is located on chromosome 3, as the marker to follow the mutation.

1.6. Introduction to the aging study:

Aging is the first and foremost risk factor for developing PD. After all, sporadic PD - like all neurodegenerative disorders-, is a disease of the aging and aged brain. There is an undeniable interrelationship between neurodegenerative disease and aging. In humans, both sporadic and familial PD forms are accompanied by a reduction of life expectancy. In *Drosophila* mutants for *α- synuclein* and *parkin*, neuronal viability and integrity is affected with age, and both mutants exhibit reduced life spans [158, 159]. Therefore, one of the most fundamental disease phenotypes to be described in the novel *DJ-1* mutant fly is the effect of *DJ-1* on aging:

Aging is conceived as a steady, progressive and irreversible decline in organismal performance. Yet a clear definition as to what aging is, does not exist. For more than two thousand years the aging process has been a focus of scientific interest, but the mechanisms as to how we age, how neurons age, and also why we age, have remained largely a mystery.

Measuring aging

Unfortunately, there is no direct assay to measure aging in the individual. Most commonly, aging is measured in an indirect demographic manner. Life span analysis is presently the primary, most commonly employed, and best assay for measuring age-related phenotypes. It is assumed that the influences that shorten lifespan- be they genetic or environmental- do so as a result on the process of aging itself.

In all aging research there is one elemental, yet unresolved, question, - and that is whether aging is an active process, and the result of a genetically determined, underlying program; or if aging is the consequence of an accumulation of random stochastic events, like toxic agents, stress, injury and disease, that eventually increase the probability of death over time.

Aging in the fly

The particular benefits of using the fruit fly *Drosophila* melanogaster for studying aging include, besides its relatively short life span and the availability of large cohort numbers, as mentioned above, its morphologically distinct life stages from embryo over larva and

pupa to the adult fly. Thus the period of aging is visually clearly distinguishable from periods of development and growth. Furthermore, the adult fly consists almost entirely of post-mitotic cells. Consistently, each cell that was present until eclosion, ages parallel to the entire fly and remains until the death of the individual.

Fig. 12: Life cycle of the fruit fly *Drosophila melanogaster*

Drosophila has already made important contributions in previous studies: A number of both genetic and non-genetic interventions are known to affect life span in the fly. Genes that are known to prolong life span in certain species, are named longevity genes.

Manipulating aging

Non-genetic manipulations include: -reproductive status (virgins live for up to twice as long as their mated equivalents), - dietary status (caloric restriction leads to an extension of life span) -ambient temperature (reduction of room temperature to 18° C can more than double life-span, probably due to the effect on metabolic rate), - mild, non-lethal stressors (probably through induction of protective heat shock proteins), - physical activity (housing flies in small containers that do not permit them to fly increases lifespan).

Genetic manipulations include the enhancement of protective and repair pathways, such as overexpression of chaperones and heat shock proteins [160, 161], methionine sulfoxide reductase [162], protein carboxyl methyltransferase [163] and others. Overexpression of histone deacetylases, -these are proteins important for the integrity and maintenance of chromatin structure-, also appears to lengthen *Drosophila's* lifespan [164-166], as well as manipulation of the insulin/IGF-like signalling pathway via mutations in the insulin receptor gene [167-170].

Decreasing or increasing antioxidant enzymes, which are important in detoxifying ROS before they cause cellular damage, influences life span [171-174]. Most intriguingly, *Drosophila's* lifespan was increased by 40-50% after overexpression of the antioxidant enzyme SOD [175].

DJ-1 and aging

DJ-1 has also been associated with the oxidative stress response: its putative function as a free radical scavenger might protect cells from life-shortening oxidative events.

In addition, the role of *DJ-1* in relation to apoptosis might directly promote cell survival. Thus, intriguingly, *DJ-1* might play a key role in cell survival and longevity in both of the basic aging theories described above and thus constitute an attractive new candidate gene as novel longevity gene.

Consequently, part of the following study aims to investigate whether manipulation of the *DJ-1* gene could exert an effect on life span.

1.7 Introduction to the drug study:

1.7.1 Possible novel drug treatments for Parkinson's disease

As described above, all present treatment for Parkinson's disease (PD) is purely symptomatic and follows the principle of either replacing, imitating or augmenting the effects of dopamine within the CNS.

In the present study, novel, potentially neuroprotective and disease modifying treatments will be tested, using the *DJ-1* fly model of PD. These treatments target main pathogenic factors, including *oxidative stress*, *mitochondrial dysfunction, excitotoxicity* and *inflammation.*

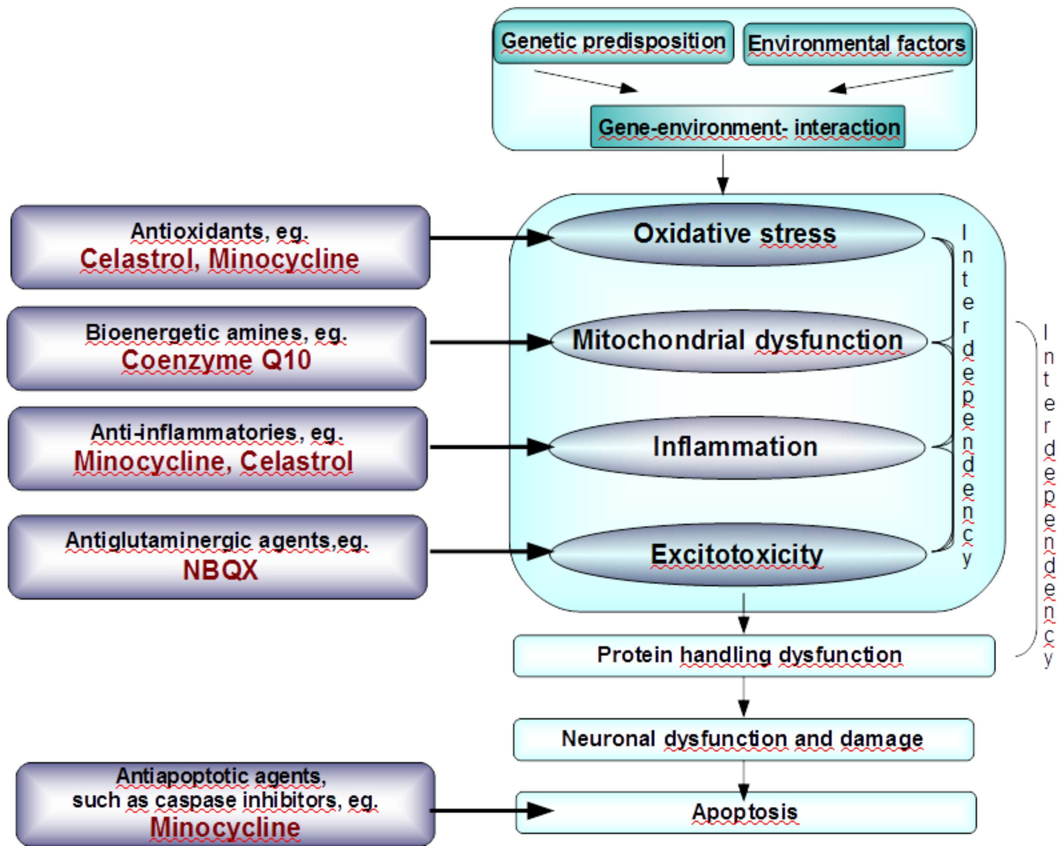


Fig. 13: Possible targets for neuroprotective and neurorestorative agents in the pathogenesis of PD

1.7.1.1 Drug 1: Minocycline

→ targeting oxidative stress, inflammation and apoptosis

Fig. 14:
Chemical structure of Minocycline

Minocycline is a member of the tetracycline group of antibiotics. It has broad spectrum antimicrobial activity and is most commonly used in the treatment of acne and rosacea

[176-177]. Recently, minocycline has been found to have additional properties that are completely independent from its antibacterial activity and that may be useful in the treatment of neurodegenerative diseases including PD: It has been shown to have advantageous effects on inflammation, microglial activation, nitric oxide production, matrix metalloproteinases and apoptotic cell death.

Minocycline has been shown to have neuroprotective effect in animal models of a number of other neurological diseases, including ischemia and stroke [178-179], traumatic brain injury, amyotrophic lateral sclerosis (ALS) [167-170, 180, 181], multiple sclerosis (MS), Huntington's disease (HD), and the MPTP mouse model of PD. Furthermore, it has been proven to be beneficial in the treatment of rheumatoid arthritis and osteoarthritis.

Minocycline has an oral bioavailability of almost 100% and its absorption, unlike with other tetracyclines, is not reduced by ingestion with food [182]. Its high lipophilicity allows it to diffuse into brain tissue easily [183]. Minocycline has a lower urinary excretion than other tetracyclines and is thus safer in elderly patients with impaired renal function. Side effects of minocycline are similar to the other tetracyclines [184]; they include nausea, headaches, dizziness, vertigo, diarrhea, rashes, stomatitis and vaginitis [185]. Cases of hepatitis, presumably due to a hypersensitivity reaction, have been reported [186]. Minocycline is harmful and not recommended for use in children [177], but Parkinson's is a disease of the elderly.

1.7.1.2 Drug 2: Celastrol

→ targeting microglial activation, inflammation, oxidative stress

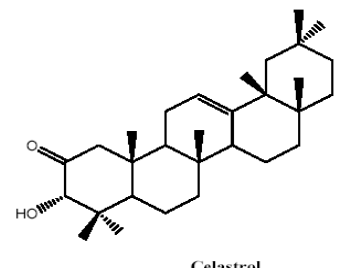


Fig.15:
Chemical structure of celastrol

Celastrol is a triterpene (see fig. 15), extracted from the root bark of an ivy-like, creeping plant, that is indigenous to a large area in Southern China, called Tripterygium wilfordii (TW) or “Thunder of God vine”, belonging to the family of Celastraceae. Extracts of the plant have a long history of use in traditional Chinese medicine to treat fever, chills, edema and joint pain [187] – all conditions, which are commonly associated with inflammation.

In a controlled double-blind trial of rheumatoid arthritis (RA) celastrol was found to have efficacy [188]. Autoimmune diseases like RA are always mediated through an inflammatory response. An extract of TW suppressed the manifestations of adjuvant arthritis in rats [189, 190]. Celastrol was found to suppress microglial cell activation, release of the inflammatory cytokines TNF-α and IL-1β by human macrophages and monocytes, and the production of nitric oxide (NO) by iNOS [187].

Furthermore, Celastrol was demonstrated to be a potent inhibitor of induced lipid peroxidation in rat liver mitochondria [191, 192], exhibiting the 15-fold antioxidant potency of (= vitamin E), which is probably the most frequently used antioxidant in clinical practice. Celastrol protected both the outer and inner mitochondrial membranes from peroxidation, which is thought to be mediated by its radical scavenging dienonephenol moiety (see fig. 15), while the anionic carboxyl group (see fig. 15) protects the inner membrane from radical attack by stabilizing its negative surface charge. In a rat model of Alzheimer's disease (AD), celastrol was shown to improve memory and learning as assessed by psychomotor activity tests (PMA) [187].

In all studies mentioned above, low, nanomolecular concentrations of the drug were sufficient to mediate efficacy. Celastrol has a high oral bioavailability and does not exhibit carcinogenicity, nor any other limiting side effects, as can be deduced from mammalian studies [189] and from the fact that it has been administered empirically to Chinese patients for centuries without apparent side effects [188]. The agent is lipophilic and easily crosses the blood brain barrier (BBB).

1.7.1.3 Drug 3: Coenzyme Q10

→ targeting mitochondrial dysfunction

Fig. 16:
Chemical structure of coenzyme Q10

Coenzyme Q 10 (coQ10, also known as ubiquinone) is composed of a quinone ring and a 10 isoprene unit tail (see fig. 16). It is an obligatory cofactor in the mitochondrial respiratory chain. As bioenergetic agent it serves as an electron acceptor of complexes I and II/III (see fig. 3).

Mitochondrial dysfunction has been well established to occur in PD, and several lines of evidence suggest its importance in the pathogenesis of the disease (see fig. 3 and ch. 1.3.1.4). The most important of these are a 30% to 40% reduction in complex I activity and the recognition, that MPTP can induce Parkinsonism by inhibiting complex I activity in the mitochondrial respiratory chain (see ch. 1.2. and ch. 1.3.1.4).

In addition, coQ10 is a potent antioxidant, distributed in all membranes throughout the cell. It is able to work in concert with α.tocopherol (vitamin E) and participate in the recovery of cells from oxidative stress [193, 194]. Levels of Co Q10, measured in mitochondria from PD patients, were found to be significantly lower than in age-matched controls [195, 196], while at the same time the percentage of oxidized coQ10 isoforms was found to be relatively increased in PD patients [197].

Based on its unique properties and function within the cell, co Q10 has attracted particular interest concerning its neuroprotective properties. In in vitro models it has been shown that co Q10 could protect against MPP+ (see ch. 1.2) [17, 40] and rotenone (see ch. 1.2) [198] toxicity. Several animal studies and pilot clinical studies (mainly phase II dosage finding and safety studies) have been conducted to evaluate the therapeutic benefit of coQ10 in PD and other neurodegenerative diseases (ALS,

HD, AD). In animal models of ALS [199] and HD [200], coQ10 treatment has shown beneficial effects.

In the MPTP mouse model of PD, it was described that oral treatment of young mice with coQ10 and nicotinamide attenuated the effect of low dose MPTP administration, while coQ10 alone reduced the dopamine depletion in the striatum [201]. However, this reduction was not statistically significant. Co Q10 treatment (200mg/kg/day) in aged (1 year old) mice, that represent a more adequate model of PD, showed that coQ10 significantly attenuated the MPTP induced loss of striatal dopamine by 37% and the loss of TH immunoreactive fibers in the mouse striatum by 62% [202]. It was also demonstrated, that oral coQ10 administration increased the mitochondrial content of coQ10 in the cortex of 1 year old rats [199]. Similar promising results have subsequently been generated using a monkey MPTP model of the disease [203].

Large clinical efficacy studies have not been completed to date and preliminary results are inconclusive [196, 204-208]. In most clinical studies coQ10 is administered together with α-tocopherol based on their collaborate effect. In the studies mentioned above, doses of up to 3000mg/day have been administered without showing intolerance or side effects. Co Q10 is extremely lipophilic, i.e. it easily crosses the BBB. Its absorption is improved by the inclusion of lipid in the formulation and by taking it with food.

In the following drug study it will be investigated, whether the results generated in MPTP models of PD can be reproduced using a genetic PD model (the *DJ-1A* RNAi fly) or whether coQ10's benefit is restricted to the toxin-induced model.

1.7.1.4: Drug 4: NBQX

→ targeting excitotoxicity

Fig. 17:
Chemical structure of NBQX

2,3-Dihydroxy-6-nitro-7-sulphamoyl-benzo[f]quinoxaline (NBXQ) is a potent competitive AMPA receptor antagonist, belonging to the group of quinoxaline diones (see fig.17). As outlined in ch.1.3.3, AMPA selective glutamate receptor antagonists constitute potential neuroprotective agents by counteracting the excitotoxic effects of excess glutamate. In the MPP+ mouse model of PD, AMPA receptor agonists were shown to have greater therapeutic potential than NMDA receptor antagonists [92].

The quinoxalindione derivates were discovered in 1988 and are still undergoing intensive study [91, 209]. NBQX exhibits improved AMPA receptor selectivity with respect to earlier quinoxalindiones [210]. NBQX has systemic activity and was first shown to have therapeutic effects in animal models of neurological disease in 1990, where it protected from cerebral ischemia after carotid artery occlusion in mice [210]. NBQX has thus been used as the antagonist of choice in many “in vivo” and “in vitro“ models. Besides its anti-Parkinson properties [211, 212], NBQX has shown efficacy in the treatment of demyelinating disorders [213], trauma [214] and stroke [210].

Despite NBQX's receptor selectivity and potency in animal models, it had to be eliminated as a drug candidate for humans based on poor pharmacokinetics. Its low water solubility at physiological pH, combined with a fast renal excretion, would cause crystallization in the kidney already at therapeutic doses [215]. The fast excretion also prevents any prolonged systemic activity in humans following oral administration.

Therefore newer compounds have been and are being designed (by computational mapping of the AMPA recognition site, in combination with x-ray crystallographic analysis of co-crystallized complexes at the receptor binding domain), with better physicochemical properties (especially higher water solubility) in humans, e.g. PNQX, YM872 [216-218], for which NBQX has served as a template.

In the following drug study the easily available NBQX is still administered to the fly PD model. Any observed neuroprotective effect in the fly might encourage the drug design and subsequent clinical testing of newer quinoxalindiones, with better pharmacokinetic properties.

2. MATERIALS AND METHODS

2.1. MATERIALS

Reagents/Chemicals

Materials	Suppliers	Lot Numbers	Additional Information
Formaldehyde Solution 37% w/w	Fisher Scientific Fair Lawn, New Jersey 07410, USA	031882	Cat-No: F79-500 Formaldehyde: CAS 50-00-0 Methanol : CAS : 67-56-1 Water : CAS : 7732-18-5
Minocycline Hydrochloride Salt	Sigma Aldrich co, P.O Box 14508, St. Luis, MO 63178 USA	014121207	Cat-No: M-9511 250mg
NBQX Disodium Salt	Sigma Aldrich co, P.O Box 14508, St. Luis, MO 63178 USA	051K 4603	Cat-No: N-183 5mg
R.T.U. Vectastain Kit	Vector Laboratories 30 Ingold Road, Burlinggame, CA 94010, USA	PK-7200	Cat-No: PK-7200 Includes: - 50ml 2,5% R.T.U. normal Horse Serum - 50ml Vectastain R.T.U Elite ABC Reagent - R.T.U Biotinylated Universal Antibody: anti-rabbit/ mouse IgG (H+2), made in horse
DAB Peroxidase Substrate Kit	Vector Laboratories 30 Ingold Road, Burlinggame, CA 94010, USA	SK-4100	Cat-No: SK-4100 Contains: - Hydrogen Peroxide Substrate Reagent - DAB Substrate Reagent - Buffer pH 7.5 - Nickel Solution
Perchloric Acid 70%	Sigma Aldrich co, P.O Box 14508, St. Luis, MO 63178 USA	244252	$HClO_4$, A.C.S reagent, CAS 7601-90-3, (FW 100.46), cat No : 244252, 100ml, Batch No : 09045AB
Permount	Fisher Scientific Fair Lawn, New Jersey 07410, USA	041295	Toluene Solution 100ml Histological Mounting Medium UNI1294 FL-08-1197 Cat No : SP15-100
Xylenes Isomers		247642	(C8H10) plus Ethylbenzene, 98,5+% A.C.S Ethylbenzene max 25% Batch No: 05350BB CAS 1330-20-7 (FW 106.17)
Ethanol	Aaper Alcohl and Chemical Co.,	03G08UA	MFD: 071603 DSP-KY-417

	Shelbyville, Kentucky 40065, USA		Absolute -200 Proof
Manganese Chloride	Sigma Aldrich co, P.O Box 14508, St. Luis, MO 63178 USA	110K0186	Cat-No: M-8054 (13446-34-9), (FW 197,90) 100g
Cupric Sulfate Pentahydrate	Sigma Aldrich co, P.O Box 14508, St. Luis, MO 63178 USA	38F-0527	1kg No. C-6283 ACS reagent [7758-99-8]
Sucrose	Fisher Chemicals Fisher Scientific Fair Lawn, New Jersey 07410, USA	006193	FL 03-0297, 3kg, CAT: S5-3 CAS 57-50-1, FW: 342.30 Sucrose Certified A.C.S (Saccharose)
Gelatin	ICN Biomedicals Inc 1263 South Chillicothe Road, Aurora, Ohio 44202, USA	6505E	100g CAT No: 960317 [9000-70-8]
Chromium Potassium Sulfate	Aqua Solutions Deer Park, TX 7736, USA	217601	Cat: S76786, 100g CAS No: 7788-99-0 FW 499.41
Yeast	Lab scientific, inc.	FLY 8040	*Drosophila* Yeast-dry, active granular
One Shot® TOP10 Chemically Competent E. coli	Invitrogen™	3079620	Cat: C4040-06 Contains: • One Shot® TOP10 Chemically Competent E. coli: 42 vials, 50 µl each • pUC19 DNA (10 pg/µl): 2 vials, 50 µl each • S.O.C. medium: 2 bottles, 6 ml each

Machines:

Materials	Suppliers	Lot Numbers	Additional Information
Bio Vortexer (Homogeniser)	Biospec Products, Bartlesville, OK, USA	1083	
Light Microscope #1	Nikon	60401	Nikon SMZ645 Model C-PS No 1018696 Carrier: Morrell, 502 Wall Whitman Road, Meltville, NY 11747, USA
Light Microscope #1	Schott-Fostec;LLC		Light tube + bulb: Fostec ACE EKE Input: 115-120V, 60Hz, 3A Output:150Watts Actual lamp: NMB Model 3115PS-12W-B20 115V-AC, 1 Phase, 50/60 Hz, 5/4.5 W, Mineba co. LTD
Light Microscope #2	ZEISS		Stemi 2000 455094
Lamp for Light Microscope #2	Technical Instruments San Francisco		Light tube: Fibre-lite EEG 2828 M Dolan Jenner Ind Lawrence MA 1-800-83 Fibre
25°C Incubator	Forma Scientific Therom Forma P.O. Box 649 Marletta, Ohio 45750, USA		Reach-in-incubator Model 3940, Rel# 8, 208-220V, 60Hz, 14A, 1PH, 4wire, 3pole S/N: 47291-1538, HI STAGE:R-134 A 382gr (13,5oz) Design Press (high):1828 kPa (250psig) (low): 710 (88psig) 4/24/01
Autoblot Micro Hybridization Oven	Bellco Glass Inc., Vineland, NY, USA		Cat No: 79-00110 Serial No: BMHOJ-1742, 115V, 60Hz (AC), 6A
29° Incubator	Sheldon Manufacturing Inc 300N 26 TH, Cornelius, OR 97113		Model No: 1500E Serial No: 04063504 Part No: 9120993, 3.0 A, 115V, 60Hz
Microscope slides	Fisher Scientific Fair Lawn, New Jersey 07410, USA		Fisher finest premium Microscope slides Superfrost 25x75x1mm,

			02/12/94, Approx. ½ gross Cat No: 12-544-7
Cover Glass	Fisher Scientific Fair Lawn, New Jersey 07410, USA		Fisher finest premium Cover Glass Cat No: 12-548P, 24x60-1 mm 02/09/04
Camera for the Light Microscope	Canon Inc, Japan		DC 8.1 No: 0620312038
+Remote Switch	Canon Inc, Japan		RS-80N3
+ Chip Card	PNY technologies		128 MB Compact Flash THNCFI28MM1
+ USB reader/writer	Made in Taiwan		P/: FPTS-C USGF2J07
High resolution Microscope Nicon Eclipse TE300	Nicon, Japan		413110 62901
+ RT Slider spoT	Diagnostic Instruments.inc		Serial No: 203424 Model No: 2.3.1
+ Light Machine	Nikon		Model: TE-PS100, 100-120V, ~2.5 A, AC IN 50/60Hz, No: 1023768, Output max 12V, 8.4A
+ spot RT Power Supply	Diagnostic Instruments. Inc		Model No: SP402-115 Input 115±5% VAC @1.05A, 50-60Hz Output:+ 15VDC@800mA maximum rating -15VDC@800mA maximum rating + 5VDC@7A maximum rating Fuse: FST 2A 250V 5x20mm Connected to Dell computer
Microtome	Leica Microsystems GmBH D-69226 Nussloch, GERMANY		Modell RM2135 Fab.Nr 5032/08.2003 Cat No: 050029802
Orbital Shaker	Bellco Glass, Inc. Vineland NJ USA		Cat.No: 7744-01000 Serial –No: UCSB-1040 Volts: 115 AC/Hz :60 Amps: 1
Nutator	Clay Adams Brands		Clay Adams Brands

	Manufactured for: Becton Dickinson Primary Care Diagnostics 7 Lovetown Circle Sparks, MD 21152-0370 Mfg by Oxis Instruments, Inc Ivyland, PA 18974 USA		Model No: 421105 Serial No: 17197 117V AC 0.03A 50-60 Hz Manufactured for: Becton Dickinson Primary Care Diagnostics 7 Lovetown Circle Sparks, MD 21152-0370 Mfg by Oxis Instruments, Inc Ivyland, PA 18974 USA
Thermolyme Type 17600 Dry Bath	Barnstead/Thermolyne, 2555 Kerper Boulevard, Dubuque, Iowa 52001, USA		On 120 Volts; 0,6 Amps; 75 Watts; 50/60 Hz Phase 1 Model No: DB 17615 Serial No: 821010330943
Kimwipes EX-L	Kimberly-Clark Inc., Mississanga, Ontario L5B3Y5 Canada		Delicate Task Wipers 280 1-PLY Wipers 11,4x21,3cm Code: 34155 Inches: 4,4x 8.4 Cm: 11.4x 21.3 280 per carton, 60 cartons
Fly Vials			Height 9.5 cm width 2.4 cm
Fly Bottles			Base diam.:6cm Upper diam.: 4cm Height: 8cm
Pestle for Homogenizer	Bellco Glass, Inc 340 Edrundo Road, Vineland, NJ 08360-3493, USA		CAT No:1980-41015 Red Pestle/ Ind Size: 1.5ml Quantity: 100EA Date: 3/04 Factory: 980052 [09041]

General fly keeping:

Preparing fly food:

Distilled H_2O 1000ml

+ Dextrose 129.4g

+ Agar 9.3g

+ Corn meal 61.2g

+ Yeast 32.4g

+ Tgosept 27ml

Preparing egg lay medium:

Distilled H_2O 1000ml

+ 650ml vinegar

+ 20g Sucrose

+ 1000g fresh live baker's yeast

Preparing fresh yeast paste:

Yeast granules

+ destilled H_2O

Mix until wet paste.

2.2. METHODS

2.2.1 **Generating the *DJ-1* mutant fly**

The author of this work only contributed parts of the necessary steps in generating the mutant *DJ-1* flies, but then used the *DJ-1* mutants for her experiments. However, the author provided essential work in the generation of two other transgenic fly models of neurodegenerative disease, namely *MARK2* and *PINK1*. Therefore, the relevant proceedings in making a mutant fly are summarized briefly:

Generating a mutant fly is based on p-element insertion. P-elements are mobile genetic elements, called transposons, which can integrate into the genome at any given site. They naturally occur in *Drosophila*; and most of the spontaneous *Drosophila* mutations have been due to transposon insertion. P-elements are autonomous, because they encode for their own transposase. P-elements are always flanked by terminal 31bp inverted repeats for insertion.

pUAST is a p-element based vector. It is used for fly transformation. It also always contains a reporter gene, which is always “red-eye” in our experiments. In principle, for generation of a mutant fly the desired gene has to be cut out and inserted into the p-element containing vector pUAST and then injected into the pre germ-cells of a fly embryo together with a transposase to insert into the genome.

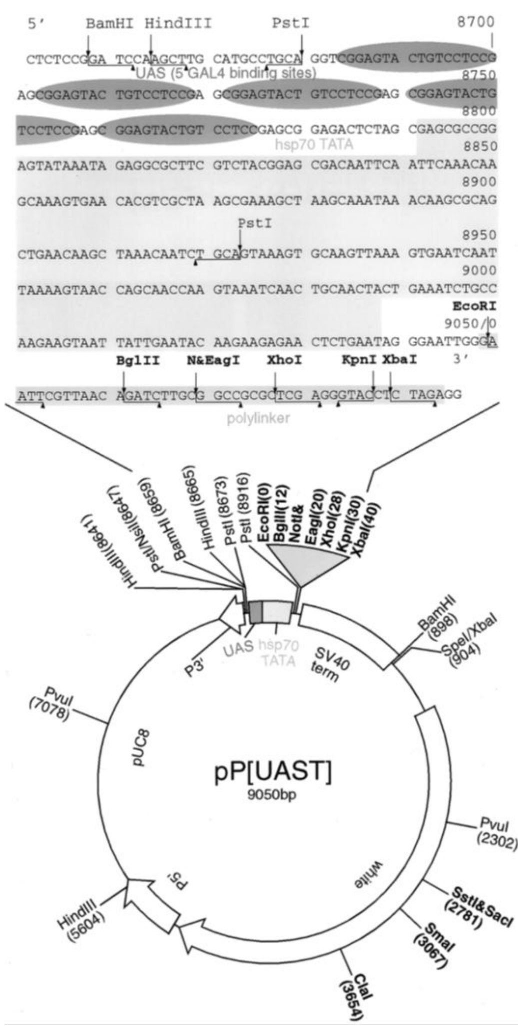


Fig.18: Sequence and restriction map of pUAST

pUAST consists of five GAL-4 binding sites (dark grey) followed by the heat shock protein (hsp) 70 TATA box and transcriptional start (light grey), a region containing restriction sites for the restriction enzymes Eco RI, Bg/II, NotI, Xho, KpnI and XbaI (medium grey). (Taken from: *snapgene.com*.)

In *Drosophila,* expression and regulation of introduced cloned genes is driven by promoter fusions. Selective tissue expression is achieved either by fusion to a defined promoter sequence or through activation of the transcription factor GAL-4. GAL-4 is a transcriptional activator from yeast. GAL-4 is driven as an enhancer trap (see fig. 18). It directs transcription of GAL-4-responsive target genes. It is only activated when it has an upstream activation sequence (UAS). It is important that the GAL-4 gene and the UAS target gene are initially (i.e. in the parental generation) separated into two distinct lines. In the GAL-4 line the activator is present, but has no target to activate. In the UAS-target line, the target gene is silent, because it has no activator to be activated by. UAS has binding sites for GAL-4 that activate the gene when they fuse (see fig.19).

Thus, crossing GAL-4 to the UAS-target line switches on the target gene in the progeny. We used the p-UAS-GAL-4 system in generating all our mutants.

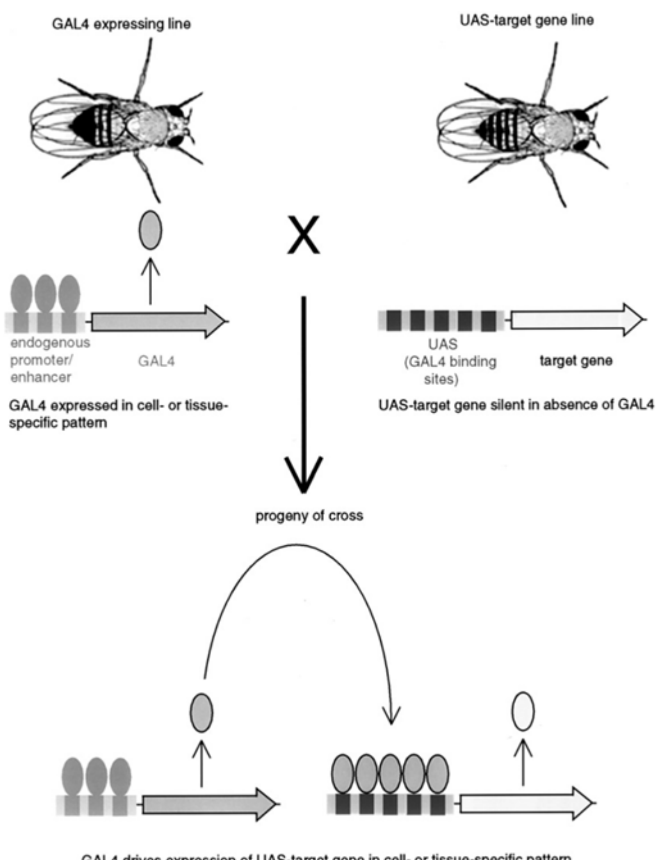


Fig.19: The principle of generating a GAL-4-expressing line
(Taken from Phelps et al 1998)

GAL-4 can be fused to tissue-specific promotors, thus expressing the gene in a cell- or tissue-specific pattern. For the following experiments, the tissue-specific promoters listed below were used.

1. Daughterless (Da) which induces the transgene expression ubiquitously in all fly tissue,

2. Elav, which induces the transgene in all neuronal tissue, and

3. DOPA-Decarboxylase (Ddc), which induces transgene expression in dopaminergic and serotonergic neurons exclusively.

The tissue specific GAL-4 lines and the w- (“white minus”) wild type stock were obtained from the Bloomington *Drosophila* Stock Center at Indiana University (www.flystocks.bio.indiana.edu).

For the following experiments, both loss of function phenotypes and ectopic expression phenotypes were generated. For generating the loss of function phenotype, double stranded (ds) RNA interference (RNAi) technique was used to reduce gene expression. The *DJ-1* RNAi constructs were generated and provided by Bingwei Lu of the Lu *Drosophila* laboratory at Stanford University [219]. To generate UAS-ds-*DJ-1A* and UAS-ds-*DJ-1B* transgenes, genomic DNA/cDNA hybrid constructs were built as described by Kalida and Smith in 1998 [220]. Genomic and cDNA fragments were amplified by PCR using the respective primers for the restriction sites. PCR products were cloned using the TA cloning kit by Invitrogen and sequenced. Analysis of the UAS-*DJ-1A* RNAi and UAS-*hDJ-1* transgenes was performed as described [219, 220].

In the sequenced *Drosophila* genome there are two gene sequences, which are equally homologous to human DJ-1: CG6646 and CG1349, henceforth named DJ-1A and DJ-1B (see Figure 8).

In generating the mutant *Drosophila* lines, the following principle steps were necessary:

A. The bacteria which contained the gene-of-interest-plasmid had to be amplified and then the bacteria had to be removed form the plasmid.
B. The gene of interest had to be isolated from its plasmid by sequential digests
C. Then the gene of interest had to be purified and amplified
D. The gene had to be cloned into the pUAST vector
E. Chemically competent cells had to be transformed with the pUAST clone and amplified
F. Next, the pUAST clone had to be isolated for sequencing and microinjection
G. The pUAST clone had to be implanted into the pre-germ cells of wild-type fly embryos together with a transposase using a microinjection technique
H. Embryos had to be cultured and flies crossed to balancer chromosome-bearing flies until stable transgenic lines were established

Since the *DJ-1* mutants were generated by other members of our laboratory, the author, by way of an example, presents the generation of another mutant fly, which was produced by the author herself: *hMARK2*. After all, the essential procedure of generating mutant flies is exactly analogous for all mutants and therefore interchangeable.

Microtubule affinity-regulating kinase 2 (*MARK2*) plays a role in another familial PD form, namely *PARK 6*. *MARK2* phosphorylates *PINK 1*(= *PARK 6*, see Introduction) and thus constitutes its regulator [221]. The *hMARK2* fly ectopically overexpresses human *MARK2.*

2.2.1.1 The cloning

A.:

1. The *hMARK2* cDNA clone (est (expressed-sequence tag) clone 3139103) was obtained from the Mammalian Gene Collection (MGC), developed by the National Institutes of Health (NIH). For plasmid purification the Quiagen plasmid Purification Kit (Midi-Prep) was used.
2. Two single cultures from the plate of *hMARK2*-plasmid containing bacteria were picked and diluted into 2ml of LB medium containing ampicillin as an antibiotic. These were incubated for 8hrs with 300bpm shaking at 37 °C. The starter culture was the diluted 1:1000 (i.e. 25µl of starter culture into 25ml of LB medium) and incubated over night at 37°C with 300bpm shaking.
3. The flask with the bacteria containing LB was then centrifuged at 4° for 15min at 6000xg. The supernatant was drained and the bacteria-containing pellet dried.
4. The bacterial pellet was resuspended in 4ml Quiagen buffer P1 by gentle vortexing.
5. 4ml of Quiagen Buffer P2 was then added, mixed, and incubated at R.T. for 5min.
6. Next 4ml of cold (on ice) Quiagen buffer P3 was added to the lysate and mixed gently.
7. The lysate was poured into the barrel of a QIA filter cartridge and incubated for 10min at R.T.

8. The outlet cap was removed, the plunger inserted and the lysate was filtered into a QUIAGEN-tip 100.
9. The QUIAGEN tip was then washed twice with 10ml Quiagen buffer QC.
10. The DNA was eluted with Quiagen buffer QF and collected in a 10ml tube.
11. DNA was precipitated by adding 3.5ml isopropanol. The mix was centrifuged at at 15000xg for 30min at 4°C. The supernatant was discarded. The pellet was dried.
12. The pellet was washed with 70% ethanol and centrifuged again at 15000xg for 10 min. at 4°C. The pellet was air dried for 10min. and then redissolved in microinjection buffer (0.2mM NaPi pH 7.8, 10mM KCl).

Next, the DNA yield was estimated by comparing to the 1kb DNA ladder (by BioLabs inc.) by gel electrophoresis.

1. Agarose gel 1,1% was prepared.
2. The 1kb standard was loaded on the gel and compared to the plasmid reaction (1µl plasmid+2 µl dye (1:6 diluted, as provided by kit) + 10 µl ddH2O.
3. 100V was applied for electrophoresis.

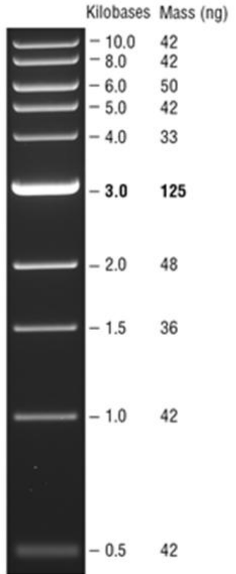


Fig. 20: The 1kb DNA ladder, as provided by BioLabs (www.neb.com)

As shown in fig. 20, the band for *hMARK2*-plasmid corresponds to 3,0kb which equals 125ng. Since the plasmid band is twice as thick and bright as in the ladder, a yield of approx. 250ng was estimated.

If 250ng are contained in 1 µl, 7.5 µg are contained in the 30 µl sample. For later insertion into pUAST 2 µg of the plasmid was requested. The size of the *hMARK2* containing plasmid (5kb) is approx. 1,5x the size of the *hMARK2* fragment (3,4kb). Therefore, for 2 µg fragment 12 µl of the plasmid solution was needed. (3 µg/ x µl=7.5 µg/30 µl).

B.:

Next, the 2,7kb *hMARK2* containing segment of the *hMARK2* plasmid had to be isolated and later inserted into compatible binding sites of the digested pUAST. Since enzyme restriction sites of the *hMARK2* fragment and the pUAST insert are not compatible, the *hMARK* plasmid was digested in two sequential digests.

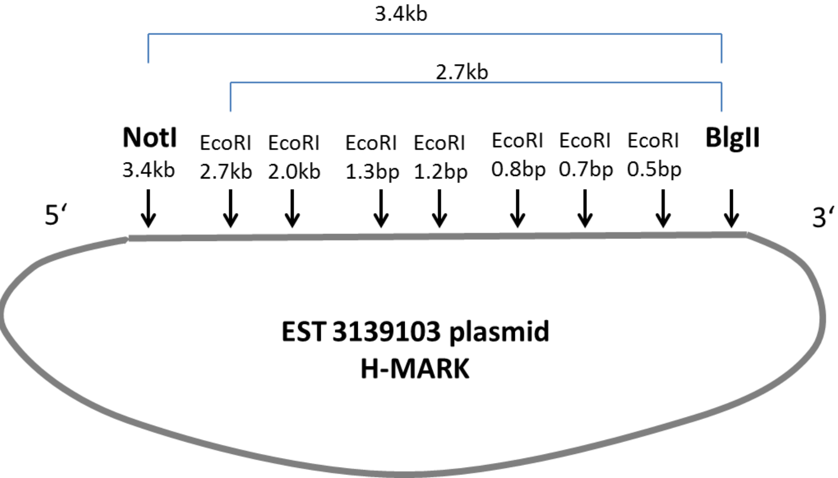


Fig.21: Map of est 3139103, the plasmid containing human *MARK2* with various restriction enzyme binding sites.

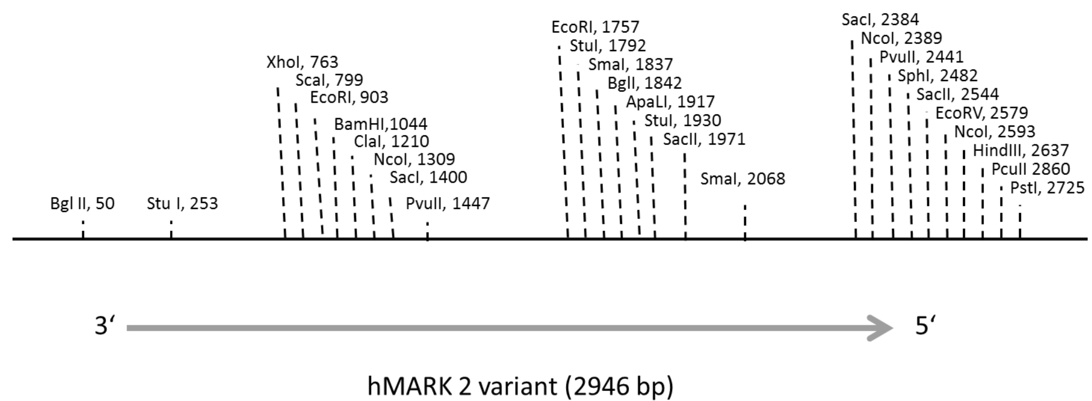


Fig. 22: Restriction map of the *hMARK2* gene

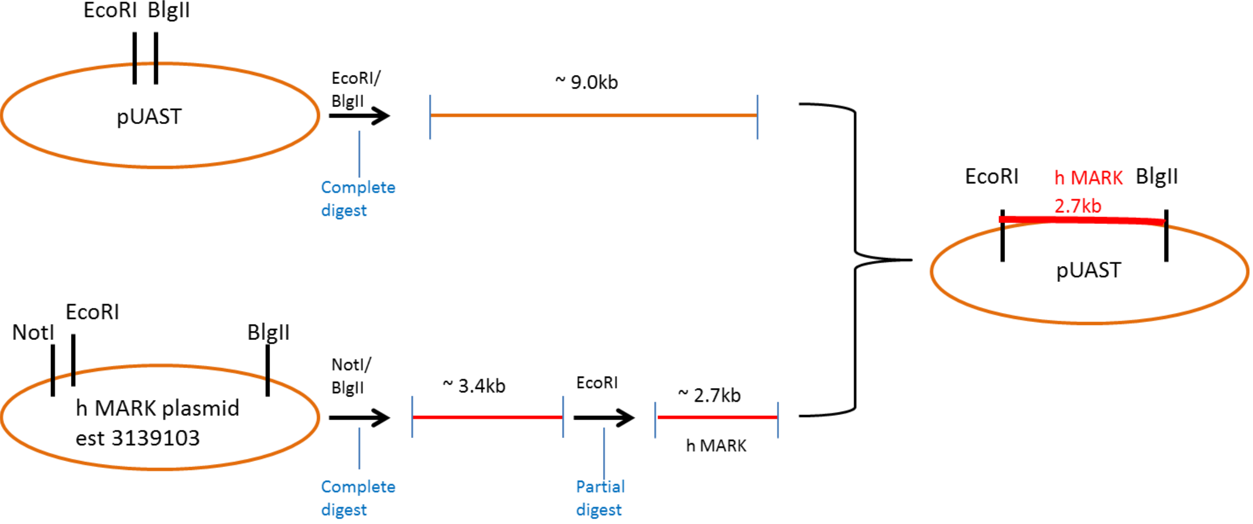


Fig.23: Concept of cloning *hMARK* into pUAST

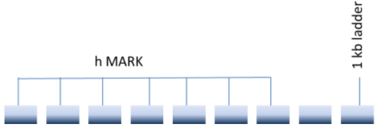
To isolate the 3.4kb fragment the first digest was prepared as follows:

12µl plasmid
+ 3µl Not I
+ 3µl Blg II
+ 10µl buffer 3
+ 72µl ddH2O

100µl end volume

This reaction was incubated at 37°C for 3hrs.

After incubation, an electrophoresis gel was run to isolate the 3.4kb fragment as described above.



Arrangement of the gel

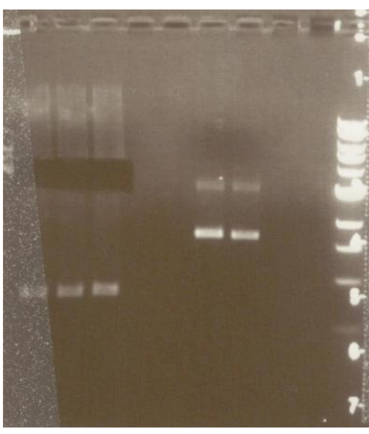


Fig.24: Southern blot after an overnight digestion of *hMARK2* with Not I and Bgl II to isolate the 3.4kb fragment. The gel fragment is already being cut.

C.:

Next the 3.4kb fragment was cut out and DNA was extracted using the QIAGEN "Quick Gel Extraction Kit Protocol".

1. First, the DNA fragment was cut out from the agarose gel with a clean scalpel.
2. The slice was weighed, 3 volumes of buffer QG were added to one volume of gel.
3. The mix was incubated for 10 min at 50°C until completely dissolved, during this time, it was vortexed every 2 min..
4. One gel volume isopropanol was added to the sample and mixed.
5. The solution was then applied to a QIAquick spin column and centrifuged for 1 min. for DNA binding.
6. The flow through was discarded. DNA was washed by adding 750µl PE to the column and centrifuging for 1 min..
7. The flow through was discarded, the DNA-containing column was centrifuged again for 1 min at 17000g.
8. The column was placed into a new tube. To elute DNA, 30µl of EB (elution buffer, 10mM Tris-Cl, pH 8.5) was added. This was left to stand for 1 min. to elute and then the tube was centrifuged for 1 min.
9. The DNA eluate was stored at -20°C.

To isolate the 2.7 fragment, the second digest was prepared as follows:

30µl plasmid 3.4kb
+ 1µl EcoR I
+ 5µl buffer EcoRI
+ 14µl ddH2O

50µl end volume

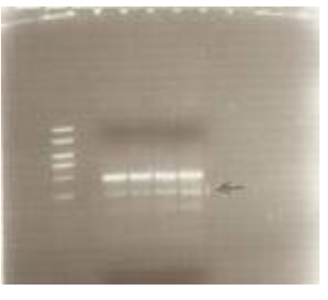


Fig. 25: Southern blot after second digestion of the 3.4kb fragment with Eco RI to isolate the 2.7kb fragment. a) before, b) after cutting of gel.

This reaction was incubated for 16min at 37°C for partial digestion. Again a gel was run and the 2.7 kb fragment cut out. The RNA was extracted again using the QIAGEN "Quick Gel Extraction Kit Protocol" (see above).

Next the pUAST was digested with BlgII and EcoI.

5µl plasmid pUASt plasmid
+ 1µl EcoR I
+ 1µl BlgII
+ 5µl buffer EcoRI
+ 37µl ddH2O

49µl end volume
+ 1µl CIP added after 2hrs

50µl end volume

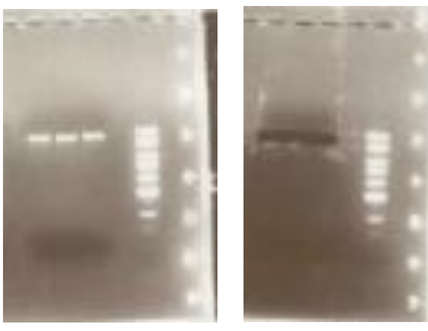


Fig.:26 Southern blot digestion of pUAST with Eco RI and Bgl II to linearize plasmid and to isolate the 9kb fragment for respective insertion of 2.7kb fragment of *hMARK2*. a) before, b) after cutting of gel.

The 49µl reaction was incubated at 37°C. After 2hrs, Calf Intestine Phosphatase (CIP, an alkaline phosphatase to catalyze the hydrolysis of phosphate groups,) was addedand incubated for 2 more hrs.

Again a gel was run, the 9kb fragment cut out and the RNA was extracted using the QIAGEN “Quick Gel Extraction Kit Protocol” (see above).

D.:

For ligation of the 2.7 kb fragment of *hMARK2* into pUAST the following ligation reaction was set up:

2µl T4-DNA ligase buffer
+ 1µl T4 DNA ligase
+ 2µl pUAST after digest
+ 10µl h MARK 2.7kb after digests
+ 5µl ddH2O

20µl end volume

This reaction was incubated o.n. at 14°C (using the PCR machine) and then frozen the next day at 20°C for transformation of chemically competent bacteria. An aliquot of the clone was taken and send for sequencing to the DNA sequencing center at Stanford (to Nahid Madani at the Foothill Research Center) to confirm correct insertion and sequence.

E.:

Next, the *hMARK2*/ pUAST clone was transformed into chemically competent bacteria (TOP 10 cells, Invitrogen) for amplification.

1. The vial containing the ligation reaction was thawed on ice and centrifuged briefly.
2. One 50µl vial of One Shot cells was thawed on ice.
3. 1µl of the ligation reaction was added to the One Shot cells and mixed by tapping gently.
4. The vial was then incubated for 30min on ice and then for 30sec. in 42°C and then put back on ice.
5. 250µl of pre-warmed S.O.C medium was added to the vial.
6. The vial was then shaken at 225rpm in a 37°C shaking incubator.
7. 10 µl of the transformation mix was then spread on a LB agar plate and incubated at 37°C o.n. and then put into the 4°C fridge.
8. Colonies could then be picked for amplification and plasmid isolation (as described above under section "A.") for later microinjection.

F.:

2.2.1.2. The embryo injection

2.2.1.2.1. DNA preparation for embryo injection

9µg pUAST plasmid containing the *hMARK2* clone was mixed with 3µg pΔ2-3 transposase. The plasmid mix was then coated onto a Quiagen plasmid preparation spin column and spun for 1 min. For purification it was then washed twice with PBS buffer and afterwards washed twice with PE buffer. The injection mix was eluted with 10µl ddH2O and then eluted again with 10µl of 2x injection buffer (0,2mM NaPi pH 7.8, 10mM KCl). The injection mix was then stored on ice, since it is not very stable at R.T.

2.2.1.2.2. Embryo preparation

4-5 day old virgin wild type flies (w- strain) were put to mate in 5-10 bottles which had been coated with egg lay medium and into which yeast paste had been added and placed in the 25°C incubator. Male: female ratio was 1:2-3; 10-15 flies per bottle. After 14-18hrs of mating, the bottles were emptied and the flies were transferred to new bottles coated with egg lay medium. The first bottles were discarded. The flies were then transferred into new bottles every 30 min. During this time, approximately 100 eggs

were laid per bottle. Each 30 min. embryos are immediately transferred to the microinjection room (22°C) for transgene injection.

2.2.1.2.3. Microinjection

Embryos were placed at the edge of a microscope coverslip, which has been coated with embryo glue. The embryos were arranged in a row of approx. 50 per plate, each posterior end facing the needle (see figs. 28 and 29). The posterior contains the germ cells and is broader than the anterior. Embryos were covered in 10S oil. Injection needles of the adequate diameter had been prepared beforehand using a needle puller (see fig. 27). The needle tip was positioned using the micrometer until its tip touched the oil. By shifting the microinjection table the embryos were approximated the needle so that the needle penetrated into their posterior end (i.e. less than 1/3 of the embryo length). The injection of the plasmid was released with approx.. 500 HPa. Cellularized embryos were eliminated.

Fig. 27: Needle puller for microinjection

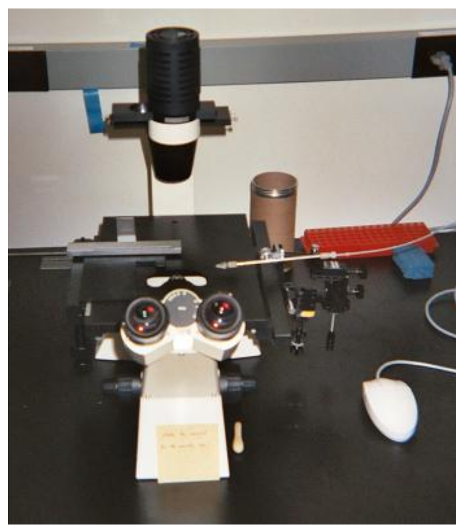


Fig28: Microinjection machine for transfection of *Drosophila* embryos

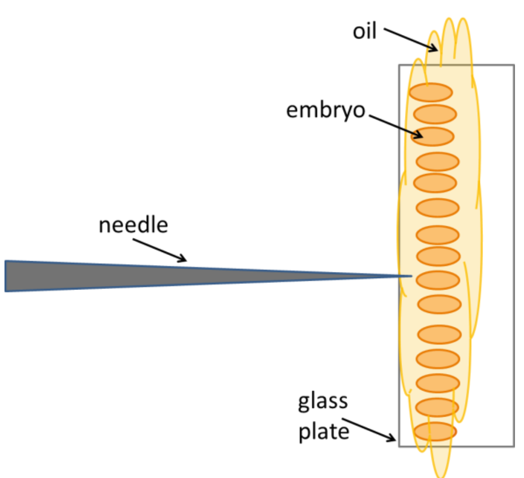


Fig. 29: Microinjection into *Drosophila* embryos, schematic

G.:

2.2.1.3. Establishing the transgenic line

After injection embryos were placed into the 18°C incubator for 48hrs. Then the larvae were collected and each larva was placed into an individual vial for approx. 2 weeks until hatching at R.T. The hatched flies were the F0 generation. They were all white eyed, since the transgene was inserted into their germ cells. Now crosses were made: Each F0 female was crossed to 2-3 w- males, and each F0 male was crossed to 2-3 w- virgin females. The progeny (F1) was then scored for eye color. If all F1 in the vial were white eyed, there would be no mutants. If the F1 showed various eye colors with

different intensities of red, multiple insertions would have occurred. If all F1 displayed the same (light red) eye color, the vials would contain a single transgenic line. These flies were chosen to be crossed to balancer chromosome flies.

In the F1 the transgene could be inserted either into chromosome X, II or III. (Chromosome IV is very small and insertions never happen here.) For crossing to F1 we used the balancer on the third chromosome TM2 (**T**hird **M**ultiple inverted 2) also carrying the dominant marker Sb (= stubbly bristles). In the balancing chromosome-containing fly, a second marker was used on the second chromosome: Cy (curly wing), resulting in the genotype: X; Cy; TM2/Sb. Depending on the phenotype of the F2 those flies containing balancer and transgene could be identified (e.g. bristle/ no bristle, curly wing/ straight wing, red eye/ white eye etc.). Within the F2 several crosses were made again. In the F3 or F4 the chromosomal location of the transgene could be identified according to the phenotype. For the *DJ-1* RNAi transgenic fly, we isolated a strain containing the transgene on chromosome III, with the balancer also on chromosome III, resulting in a red eye, stubbly bristle, straight wing mutant fly of the genotype UAS- IR-6646-6.3/TM2. This procedure was done for the IR-*DJ-1A* (IR6646-6.3) transgene, the IR *DJ-1B* (IR 1349) transgene, the UAS *DJ-1A* transgene the UAS *DJ-1B* transgene and the UAS *hDJ-1* transgene (i.e. overexpression of human *DJ-1*).

Fig.30: Flies exhibiting dark cuticle/light cuticle used as marker

Fig.31: Flies exhibiting white eye/red eye used as marker

2.2.2. Methods used in the neurodegeneration study

2.2.2.1 Drosophila Genetics and Molecular Biology

Female adult *Drosophila* of the genotypes Ddc-GAL4>*DJ-1A* RNAi and Ddc-GAL4/+ were used in all experiments. The Ddc-GAL4 line and the w- wild type stock were obtained from the Bloomington *Drosophila* Stock Center. Genomic DNA/cDNA constructs were generated to create UAS-*DJ-1A* RNAi transgenics, as described earlier [220]. Analysis of the transgenes was performed as described [133, 157].

2.2.2.2. Histology and Immunohistochemistry

For DA-neuron count, the heads of 1 day- and 25 day-old adult flies were dissected, formaldehyde-fixed and paraffin-embedded as described in the protocol below.

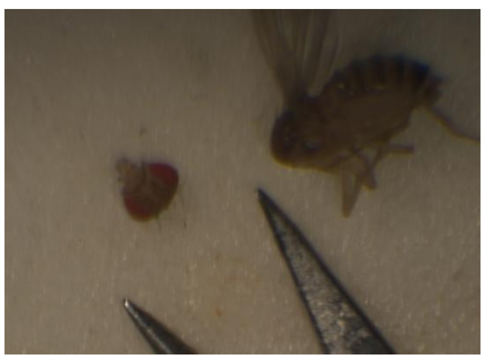


Fig.32: The heads of the flies are cut off using micro-scissors under mild CO_2 anesthesia

1. **Cut Heads** off under CO_2 anesthesia.

2. **Fixation**:

   FA (4%) / PBS-Triton (0,1%) 150 min- 240 min. at 4°C

3. **Dehydration**:

PBS-Triton (0,1%)	10(-20) min	R.T.
50% EtOH / PBT	10 min	R.T.

70% EtOH / PBT	10 min	R.T
90% EtOH / PBT	10 min	R.T
100% EtOH	10 min	R.T
100% EtOH	10 min	R.T
100% Xylenes	10 min	R.T
100% Xylenes	10 min	R.T

4. **Embedding**:

   Xylenes:Paraffin (1:1) 60 min 37°C

   Paraffin 60 min 60°C

   Paraffin 60 min 60°C

5. **Molding**

   Anterior facing up in paraffin

6. **Mounting**
7. **Microtome sectioning** 8 - 10 μm:

   Serial 10 μm frontal sections were cut from anterior to posterior to include the entire brain.

8. **Rehydration**

100% Xylenes	10 min R.T
100% Xylenes	10 min R.T
100% EtOH	10 min R.T
100% EtOH	10 min R.T
90% EtOH / PBT	10 min R.T
70% EtOH / PBT	10 min R.T

50% EtOH / PBT	10 min R.T
PBS-Tween (0,1%)	10 min R.T.

Anti-TH polyclonal antibody (1:100, Pel Freez Biologicals) served as primary antibody. Incubation was carried out overnight at 4°C. For subsequent processing, the Vectastain Universal Elite ABC Kit (Vector Laboratories) was used as described below:

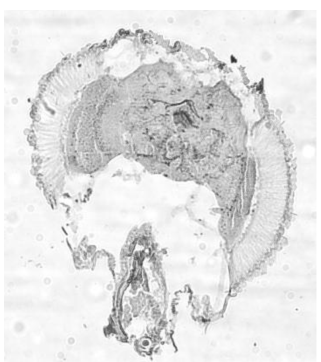


Fig.33: Complete section of fly head after paraffin embedding. Anterior shows mouthparts, lateral compound eyes. In the posterior, TH^+ neurons and great interneuron commissure can be detected.

9. Sections were incubated for 20 min. with diluted normal horse serum.
10. Excess serum was blotted from sections.
11. Sections were incubated with primary antibody: Rabbit α TH (peel freez) prepared in 2,5% normal horse serum 1:50. Incubate 1 h at room temperature, then o.n. at 4C, next morning again 1h at room temperature.
12. Sections were washed 3x for 5-10 min. in 1x PBS-Twin.
13. Sections were incubated for 45 min. with diluted biotinylated universal secondary antibody.
14. Sections were washed 3x for 5 min. in 1x PBS-Twin.
15. Sections were incubated for 90 min. with R.T.U. Vectastain ABC Reagent.
16. Sections were washed 3x for 5 min. in 1x PBS-Twin.

Immunopositive cells of the dorsomedial cluster at the level of the giant interneuron commissure (as described in the chapter "Dopamine containing neurons in *Drosophila* melanogaster" of the Introduction) were counted in all sections, using a light microscope (Nikon Eclipse 3000, Japan). Similar methods have been used previously to analyze dopaminergic degeneration in various *Drosophila* models of PD [133, 150].

2.2.3. Methods used in the life span assays

2.2.3.1. Drosophila Genetics

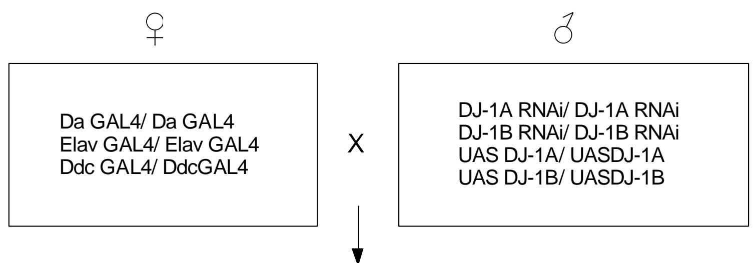
DJ-1 function was either overexpressed or downregulated in 3 specific body tissues. Three kinds of GAL-4 drivers were employed to induce RNAi or high level expression after *P* element insertion, respectively:

1. ***Da-GAL4 (Daughterless-GAL-4)*** which induces UAS/RNAi expression ubiquitously in all fly tissue,
2. ***Elav-GAL-4***, which induces UAS/RNAi panneuronally, and
3. ***Ddc-GAL- 4 (DOPA-Decarboxylase-GAL4)***, which induces UAS/RNAi expression in dopaminergic and serotonergic neurons exclusively

The DaGAL4, DdcGAL4, Elav GAL4 and the white minus wild-type streak were obtained from the Bloomingdale *Drosophila* Stock Center. Genomic DNA/cDNA constructs were generated to create UAS-ds-*DJ-1* transgenics as described earlier [220] and cloning of amplified full-length cDNA was performed to generate UAS-*DJ-1* transgenics [133].

RT/PCR and Western Blot analysis of the constructs was performed as described [133, 157]. The same set of experiments was performed for each homologue of the *DJ-1* gene separately.

Experimental flies were obtained from crosses of virgin females from the specified GAL 4 strains with males of each *DJ-1* responder transgene strain:



Da GAL4>DJ-1A RNAi; Da GAL4>DJ-1B RNAi; Da GAL4> UAS DJ-1A; Da GAL4>UAS DJ-1B
Elav GAL4>DJ-1A RNAi; Elav GAL4>DJ-1B RNAi; Elav GAL4> UAS DJ-1A; Elav GAL4>UAS DJ-1B
Ddc GAL4>DJ-1A RNAi; Ddc GAL4>DJ-1B RNAi; Ddc GAL4> UAS DJ-1A; Ddc GAL4>UAS DJ-1B

2.2.3.2. Stocks

Crosses (10 healthy young female virgins x 10 healthy young male flies) were carried out in 325ml fly bottles containing approximately 40ml of standard fly food. Flies were allowed to lay eggs within a 48hr period; then bottles were emptied, so that eggs could develop in non crowded conditions.

Bottles of all crosses were transferred to a 25°C incubator with constant 5% humidity and flies were allowed to develop.

After eclosion, all flies were held at 25C.

Of the F1 progenies, virgin females and males were collected within a 24hr period of eclosion and passed to standard vials (~ 40ml) containing ~ 7ml standard fly food, at initial densities of 25 to 35 flies per vial.

Females and males were henceforth kept separately. All vials containing adult flies were kept at 25°C in constant 5% humidity.

Flies were transferred to fresh vials every 2-3 days and the number of dead flies was scored until all flies were dead.

For each genotype and gender between 80 and 110 flies were investigated.

(Approximately 2500 flies in total were included in the study).

2.2.3.3. Data analysis

All data were analysed using Microsoft Excel and GraphPad InStat 3 software.

Life spans were plotted to construct survivor curves and box and whiskers diagrams.

The mean (50%) and maximum (90%) lifespans were calculated as the age in days required to reach 50% and 10% survivorship, respectively. Kaplan-Meyer analysis of the results was performed using semi-parametric log-rank-tests. Flies that escaped during vial changes were kept track of through log rank analysis. However the number of escaping flies was negligible.

2.2.4. Methods used in the toxicology study

2.2.4.1. Drosophila stocks

For methods of setting up the necessary crosses and fly culturing, see the methods section of the study on aging, as those are analogous.

For the toxicology study, only female virgins were used. The pan-cellular driver daughterless (Da) was used in all experiments. Since it was suggested by the results of the study on aging that the *DJ-1A* homologue displays a stronger phenotype, *DJ-1A* mutants were used for all following experiments. Since it has been shown previously that human *DJ-1* protein was able to neutralize H_2O_2 in vitro, a mutant overexpressing *hDJ-1* was also included into the assay. Thus, the following genotypes were produced:

1) Da GAL4>*DJ-1A* RNAi (*DJ-1A* downregulation phenotype)
2) Da GAL4>UAS *DJ-1A* (*DJ-1A* overexpression phenotype)
3) Da GAL4>UAS *hDJ-1* (*hDJ-1* overexpression phenotype)
4) Da GAL4> *w-* (control fly, driving the wild-type gene)

2.2.4.2. Preparation of toxins

As toxins and ROS (H_2O_2), a catalase inhibitor (3-AT) and two metal compounds were chosen. Suitable toxin concentrations were estimated by serial dilutions (first by factor 100, then by factor 10, then smaller, until concentrations that would not lead to immediate lethality were found). Toxins were dissolved in 2% sucrose solution, which flies like to feed on.

2.2.4.3. Oxidative Stress Assay

For oxidative stress assay, flies were kept in plastic vials with Kimwipe paper soaked in 1% H_2O_2, 100 mM 3-amino-triazole (3-AT), 0.03% Cu_2SO_4 or 0.03% $MnCl_2$ in 2% sucrose solution (2 Kimwipes per vial) . The vials were kept at 25°C in a shielded box. Fresh sucrose/toxin solution was added to the paper daily with a syringe. Mortality was recorded every 12 h.

2.2.4.4. Statistics

Survival curves were plotted using Microsoft excel. For comparison of differences the time spans to reach 50% mortality were calculated using Graph Pad Instat software.

2.2.5. Methods used in the drug study

2.2.5.1. Drosophila Genetics and Molecular Biology

Female adult *Drosophila* of the genotypes Ddc GAL4>*DJ-1A* RNAi and DdcGAL4/+ were used in all experiments. The Ddc-GAL4 line and the white minus wild type stock were obtained from the Bloomingdale *Drosophila* Stock Center. Genomic DNA/cDNA constructs were generated to create UAS-*DJ-1A* RNAi transgenics, as described earlier [220]. Analysis of the transgenes was performed as described [133, 157].

2.2.5.2. Animals and Drug Feeding

Drugs, dissolved in 10% DSMO, were mixed into wet yeast paste. Drug-containing yeast paste was offered together with standard fly food in standard vials containing approximately 10 flies each. All drug preparations were mixed freshly every 2-3 days. Flies were administered celastrol (1 µg/ml, 5 µg/ml, and 20 µg/ml), minocycline (25 mg/ml, 50 mg/ml), NBQX (30 mg/ml), coenzyme Q10 (100 mg/ml), or yeast paste alone as no-treatment control. Drug feeding was carried out at 25°C for 25 days from day 1 after eclosion. Ddc-GAL4>*DJ-1A* RNAi flies and DdcGAL4/+ control flies were treated. Approximately 21-24 flies for each drug and genotype were processed for HPLC DA measurement after 10 days of treatment.

10- 25 heads per group were evaluated by immunohistochemistry after 25 days of treatment.

Therapeutic doses were based on dose-finding studies, which compared known oral doses in mammals based on weight, metabolic rate etc. and parallels to substances previously administered to the fly (e.g. cocaine and biogenic amines) [222-227].

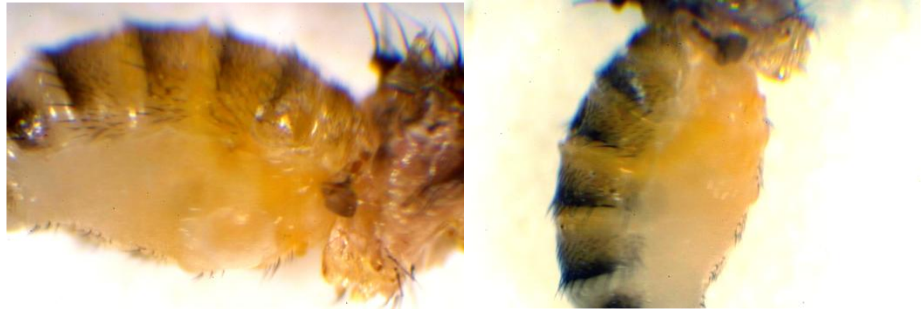


Fig.34: Photograph demonstrating that the drugs are indeed taken up by the fly
Minocycline, which is a strong yellow chromophore, can be detected through the chitin belly of the fly. Wings and extremities have been removed for better visualisation. Light microscopy.

2.2.5.3. Histology and Immunohistochemistry

For DA-neuron count, the heads of 1-day and 25-day-old adult flies were dissected, formalin-fixed and paraffin embedded as described above under “methods used in the neurodegeneration study”. Serial 10 µm frontal sections were cut from anterior to posterior to include the entire brain. Anti-tyrosine-hydroxilase polyclonal antibody (1:100, Pel Freez Biologicals) served as primary antibody. Incubation was carried out

overnight at 4°C. For subsequent processing the Vectastain Universal Elite ABC Kit (Vector Laboratories) was used. Immunopositive cells of the dorsomedial (DM) cluster at the level of the giant interneuron commissure were counted in all sections using a light microscope (Nikon Eclipse 3000, Japan). Similar methods have been used previously to analyze dopaminergic degeneration in various *Drosophila* models of PD [133, 150, 228].

2.2.5.4. HPLC Measurement of Dopamine

For sample preparation, 10-day-old female fly heads were cut off under mild CO_2 anaesthesia and quickly homogenized in chilled 0.1 M perchloric acid using a motorized hand held tissue homogenizer. 3 heads were used per 50 µl perchloric acid sample. Homogenates were frozen immediately on dry ice and stored at – 80 degrees prior to HPLC measurement. 21-24 fly heads were measured per genotype and per experiment.

HPLC was performed in a facility of the F. Beal laboratory at the Weil Cornell University in New York. For HPLC frozen samples were shipped to NYC.

For measurement of dopamine (DA), a modified HPLC protocol for catecholamine measurement was used as described earlier [229].The chilled homogenates of fly heads were centrifuged and 10 µl supernatant fluid was eluted through an 80 x 4.6 mm C18 column (ESA Inc, Chelmsford, MA, USA). The mobile phase contained 75 mM of NaH2PO4, 1.5 mM Octanesulfonic acid (OSA), 5% acetonitrile (pH 3.0). For coulometric electrochemical detection, a two-channel Coulochem II electrochemical detector (ESA, Inc) was used. The flow rate was 1 ml/min. Concentrations of DA were expressed as nanograms per milliliter (ng/ml).

2.2.5.5. Data Analysis

All data were analyzed using Microsoft Excel and Graph Pad InStat3 software.

The effects of minocycline, celastrol, NBQX and coenzyme Q10 ingestion on neuronal survival within the DMC of sectioned whole-mount fly brains were analyzed with a one-way analysis of variance (ANOVA). Multiple pair-wise comparisons of the means were

performed using Dunnett's Multiple Comparison Tests: The effects of the respective drugs on dopamine concentration within the fly head were also analyzed with a one-way analysis of variance (ANOVA) and the Dunnett's Multiple Comparison as post-test. Treatment differences for all data were considered statistically significant at P<0.05. For both studies, mean numbers of surviving neurons or dopamine content, respectively, were plotted as columns. Error bars indicate the standard deviation of the mean (SD). Number of asterisks indicates statistical significance. ***, P<0.0001; ** P<0.01; * P< 0.05; no asterisk, P>0.05.

3. RESULTS

3.1. Results of the neurodegeneration assay

3.1.1. Age-dependent loss of dopaminergic neurons in Ddc-Gal4>*DJ-1A* RNAi flies

The main pathological hallmark of PD is an age dependent degeneration of dopaminergic neurons. It was determined if the *Drosophila DJ-1* model could reproduce a phenotype of dopaminergic neuronal degeneration.

The age-dependent loss of dopaminergic neurons in the *DJ-1A*-deficient fly as compared to w^--controls was described using the DA-specific driver dopa decarboxylase (Ddc). A stronger neuronal degeneration in the Ddc-Gal4 driven *DJ-1A* RNAi flies was confirmed. Young, 1-day-old *DJ-1A* RNAi flies and 1-day-old control flies (Ddc-Gal4/+) showed no significant difference in their numbers of TH-positive (TH^+) neurons within the DMC (22.6 ± 1.98 in *DJ-1A* RNAi flies, vs. 21.20 ± 1.97 in controls, P= 0.98) (fig.35, fig. 36). With age, both *DJ-1A* RNAi flies and control flies showed a reduction in TH^+ neurons (fig. 35, fig. 36). The age-dependent loss of TH^+ neurons was more substantial when *DJ-1A* was deficient: *DJ-1A* RNAi flies aged 25 days sustained a 55.3% decrease of TH^+ neurons within the DMC, as compared to 1-day-old flies (22.6± 1.98 vs. 10.1± 1.31), whereas 25-day-old control flies only sustained a 24.3% loss compared to 1-day-old flies (21.2 ± 1.97 vs. 16.1 ± 1.95). Thus, at 25 days of age, Ddc-Gal4>*DJ-1A* RNAi flies contained 62.8% of the DMC TH^+ neurons of age matched controls (P < 0.0001 in ANOVA with Dunnett's post test).

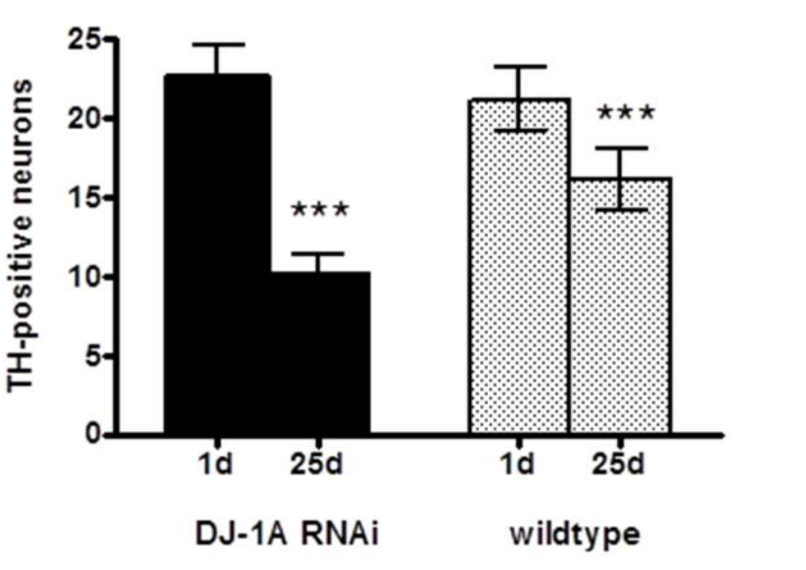


Fig.35: Number of dopaminergic neurons, as counted by TH- immunostaining, within the DM cluster of wildtype flies and *DJ-1A* deficient flies, driven with Ddc-GAL4, 1-day-old and 25-day-old flies.

Sections of 15 to 25 heads for each genotype and age were evaluated. Columns depict mean with standard deviation of the mean (SD)***: p< 0.0001 in student's t-tests, indicates statistical difference in cell count.

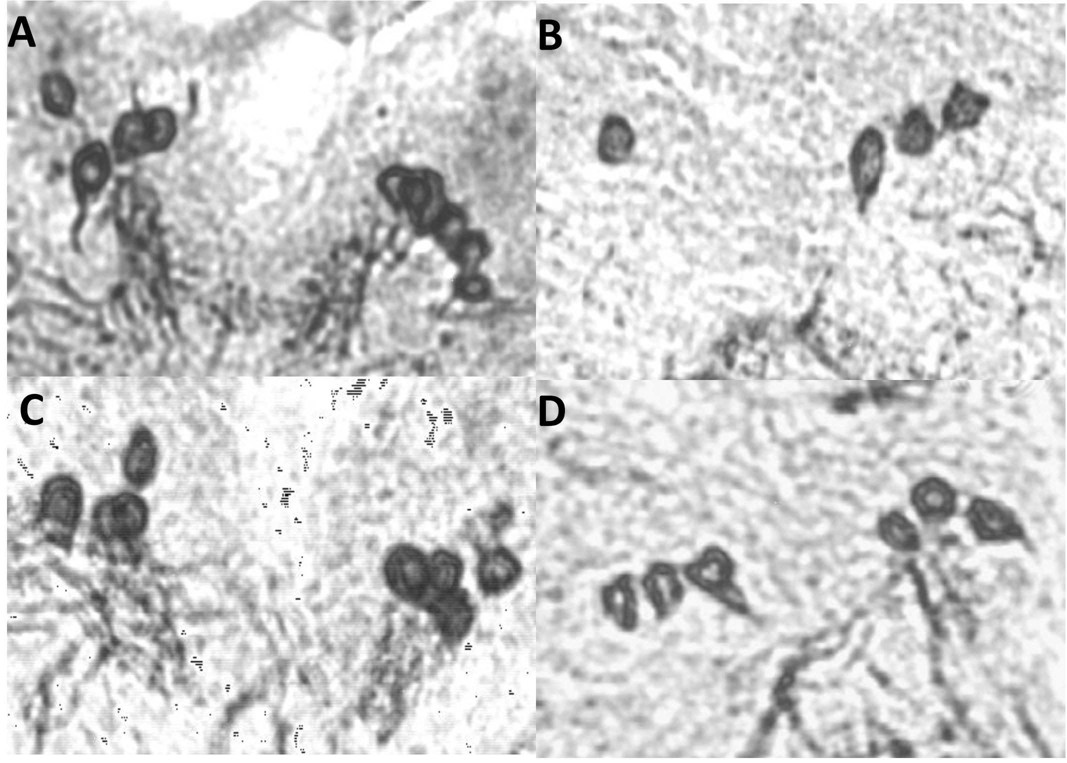


Fig.36: Number of TH-positive neurons in the DM cluster of *DJ-1A* RNAi flies and wildtype flies. Age-dependent loss. A+B: *DJ-1A* RNAi flies, C+D: wildtype flies. A+ C: TH-immunostaining of 1-day-old flies, B+ D: TH-immunostaining of 25-day-old flies.

3.2. Results of the life span study

DJ-1 exhibits differential effects on aging and longevity in different body tissues of *Drosophila* melanogaster

Since aging is the first and foremost risk factor of developing sporadic PD and PD is associated with a reduction of life expectancy, the effect of *DJ-1* deficiency and overexpression was investigated in the fly by life span assays. Gene expression was targeted at specific body tissues using different designated drivers.

3.2.1. The effect of DJ-1 *downregulation on life span*

3.2.1.1. Using the daughterless-GAL-4-system: global downregulation in all body cells

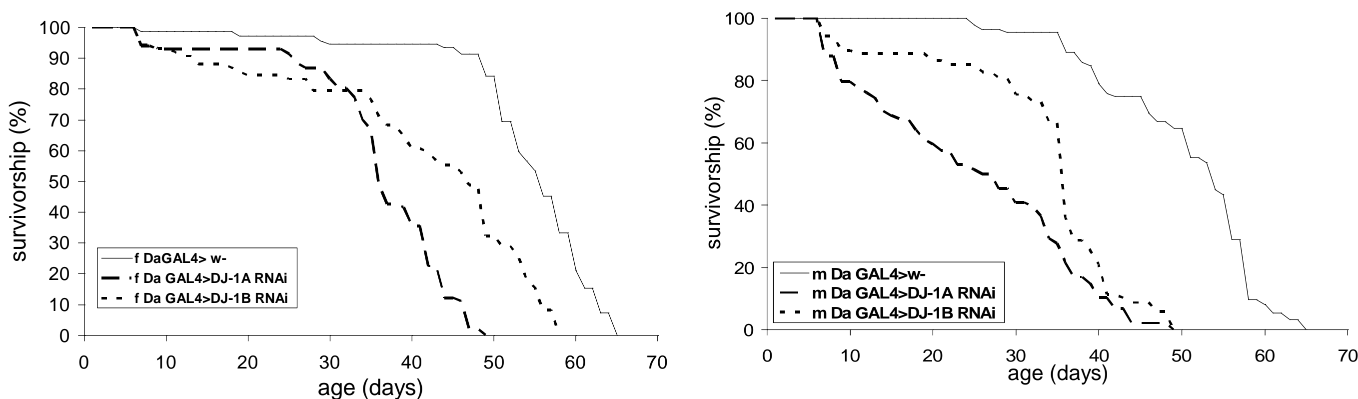


Fig. 37: Survival curves of *DJ-1A.* or *B*-deficient transgenic flies (Da>*DJ-1A*, Da>*DJ-1B*) compared to wildtype flies (Da>w-). *DJ-1A* or *B* was downregulated ubiquitously in experimental flies using the daughterless (Da) driver. Curves were generated using adult females (left) and males (right). In *DJ-1A*-reduced flies, the mean life span was 34% shorter in females ($p < 0.0001$) and 51% shorter in males ($p < 0.0001$). In *DJ-1B*-reduced flies, the mean life span was 24% shorter in females ($p < 0.0001$) and 35% shorter in males ($p < 0.0001$).

In the first experiment, employing transcript expression in all body tissue, both *DJ-1A* and *DJ-1B*-downregulated flies showed a statistically significant decrease in life span (see fig. 37).

Mean life spans in *DJ-1A* RNAi females and males were 35.26 days and 24.17 days, respectively, while the mean life spans of the control were 53.34 days in females and 49.40 days in males, respectively. Thus, loss of *DJ-1A* function reduced life span to 66.1 % of the control in females and 48.9% in males, respectively.

In *DJ-1B* downregulated flies life spans were reduced to 40.36 days in females and 32.26 days in males, equalling 75.7% and 65.3%, respectively, of the controls.

3.2.1.2. *Using the Ddc-GAL-4-system, downregulation of DJ-1 in dopaminergic neurons*

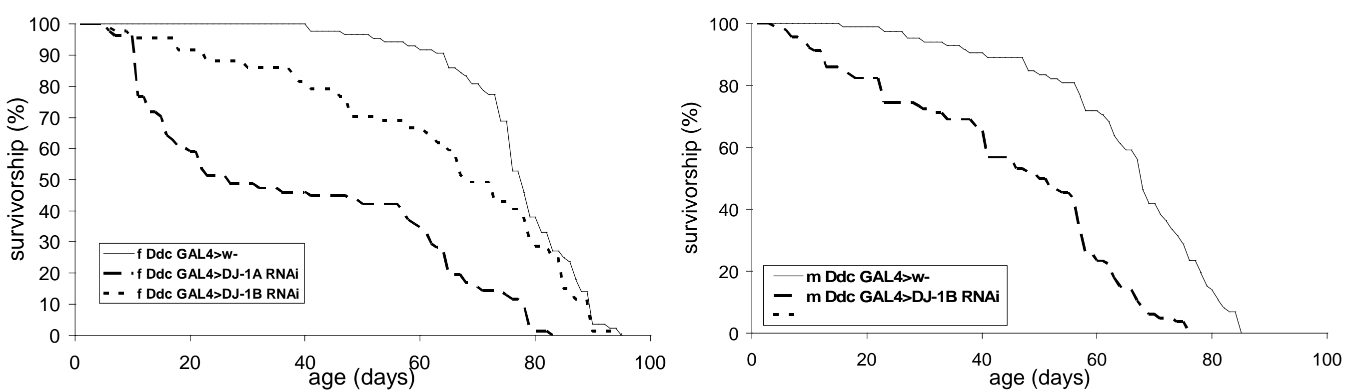


Fig. 38: Survival curves of transgenic flies, lacking *DJ-1A* or *B* in their monoaminergic neurons for dopamine and serotonin (Ddc>*DJ-1A*, Ddc>*DJ-1B*), compared to wildtype flies (Ddc> *w-*).

Curves were generated using adult females (left) and males (right). In flies with *DJ-1A*-reduced dopaminergic and serotonergic neurons, the mean life span was 50% shorter in females (p< 0.0001). In males, *DJ-1A* downregulation resulted in a lethal phenotype (no curve in D). Flies carrying the *DJ-1B* downregulation in serotonergic and dopaminergic neurons exhibit mean life spans shortened by 18% in females (p< 0.0001) and by 32% in males (p< 0.0001).

When downregulating *DJ-1* function in dopaminergic and serotonergic neurons exclusively, flies exhibit a statistically significant reduction in life span when compared with controls, despite the tissue selectivity of the driver employed (Ddc). In males, *DJ-1A* downregulation in Ddc neurons results in a semi-lethal phenotype at 25°C. In female Ddc> *DJ-1A* RNAi flies mean life span was 37.98 days, while that in the controls 76.23 days.

Hence, *DJ-1A* downregulation in a small subset of neurons is able to produce a reduction of life expectancy to 50% of the control. *DJ-1B* downregulation in Ddc neurons leads to a reduction from 76.23 days to 62.5 days of lifespan in females (equalling 82.0%) and from 64.23 days to 43.64 days (equalling 67.9%) in males. Hence, the effect of *DJ-1B* downregulation in Ddc neurons was smaller, but also significant.

Different shapes of survival curves were observed for the two lines Da > *DJ-1A* RNAi and Ddc> *DJ-1A* RNAi. In flies containing the Ddc-driven construct survivorship was decreased greatly in relatively young flies, indicated by the steepest decline in the survivorship curve during the ages between 10 and 30 days (see fig. 38). 50% of all flies (T50) were dead after only 25 days (equalling 33% of the respective control T50), while maximum life span was still around 80 days.

In flies containing the Da driven construct, the mortality in young flies was low, so that the first half of the curve very much resembles the control curve. (T50 in Da> *DJ-1A* RNAi flies was 54% of the respective control.) The shape of the two curves (see figs. 37 and 38) is then reversed with advancing ages of the flies. Here, the Ddc curve approaches the control curve again, while the Da curve shows its steepest decline. Maximum life span is greatly reduced in Da-driven flies while the effect of Ddc driving on maximum life span is not significant.

Overall, the effect of *DJ-1A* RNAi on life span was more significant in Ddc driven flies than in flies of the Da line. (Mean life span of Ddc> *DJ-1A* RNAi females was 50% of the respective controls, while the mean live span of Da driven females was only 66% of the controls in females). In addition, the semi-lethal phenotype we observed in Ddc> *DJ-1A* RNAi males did not occur in Da-driven males.

In contrast, the effect of *DJ-1B* on life span was weaker in the Ddc-line compared to Da-driven flies (in females a reduction to 82.0% versus 75.7%)

3.2.1.3. *Using the elav-GAL-4-system: panneuronal downregulation of DJ-1*

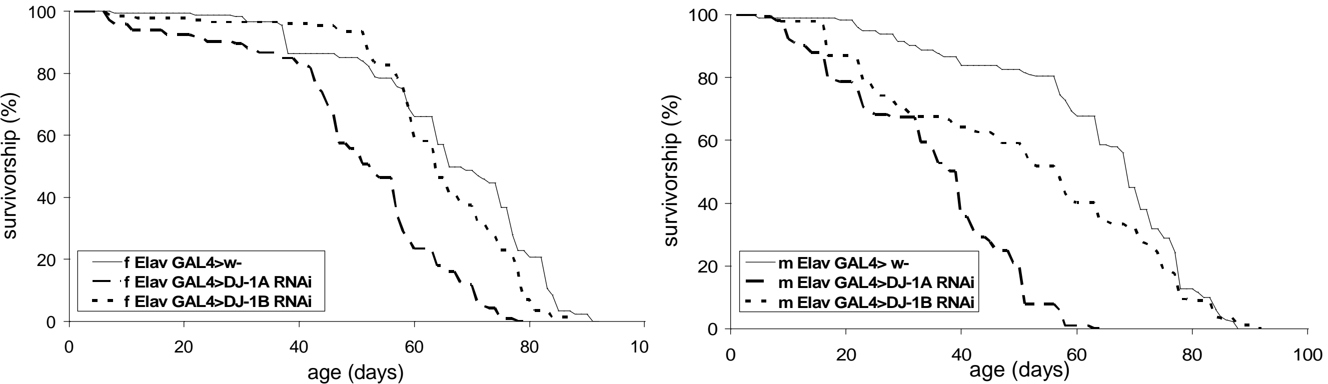


Fig. 39: Survival curves of transgenic flies, lacking *DJ-1A* or *B* in all their neurons (Elav>*DJ-1A*, Elav>*DJ-1B*), compared to wild-type flies (Elav> *w-*).

Curves were generated using adult females (left) and males (right). In flies with *DJ-1A* reduced neurons mean life span was 21% shorter in females (p< 0.0001) and 45% shorter in males (p< 0.0001). In flies with *DJ-1B* downregulated neurons the mean life spans are shortened by 11% (p> 0.5) in females (p< 0.0001) (E) and 24% in males (p< 0.0001).

When using the Elav GAL 4 system to activate (induce) *DJ-1* RNAi in all neurons, mean life spans were reduced from 62.04 to 49.17 days (reduction to 79.3%) in females, and from 61.39 to 33.75 days (reduction to 55.0%) in males, respectively. *DJ-1B* RNAi produced a reduction to 46.85 days (reduction to 76.3%) in males, while female Elav GAL 4> *DJ-1* RNAi life spans showed no significant difference compared with the control.

Overall, a rank order in the three different drivers' effect on life span was observed, with downregulation in Ddc neurons having the strongest effect, followed by ubiquitous downregulation in all body tissues, and with panneuronal downregulation exhibiting the weakest effect: (Ddc GAL 4 -> Da GAL 4 -> Elav GAL 4.)

3.2.2. *The effect of DJ-1 overexpression on life span*

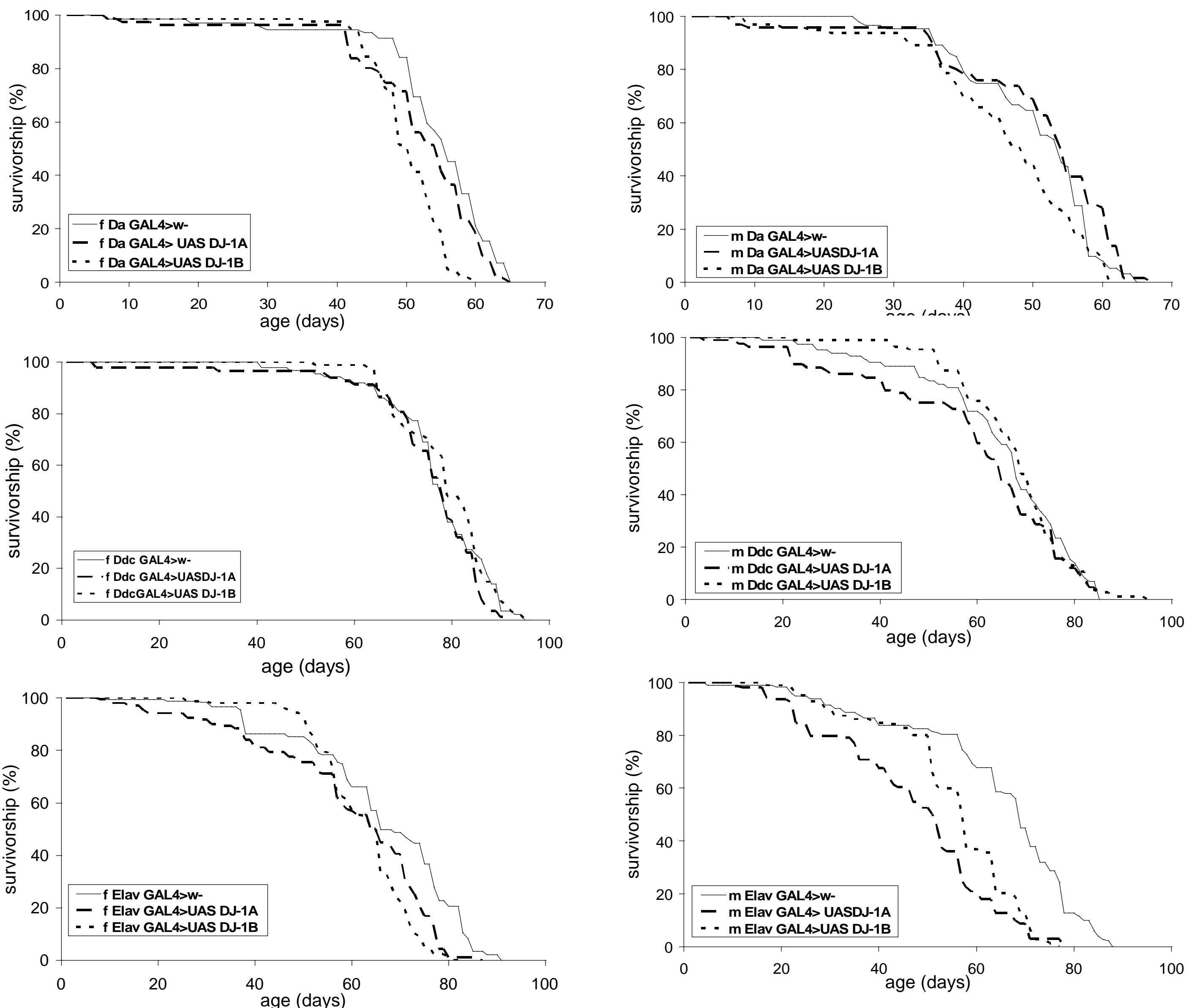


Fig. 40: Survival curves of *DJ-1A* or *B* overexpressing transgenic flies.

DJ-1 transcription is upregulated in different body tissues: Da (global overexpression); Ddc (overexpression in dopaminergic and serotonergic neurons); Elav (overexpression in all neuronal cells). Left side: females, right side: males.

Overexpression of *DJ-1* has no positive effect on mean life spans in any of the systems tested. Global overexpression of *DJ-1A* has no effect on lifespan in either gender (p> 0.05 for both genders.) Global overexpression of *DJ-1B* has a slightly negative effect on mean lifespan in both genders (reduction by 9% in females (p= 0.001) and 10% in males (p= 0.006)). Overexpression of either *DJ-1A* or *B* in monoaminergic neurons selectively has no effect on life span at all (all p-values for either gender > 0.05).

Overexpression of *DJ-1A* in all neurons leads to a certain reduction of life span in females and males by 11% (p= 0.06) and 19% (p< 0.0001). Overexpression of *DJ-1B* in neurons leads to a reduction of life span by 16% in males (p< 0.0001), while it has no effect on female mean life span (p<0.05). The experimental groups overexpressing *DJ-1* either ubiquitously (Da GAL 4) or selectively in dopaminergic neurons (Ddc GAL 4) exhibited no statistically significant prolongation in life span. In Elav driven flies a reduction in lifespan was observed in male Elav GAL 4> UAS *DJ-1A* flies to 71.4% of the control and in male Elav GAL 4> UAS *DJ-1B* flies to 83.6% of the control. This effect was only present in males, while females showed normal life span.

No extension of lifespan was observed in any of the UAS *DJ-1* genotypes.

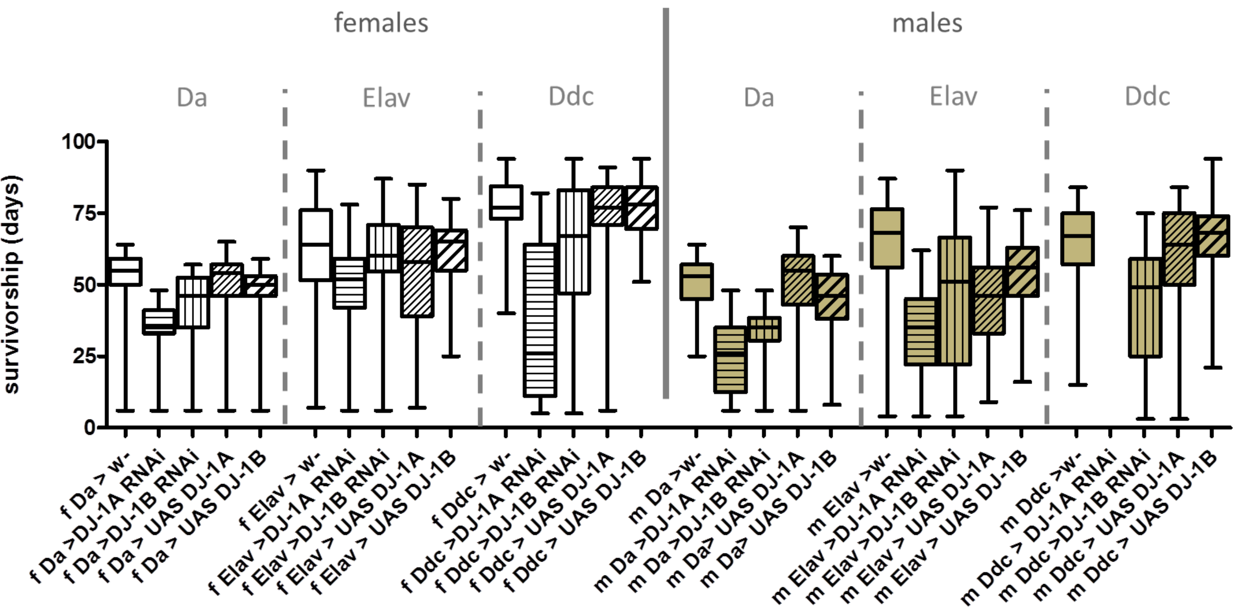


Fig. 41: Comparison of mean life spans plotted as box and whiskers diagram.

Light columns on left half: females, dark columns on right half: males. Qunitets represent different drivers. Left quintet each: Da-driven, middle quintet each: Elav- driven, right quintet each: Ddc- driven. Male transgenics generally exhibit shorter lifespans than females (compare left half/ right half of diagram). As depicted by the biggest drop in median from the first to the second box-and-whiskers-plot in each quintet, *DJ-1A* RNAi leads to the greatest reduction in lifespan as compared to w$^-$-controls. The median and inter-quartile range (IQR) of overexpression phenotypes do not differ greatly from the respective w$^-$-control-plots (compare 1st box-and-whiskers-plot to 4th and 5th of each quintet), indicating *DJ-1* is no longevity gene.

In summary, a ubiquitous decrease of *DJ-1* expression in the entire individual, as well as a tissue specific decrease of *DJ-1* function in selective neuronal tissues, led to a

significant reduction of life span, both for the *DJ-1A* orthologue and the *DJ-1B* orthologue.

Ddc-driven flies exhibited the strongest reduction of lifespan overall, when compared to the other drivers.

The effect of *DJ-1A* function on life span was markedly greater than the one of *DJ-1B*. The finding of a stronger physiological consequence of *DJ-1A* function over *DJ-1B* function, as assessed by student's t-tests, was consistent in all of our studies, irrespective of whether we reduced *DJ-1* function in all body cells or in different neuronal tissues selectively.

Males expressing the RNAi transgene generally exhibited a relatively stronger reduction of lifespan than females. In Ddc> RNAi flies, males exhibited a semi-lethal phenotype.

Expression of the *DJ-1* RNAi constructs in different body tissues led to divergent shapes of survival curves. A rank order of the three drivers in terms of their effect on life span can be extrapolated: Ddc GAL 4 -> Da GAL 4 -> Elav GAL 4.

However, overexpression of *DJ-1* did not prolong life spans in any of the systems tested.

3.3. Results of the toxicology study

***DJ-1* lack of function mutants exhibit increased sensitivity to exogenous toxins and oxidative stress**

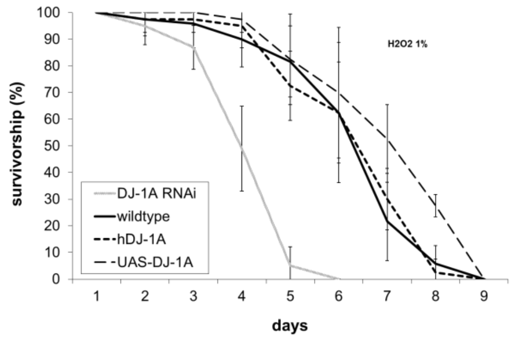
DJ-1 has been hypothesized to play a role in oxidative stress response and in cellular protection mechanisms. Therefore, the response of various *Drosophila DJ-1* mutants to exogenous stressors was investigated.

When downregulating the *DJ-1A* function in all body tissue using the daughterless driver flies have significantly shortened lifespans as compared to wildtype controls when exposed to various environmental toxins. When exposed to the ROS H_2O_2, the time to reach 50% mortality was shortened by 37% (4 days) in *DJ-1A* RNAi flies (see fig.42) as compared to control flies. When treated with the catalase inhibitor 3-AT, the time to reach 50% mortality was shortened by 25% (7.3 days). Treatment with the metal compounds Cu_2SO_4 and $MnCl_2$ resulted in a time reduction of 18% (4.6 days) and 39% (6.1days) respectively (see fig. 42).

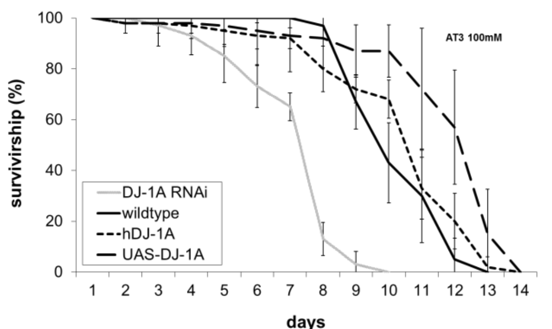
***DJ-1A* upregulated flies show reduced sensitivity to exogenous toxins and oxidative stress**

The overexpression of *DJ-1A* led to a markedly stronger resistance to exogenous toxins, with a prolongation of the 50%-mortality by + 13% for H_2O_2, +25% for 3-AT, 9% for Cu_2SO_4 and 11% for $MnCl_2$.

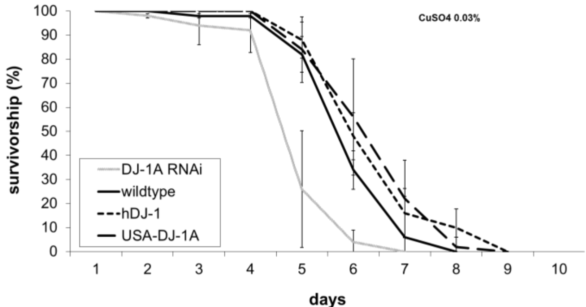
Overexpresssion of human *DJ-1* also resulted in a prolongation of the 50%-mortality as compared to wildtypes. However, this was less pronounced as with the *DJ-1A* overexpressing phenotype. Prolongation of 50% mortality in the DaGAL4>UAS *hDJ-1* was +2% for H_2O_2, +8% for 3-AT, 5% for Cu_2SO_4, and 10% for $MnCl_2$.



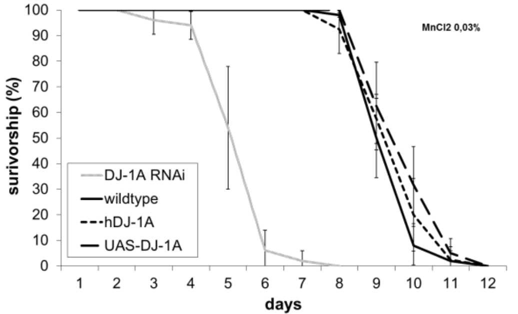
50% mortality on H_2O_2 1%				
	Wildtype (w-)	DJ1-A RNAi	UAS h-DJ1	UAS-DJ1-A
Days until 50% mortality	6.31	3.96	6.41	7.10
Δ % of wildtype mortality		- 37.2 %	+ 1,6 %	+ 12.5 %



50% mortality on AT3 100mM				
	Wildtype (w-)	DJ1-A RNAi	UAS h-DJ1	UAS-DJ1-A
Days until 50% mortality	9,71	7.29	10.51	12.17
Δ % of wildtype mortality		- 24.9 %	+ 8.2 %	+ 25.3 %



50% mortality on $CuSO_4$ 0.03%				
	Wildtype (w-)	DJ1-A RNAi	UAS h-DJ1	UAS-DJ1-A
Days until 50% mortality	5.67	4.64	5.95	6.18
Δ % of wildtype mortality		- 18.2 %	+ 4.9 %	+ 9.0 %



50% mortality on $MnCl_2$ 0.03%				
	Wildtype (w-)	DJ1-A RNAi	UAS h-DJ1	UAS-DJ1-A
Days until 50% mortality	10	6.13	10.20	10.60
Δ % of wildtype mortality		- 38.7 %	+ 10.2 %	+ 10.6 %

Fig. 42: Survival curves of *DJ*-1A-deficient transgenic flies (Da>*DJ-1A* RNAi) and of *DJ-1A* overexpression flies (Da>UAS *DJ-1A*) and of human *DJ-1*-expressing flies (Da>UAS *hDJ-1*) compared to wildtype flies (Da>*w*-), when exposed to various exogenous toxins. A) 1% H_2O_2, B) 100mM 3-AT, C) 0.03% Cu_2SO_4, D) 0.03% $MnCl_2$. Corresponding tables demonstrate time difference to reach 50% mortality as compared to wildtypes. *DJ-1A* was downregulated or overexpressed ubiquitously in experimental flies, using the daughterless (Da) driver. Curves were generated using adult females in quadruple vials of 10 flies per vial (=40 flies per toxin) at 25°C. The number of dead flies was recorded every 12 days. Curves demonstrate higher sensitivity to toxins in the RNAi flies and resistance in UAS flies.

3.4. Results of the drug feeding study

Neuroprotective effects of compounds with antioxidant and anti-inflammatory properties in the *Drosophila DJ-1* model of PD

To evaluate the effects of potential neuroprotective drugs *in vivo*, dopaminergic neuron survival and dopamine content of the brain were measured in a fly model of PD. The effects of the drugs Minocycline (M), Celastrol (C), NBQX, and Coenzyme Q10 (CoQ10) on *DJ-1A* RNAi–induced dopaminergic dysfunction and degeneration were investigated. One pathological hallmark of PD is a progressive loss of dopaminergic (DA) neurons in the substantia nigra. It has been shown that dopaminergic neurons in a circumscribed brain region, the dorsomedial cluster (DMC), selectively degenerate in *Drosophila* models of PD (see above and also Feany MB et al. 2000, Yang Y et al. 2005). Thus, serial frontal tissue sections across the brains of aged flies were first stained using the anti-tyrosine hydroxylase (TH) antibody that specifically detects dopamine-synthesizing neurons. Immunopositive neurons of the DMC were quantified. The age-dependent loss of DA-neurons is accompanied by a decrease of dopamine levels in the brain. Therefore, in the second set of experiments we studied whether drugs would also affect the brain's content of the transmitter dopamine, as measured by HPLC analysis of whole head homogenates.

3.4.1. The effect of minocycline on dopaminergic neuron loss and brain dopamine content

Treatment of Ddc-Gal4>*DJ-1A* RNAi flies with minocycline resulted in both the attenuation of dopaminergic neuronal loss and dopamine depletion in the brain (fig. 43 and fig. 44). *DJ-1A* RNAi flies that received daily feeds of minocycline-containing yeast paste (50 mg/ml) for 25 days showed a significant increase of TH^+ neurons within the DMC (167% of the yeast fed controls, $P<0.01$) (fig.44). The mean number of TH^+ neurons in the DMC of minocycline (50 mg/ml) treated *DJ-1A* RNAi flies was 16.8 ± 2.0 (mean ± SD), as compared to 10.1 ± 1.3 in yeast-fed control *DJ-1A* RNAi flies. Thus, treatment with 50 mg/ml minocycline resulted in a complete recovery of the DMC TH^+ neurons to wild type levels (16.1± 2.0 at 25 days).

We further examined the effects of minocycline on dopamine concentrations in head homogenates of 10-day-old Ddc-Gal4>*DJ-1A* RNAi flies. Daily oral uptake of minocycline attenuated dopamine depletion in a dose-dependent manner (fig. 45). Feeds of 25 mg/ml and 50 mg/ml minocycline resulted in significant increases of mean dopamine concentrations of 12.4 ± 2.3 ng/ml (*P*<0.01) and 14.1 ± 3.7 ng/ml (*P*<0.01) respectively, while dopamine levels of *DJ-1A* RNAi flies fed with yeast alone (no drug) were 7.5 ± 2.1 ng/ml. Age-matched wild type levels were 16.7 ± 5.0 ng/ml. Hence, as compared to yeast-fed, no-drug condition, high dose minocycline feeding almost doubled dopamine levels in Ddc-Gal4>*DJ-1A* RNAi flies, attaining 85% of wild type levels. Lower dose feeding (25 mg/ml) still increased dopamine levels to 166% of the untreated *DJ-1A* RNAi flies, reaching 71% of wild type levels. Minocycline had no effect on control *Ddc-Gal4/+* flies when fed in high doses (50 mg/ml). Neither TH^+ neurons (17.0 ± 1.0 vs. 16.1 ± 2.0 TH^+ neurons, *P*>0.05), nor mean dopamine levels (16.7 ± 3.1 ng/ml vs. 16.7 ± 5.0 ng/ml, *P*>0.05) were significantly different from those in flies treated with yeast only.

3.4.2. The effect of celastrol on dopaminergic neuron loss and brain dopamine content

Celastrol treatment both prevented dopamine neuronal loss and protected from transmitter depletion. The effects of celastrol are shown in figs. 3 and 4. Immunostaining of dopaminergic neurons in Ddc-Gal4>*DJ-1A* RNAi flies displayed a significant increase in the number of TH^+ neurons by 41 % (*P*<0.01) on average, when treated with celastrol at 5 µg/ml (from 10.1 ± 1.3 in untreated flies to 14.3 ± 1.0 in treated flies). When treated with a higher dose of celastrol (20 µg/ml), a mean increase by 48% or 4.8 neurons was observed. Thus, low dose celastrol treatment (5 µg/ml) restored the DA neuron content to 83% of age-matched control level (16.1 ± 2.0), whereas higher dose (20µg/ml) treatment resulted in the attainment of 93% of control levels.

To further ascertain the neuroprotective effects of celastrol, HPLC measurement of head dopamine levels was conducted in 10-day-old flies treated with two different doses: 1 µg/ml and 5 µg/ml. After feeding with 1 µg/ml celastrol, mean dopamine levels in the Ddc-Gal4>*DJ-1A* RNAi strain increased by 30% (9.7 ± 1.5 ng/ml in treated vs. 7.5 ± 2.1 ng/ml in untreated animals). These results showed no statistical significance (P>0.05). However, treatment of flies with a higher dose of celastrol (5 µg/ml) led to a more significant increase in dopamine levels by 92% to 14.4 ± 3.4 ng/ml. Hence,

treatment with 1 μg/ml celastrol preserved 58% of the wild type dopamine level, whereas treatment with 5 μg/ml celastrol maintained 86% of wild type levels. Both confocal microscopy of TH^+ neurons (figs. 43 and 44) and HPLC measurement of dopamine levels (fig. 45) suggest a dose-dependent effect of celastrol.

The protective effects of celastrol appeared to be selective for the *DJ-1A* RNAi flies, since celastrol treatment in control flies did not produce any significant alterations in TH^+ neuron number or brain dopamine levels. TH^+ neurons numbers were 17.1 ± 1.0 in 5 μg/ml celastrol-fed vs. 16.1 ± 2.0 in untreated *Ddc-Gal4/+* flies ($P>0.05$), and 16.4 ± 1.4 in 20 μg/ml celastrol-fed vs. 16.1 ± 2.0 in untreated flies ($P>0.05$). Brain DA levels were 18.8 ± 2.4 ng/ml in 1 μg/ml celastrol-fed and 16.7 ± 2.1 ng/ml in 5 μg/ml celastrol-fed flies, compared to 16.7 ± 5.0 ng/ml in untreated *Ddc-Gal4/+* flies ($P>0.05$ in both conditions).

3.4.3. The effects of NBQX on dopaminergic neuron loss and brain dopamine content

In contrast to minocycline and celastrol, NBQX attenuated TH^+ neuron loss, but failed to impede dopamine depletion. The number of TH^+ neurons in the DMC of Ddc-Gal4>*DJ-1A* RNAi flies treated with NBQX (30 mg/ml) averaged 176 % of their untreated control (17.7 ± 1.2 in NBQX fed *DJ-1A* RNAi flies vs. 10.1 ± 1.3 in untreated animals, $P<0.01$). Thus NBQX treatment was sufficient to fully restore dopamine neuronal numbers to wild type levels (10.1 ± 2.0) at 25 days of age (fig. 43 and fig. 44).

Surprisingly, the NBQX-induced increase in surviving DA neurons determined by immunohistochemistry was not corroborated by neurochemical analysis of DA content (fig. 44 and fig. 46). HPLC measurement of head DA concentrations showed only a slight increase in NBQX-treated Ddc-Gal4>*DJ-1A* RNAi flies (from 9.6 ± 3.4 ng/ml in the untreated controls to 10.6 ± 3.1 ng/ml in treated animals). Furthermore, in contrast to the findings in *DJ-1A* RNAi flies, NBQX significantly diminished DA levels in control *Ddc-Gal4/+* flies by 33% on average (from 13.1 ± 2.9 ng/ml in untreated to 8.7 ± 1.1 ng/ml in NBQX-treated animals, $P<0.05$) (fig. 46). In addition, a statistically non-significant small decrease of TH^+ neurons was observed in NBQX-fed *Ddc-Gal4/+* control flies (from 16.1 ± 2.0 in untreated to 14.8 ± 1.2 in treated animals) (fig.44).

3.4.4. The effects of coenzyme Q10 on dopaminergic neuron loss and brain dopamine content

Coenzyme Q10 (CoQ10) fed at 100 mg/ml showed no significant effect on dopaminergic neurons or dopamine levels in *DJ-1A* RNAi flies or controls. The mean number of TH^{+} neurons in *DJ-1A* RNAi flies when treated with 100 mg/ml CoQ10 was 9.5 ± 1.5 vs. 10.1 ± 1.3 in untreated flies (P>0.05). In *Ddc-Gal4/*+ control flies, 100 mg/ml CoQ10 treatment resulted in 16.9 ± 2.0 dopaminergic neurons vs. 16.1 ± 2.0 in untreated controls (P>0.05) (fig. 43, fig. 45). As shown in fig. 46, mean dopamine levels were 12.1 ± 1.9 ng/ml in 100 mg/ml CoQ10 treated vs. 11.4 ± 3.1 ng/ml in untreated *DJ-1A* RNAi flies (P>0.05). In *Ddc-Gal4/*+ control flies, dopamine levels were 17.0 ± 3.8 ng/ml in 100 mg/ml CoQ10-treated vs. 16.2 ± 2.3 ng/ml in untreated flies (P>0.05) (fig. 46).

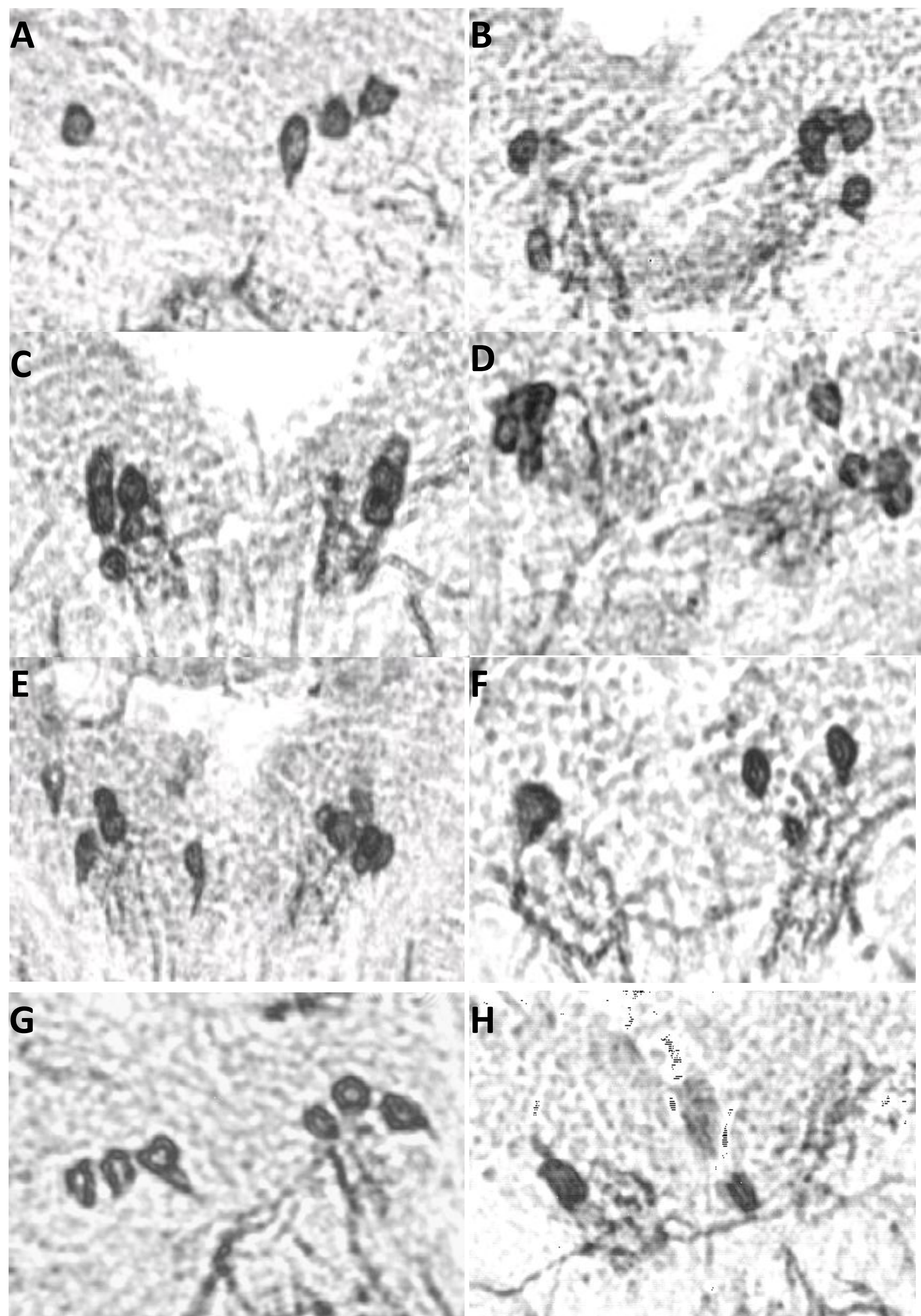


Fig. 43: Number of TH-positive DA neurons in the DM clusters of 25 day old *DJ-1A* RNAi and wildtype flies: Effect of drug feeding.
Representative slides are compared by way of example. A-F: Ddc>*DJ-1A* RNAi flies. A: yeast-fed control, B: Celastrol 20µg/ml, C: Minocycline 50mg/ml, D: Celastrol 5µg/ml + Minocycline 25mg/ml, E: NBQX 30 mg/ml, F: Coenzyme Q10 100mg/ml. G+H: wildtype flies. G: yeast fed control, H: NBQX 30 mg/ml.
When comparing numbers of TH^{+} neurons of 25d old *DJ-1A* control-fed flies (photo A: 4 neurons detectable) to age-matched flies treated with celastrol, minocycline, a mixture of celastrol and minocycline, and NBQX (photos B to E, between 8 to 12 neurons counted, respectively) a neuroprotective effect of these drugs is observed. Treatment with Coenzyme Q10 has no effect on neuron numbers (4 neurons in F, - same as in A).Contrary to in *DJ-1A* transgenics, NBQX promotes neuronal loss in w^{-}-controls (2 neurons counted in H, as compared to 6 in G).

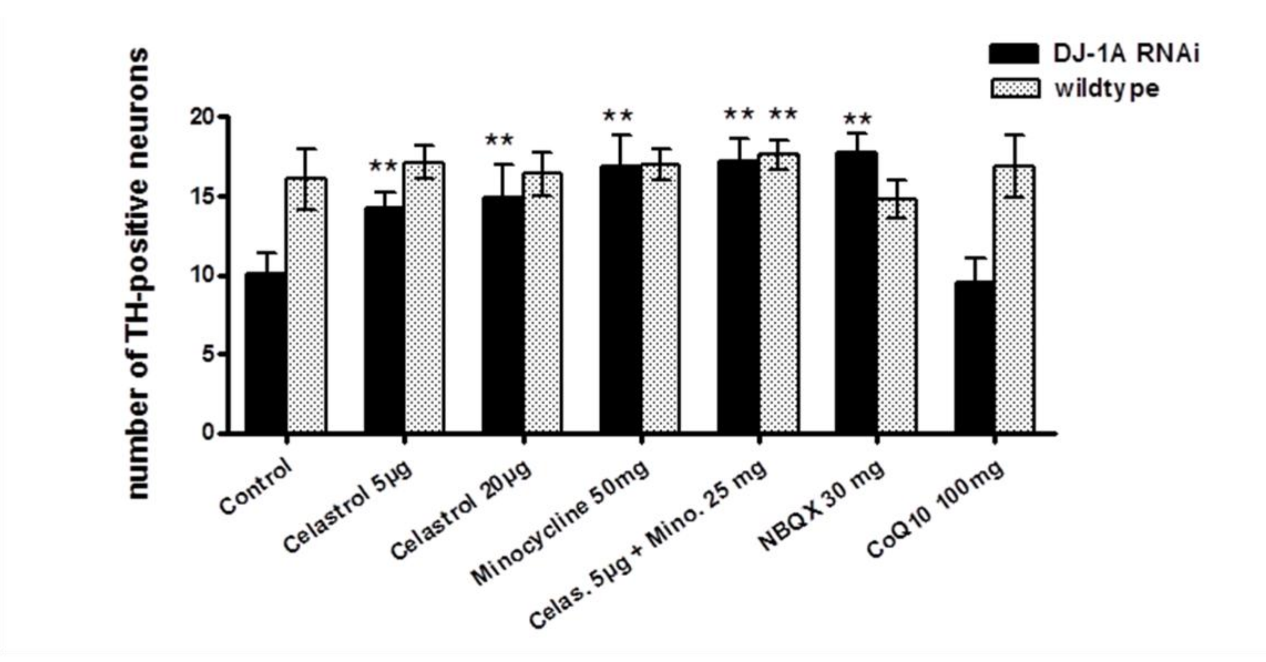


Fig. 44: Number of dopaminergic neurons, as counted by TH- immunostaining, in the DM cluster of drug treated 25 day old *DJ-1A* RNAi flies. These were compared with untreated *DJ-1A* RNAi flies and with wildtype control flies. Results are the mean + SD of 8-25 sectioned heads for each genotype and drug treatment. **: p< 0.01 in student's t-tests, indicates statistical difference in cell count between drug-treated and untreated flies. As depicted, feeding of celastrol (dose-dependent), minocycline, a combination of celastrol and minocycline, and NBQX leads to significantly higher numbers of TH^+ neurons in aged flies, respectively, as compared to control-fed flies. Feeding of Coenzyme Q10 shows no effect on neuronal numbers.

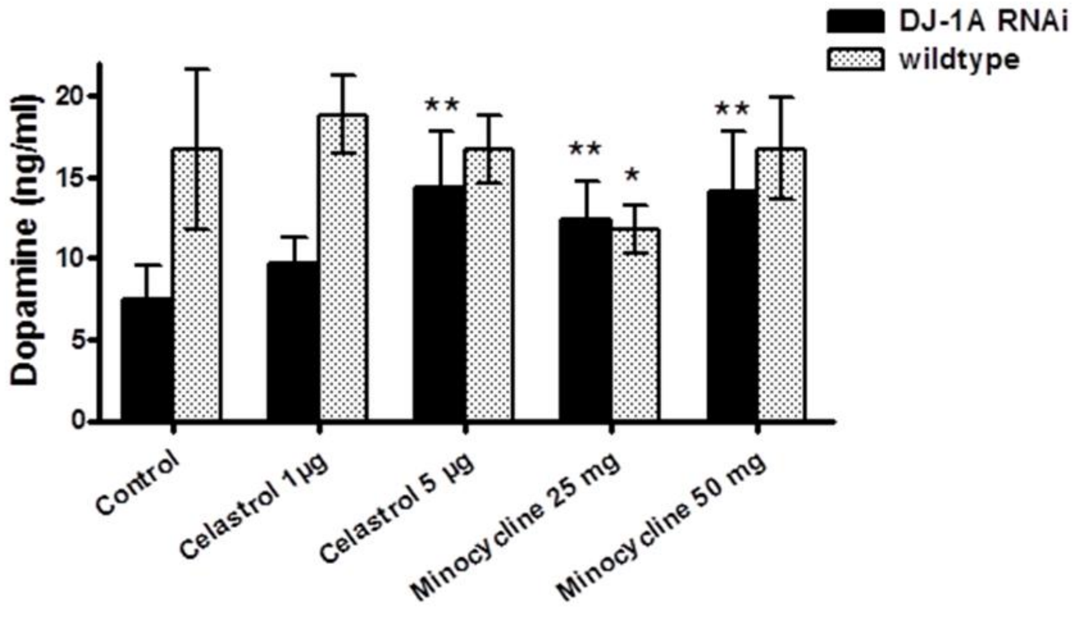


Fig. 45: Dopamine concentrations of head homogenates of 10 day old, celastrol- and minocycline-treated *DJ-1A* RNAi flies, as measured by HPLC. These were compared with untreated *DJ-1A* RNAi flies and with wildtype control flies. mean ± SD of 21 homogenised heads for each genotype and drug treatment. *: p< 0.05 and **: p< 0.01 in student's t-tests, indicates statistical difference in head dopamine content between drug-treated and untreated flies. Feeding of celastrol and minocycline leads to significantly higher dopamine levels in aged flies, compared to control-fed flies. A dose-dependency effect is observed.

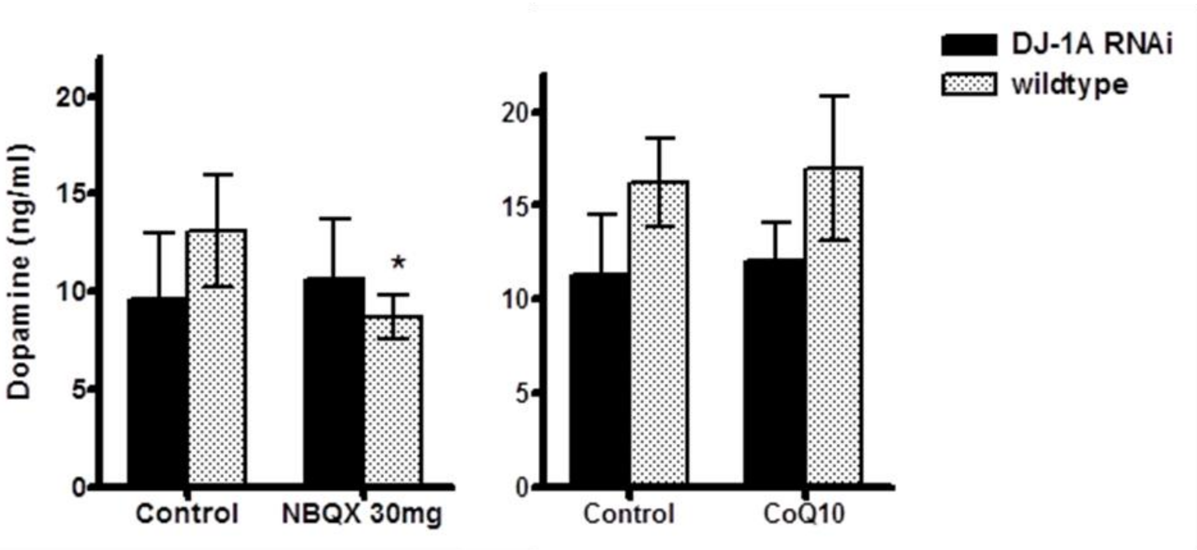


Fig. 46: Dopamine concentrations of head homogenates of 10 day old NBQX and coenzyme Q 10 treated *DJ-1A* RNAi flies, as measured by HPLC. These were compared with untreated *DJ-1A* RNAi flies and with wildtype control flies. Results are the mean + SD of 21 homogenised heads for each genotype and drug treatment. *: p< 0.05 in student's t-tests, indicates statistical difference in head dopamine content between drug-treated and untreated flies. Feeding of NBQX leads to insignificantly higher dopamine levels in aged flies, compared to control-fed flies (left diagram). Coenzyme Q10-feeding shows no effect (right diagram).

4. Discussion

In the present study the newly generated *Drosophila DJ-1* model of PD is established and described. For this purpose, assays of aging, neurodegeneration and resistance to oxidative damage have been employed. Secondly, the neuroprotective effects of four drugs, celastrol, minocycline, coQ10 and NBQX, are studied in the established *Drosophila DJ-1* model of PD. The parameters used here, brain dopamine level and the survival of dopaminergic neurons within the DMC, are relevant to the pathological hallmarks seen in PD patients. Therefore, the results shown here have implications for the introduction of new neuroprotective drugs into clinical practice as well as for further understanding the role of DJ-1 in normal dopaminergic physiology and PD pathogenesis.

4.1. Discussion to the life span study

Life span studies are always instructive when investigating genes with strong influence on longevity and aging. This is the case in all neurodegenerative disease.

DJ-1's function in the cell has previously been linked to anti-apoptotic cell survival pathways [119, 128, 132, 133] and oxidative stress defence [121, 124, 138-140], - both being cellular functions which might directly or indirectly influence organismal longevity. In the present study the effects of decreased and increased *DJ-1* function on the life span of *Drosophila melanogaster* was examined. Since two orthologues of *DJ-1* in the fly exist (*DJ-1A* and *DJ-1B*), they were examined separately. Our results suggest that both *DJ-1A* and *DJ-1B* may play important roles in development and in life span.

The first main finding of this study was that reduced expression of *DJ-1* protein in various tissues is correlated with lower viability and significantly shortened mean life spans by up to 50%. This finding demonstrates that *DJ-1* is an essential and indispensable protein required for survival and normal life span in the fly. There are many genes in the genome of an organism, which when silenced result in an altered phenotype only, but do not affect viability or life span. These genes usually have a highly specialized function within the cell. In contrast, *DJ-1* appears to play a more fundamental role. The fact that *DJ-1* is a phylogenetically old protein, that is present

even in prokaryotes, and has undergone only very little change during the evolution to humans, underlines its essential physiological role in cellular functioning and survival.

Even though the suppression of both orthologues, *DJ-1A* and *DJ-1B*, led to a significant reduction of life span, the effect of *DJ-1A* function was markedly greater in all of the lines we used, whether we reduced *DJ-1* function in all body cells or in different neuronal tissues selectively. This differential effect might be due to a gene dosage effect, i.e. the remaining amount of *DJ-1B* after RNAi in the cell might be higher than the amount of *DJ-1A* after RNAi. Yet quantitative PCR and Western Blot analysis (data not shown) indicate a similar effectiveness of RNAi.

It is therefore concluded that *DJ-1A* has a greater differential relevance on viability and aging in various tissues than *DJ-1B*. This deduction is further supported by previous studies which describe a stronger physiological role of *DJ-1A* function than *DJ-1B* in different contexts. Expression of *DJ-1A* RNAi in the *Drosophila* eye led to a severe loss of photoreceptors, while *DJ-1B* RNAi did not produce any obvious eye abnormalities [133]. Expression of *DJ-1A* RNAi or *DJ-1B* RNAi, respectively, in dopaminergic (DA) neurons resulted in a relatively strong age dependent loss of DA neurons in the case of *DJ-1A* RNAi [133].

The second important finding of this study was that overall the effect of *DJ-1A* RNAi on life span was strongest in Ddc-driven flies, as compared to the other drivers. Physiologically, *DJ-1* (both *A* and *B*) is expressed ubiquitously in all cells of the body and not confined to e.g. a neuronal sub-population. While the Da (daughterless) driver expresses RNAi in all tissues, Ddc expresses it only in a very small subset of cells: the dopaminergic and serotonergic neurons. Therefore, the effect of Da>*DJ-1A* RNAi on life span might have been expected to be strongest, while the one of Ddc>*DJ-1A* RNAi might be assumed to be rather negligible. Yet the opposite was observed here. In addition, a semi-lethal phenotype was observed in Ddc> *DJ-1A* RNAi males only, and not in Da driven males nor any other genotype.

In contrast, the effect of *DJ-1B* RNAi on life span was smaller in Ddc driven (and also Elav driven) flies than in Da driven flies. We therefore conclude that *DJ-1B* might occupy a more relevant role in tissues other than neurons, while the differential relevance of *DJ-1* for dopaminergic neurons is mainly mediated by the *DJ-1A* orthologue. The smallest effect on life span was observed when expressing the *DJ-1*

RNAi (both A and B) constructs with the Elav driver, which is a pan-neuronal driver. This result might again be explained by *DJ-1*'s differential relevance in different cell types. While *DJ-1A* is essential for dopaminergic neurons, it appears to be rather dispensable for other neuronal populations. Considering *DJ-1*'s hypothesized function in the oxidative stress response, its loss will affect neurons, which are not involved in dopamine metabolism, to a much smaller extent. Proceeding from this, other non-neuronal tissues, e.g. the highly energy consuming muscle cells of the fly's flight apparatus, might generate higher levels of oxidative stress than non-dopaminergic neurons, leading to a greater effect on life span in Da driven flies than in Elav flies. However, the reduction in life span of flies might not be due to *DJ-1*'s anti-oxidant property only, but to other hypothesized functions, such as changes in transcriptional regulation, cellular trafficking, fertilization or others [37, 119, 122, 123].

Interestingly, downregulation of *DJ-1A* in different tissues produced different shapes of survival graphs. While loss of *DJ-1A* function in all body tissue (Da-driver) resulted in an exponential survival graph (which very much resembled the control survival graph, being shifted as a whole to the left on the time axis), downregulation of *DJ-1* exclusively in those cells that are affected in Parkinson's disease, namely the dopaminergic neurons (Ddc-driver), produced a sigmoid survival graph. In Ddc driven flies, survivorship was most decreased in young flies, already soon after eclosion, resulting in a great reduction of mean and median life spans, with comparably less effect on maximum life span. In Da driven flies the majority of deaths still occurred at older ages, resulting in both a major reduction of mean and maximum life spans.

In a simplified concept life span and death are determined by two major components: The first is an intrinsic process which leads to a deterioration of organismal homeostasis and metabolism. The second component affecting aging and life span is external insults such as oxidative stress and infection. A lack of defence against these external insults, possibly genetically determined, will also increase the probability of death over time. Importantly, this second component is non-age dependent, i.e. death can also occur at early ages of the individual; whereas the first component is age dependent, resulting in the highest incidence of deaths at progressed ages of the cohort. In our study, the Da> *DJ-1A* RNAi flies give a typical example of age-dependent reduction in life span, while the Ddc>*DJ-1A* RNAi flies present a non-age dependent reduction of life span.

Our results are therefore compatible with *DJ-1*'s hypothesized role in oxidative stress defence, which has been described in previous cell culture studies [121, 127, 138, 139, 141]. A flaw in the protection against external (and internal) stressors can lead to death at any ages of the individual. The fact that the effect described above on survival curves is restricted to the Ddc driver might be attributed to the cell specific properties of dopaminergic (DA) neurons, including their physiologically increased vulnerability to oxidative events caused by the free-radical producing dopamine metabolism, and the high content of catalysing iron and other metals. Taken together, the physiological relevance of *DJ-1A* might be strongest in DA neurons.

However, the Da driver and also the Elav driver downregulate *DJ-1* in DA neurons also (amongst other cells). So why is there an apparent difference in the effect on DA-neurons between these drivers? It is assumed that the concentration of RNAi in each specific cell type is relatively lower after global expression than it is when targeted exclusively at that cell type (dilution effect). This might explain the low juvenile mortality in Da driven flies. At the same time it suggests that the life span reduction observed in Da driven flies may be mediated by *DJ-1* functions other than the one in DA neurons (e.g. its function for muscle tissue, circulation etc.).

The rates of viability and the life spans of our transgenic flies were generally lower in males than in females. This difference may be due to different expression levels (however, quantitative PCR and western blotting results did not suggest this- data not shown) or different function of the *DJ-1* gene in males and females. In fact, *DJ-1* has a role in male fertility and spermatogenesis, which is clearly gender specific [37, 121-123]. On the other hand, in *Drosophila*, males are generally less resistant to stressors than females [230]. Thus genetic manipulation that has negative effects on an individual's resistance to external stressors is expected to show more pronounced consequences in the more fragile males [231]. In addition and for similar reasons, male and female life spans generally differ in flies. This can be observed already when comparing male and female control flies. Even though maximum life spans do not differ between the two genders, deaths start to occur at younger ages in the male population than in the female one, leading to flatter survivor curves. This finding is consistent in all our experiments and therefore unlikely to be due to genetic manipulation. Reduced life spans in males can be explained by higher levels of social stress within the male cohort and male fighting behaviour [230].

The third important finding of our study was that overexpression of the *DJ-1* gene had no positive effect on the survival time in any of the systems tested. Thus, it can be concluded that *DJ-1* is not a member of the so called longevity genes. Longevity genes are genes which have the ability to regulate life span, such as some heat shock proteins, chaperones and SOD. But *DJ-1* does share properties with several other longevity genes, including its involvement in transcriptional regulation and tumorigenesis, as well as its proposed function as molecular chaperone and its role in the oxidative stress response. Therefore, *DJ-1* constituted a possible candidate for a new longevity gene. Other genes, which have been shown to prolong life span in *Drosophila* and also in the nematode *C. elegans,* include transcriptional regulators [164-166], chaperones [160, 160] and anti-oxidants [171-175]. Taken together, these results show that even though *DJ-1* is essential for life span and viability, it does not have the property to extend life span.

Interestingly, overexpression of *DJ-1* appeared to have negative effects on life span in Elav driven males. It might be assumed that excess *DJ-1* protein possibly has toxic properties for certain subtypes of neurons. We know that *DJ-1* has the propensity to form dimers and to aggregate [230]. Thus, the accumulation of *DJ-1*, for example, could interfere with cellular trafficking or metabolism. In certain subtypes of cells a tight regulation of transcriptional levels might be required for proper functioning.

It should be mentioned that Da driven flies generally exhibit shorter life spans than Ddc- and Elav-driven ones. This phenomenon is most likely due to manipulation of the genetic background of the fly through the DaGAL4 system [235]. Since the effect was equally present in control and experimental flies (we used the Da driver for control flies also (Da>*w*-)), study conditions remained the same for the groups compared. The slope of the curve in Da driven controls was no different from Ddc and Elav driven controls.

In conclusion, the differential effect of *DJ-1* overexpression and downregulation on life span in three different body tissues was analyzed employing genetically constructed insect models. It was demonstrated that normal *DJ-1* function is indispensable for organismal longevity and viability. However, *DJ-1* does not appear to constitute a novel longevity gene that could prolong life span when overexpressed. *DJ-1* has a greater differential physiological relevance for dopaminergic neurons than for any other cell

type. The findings of the aging study are consistent with *DJ-1*'s hypothesized function as free radical scavenger, as shown by KimRH et al. in 2014 [127].

4.2. Discussion of the toxicology study

After having demonstrated shortened lifespans of *DJ-1* RNAi flies in the aging study, the toxicology study aims at investigating the mutants' resistance to exogenous toxins, specifically to ROS and to metals. Since the results of the aging study suggest that the phenotype of the *DJ-1A* mutant is stronger than that of the *DJ-1B*, it was decided to concentrate on *DJ-1A* in the subsequent experiments. Two oxidative stress essays employing H_2O_2 and 3-AT, and two metal toxicity essays, employing $MnCl_2$ and Cu_2SO_4 were conducted. The results indicate an increased sensitivity of the *DJ-1A* loss of function phenotype and an increased resistance of the *DJ-1A*-overexpressing mutant to exogenous toxins.

Increases in oxidative stress and mitochondrial dysfunction are being increasingly recognized as common pathogenic contributors to many neurodegenerative diseases, including PD [23-27, 41], as also outlined in the chapter on the pathogenesis of PD of the introduction. The results of the oxidative stress essays provided here (see fig. 42) strongly support this theory and suggest that *Drosophila DJ-1A* might play a role in the oxidative stress defence. Since overexpression of *DJ-1-A* was shown to confer protection against ROS, it might be assumed that *DJ-1* protein has a direct function as a free radical neutralizer.

Hydrogen peroxide was chosen as a toxin, because it is a highly reactive oxygen species, which is also naturally produced by most living organisms, - also by *Drosophila*, as a side product of oxidative metabolism. Therefore, organisms have developed various radical scavenging strategies, including H_2O_2 -degrading enzymes, such as catalase [236].

3-amino-triazole (3-AT) is a cell-permeable catalase inhibitor, which thus indirectly increases the concentration of ROS, as it inhibits the conversion of H_2O_2 (to H_2O and O_2). Furthermore, 3-AT is being used as a non-selective triazole herbicide. As outlined in the epidemiology chapter of the introduction, the incidence of sporadic PD has been linked to chronic pesticide exposure [18, 19].

Here it is shown that *DJ-1A* RNAi flies show hypersensitivity to oxidative stress. The results are also consistent with previous in vitro data: Cell culture studies have provided many valuable clues as to the potential radical salvaging properties of *DJ-1* protein: *DJ-1* deficient neuronal cells exhibit an increased susceptibility to H_2O_2, MPP^+, 6-hydroxydopamine, and rotenone toxicity [121, 139, 140], whereas DJ-1 overexpression could dramatically reduce H_2O_2-induced cell death in neurons [127, 139]. Mitsumoto A et al. have shown in 2001 in *in vitro* studies using human neuronal cell cultures that the expression of *hDJ* increases in response to ROS, and that *DJ-1* might thus serve as an endogenous indicator of oxidative stress status [124]. Human *DJ-1* protein was previously shown to be able to eliminate H_2O_2 *in vitro* by oxidizing itself at specific Cys residues [121, 138].

However, in order to describe loss of function phenotypes, the expression in an integral, live organism is indispensable. We therefore created animal models of *DJ-1* loss of function and *DJ-1* overexpression genotypes, employing the fruit fly *Drosophila melanogaster*.

Other groups later independently produced very similar results using different loss *Drosophila* loss of function mutants. Lavera-Culebras E. et al., who produced *DJ-1A* and *DJ-1B* loss of function phenotypes by insertional mutation, have demonstrated increased sensitivity to paraquat and also reduced life spans [237]. Meulener M. et al. who created *DJ-1A* and *DJ-1B* double knockouts and exposed them to paraquat, rotenone and H_2O_2 again observed increased sensitivity to these exogenous toxins [238].

Secondly, the sensitivity of *DJ-1A* mutants to metal toxicity was here tested in the *Drosophila* model. Again, it is shown that *Drosophila DJ-1A* RNAi show markedly increased sensitivity to metal toxicity. Epidemiological studies have linked the incidence of PD to chronic exposure of transition metals, such as manganese (Mn) and copper (Cu) [21]. Welders and miners exposed to manganese containing fumes show a higher incidence of PD. Sriram K. et al have shown in 2010 that the instillation of Mn containing fumes leads to mitochondrial impairment, reduction of the tyrosine hydroxylase enzyme and an altered expression of *DJ-1* [22]. In vitro studies have demonstrated a selective manganese-induced cytotoxicity to dopaminergic neurons [239]. Here, exposure to $MnCl_2$ resulted in a downregulation of *DJ-1* in a rat model, as shown both by PCR and

by western blotting, suggesting that *DJ-1* might play a critical role in Mn-mediated dopaminergic dysfunction.

Similarly, Parkinson-like motor symptoms are associated with Wilson's disease, a condition involving abnormal Cu storage in body tissues, including the brain [240]. The heightened vulnerability of *DJ-1A* RNAi flies to metals might again be explained by an (indirect) increased ROS exposure. If metal compounds lead to the downregulation of *DJ-1*, as suggested by Sriram K et al. [22], and *DJ-1* acts as a free radical catcher, ROS will accumulate by inactivation of *DJ-1*. A disabling of *DJ-1* by metals would also be consistent with the finding that UAS-*DJ-1A* phenotypes did not result in as strong a resistance to metals as to H_2O_2 and 3-AT. On the other hand metal ions always act as chemical catalysts, thereby generally producing ROS as a by-product. However, further studies would be required to investigate *DJ-1*-metal interactions.

4.3. Discussion of the drug study

The phenotype of the newly produced mutant *DJ-1A* RNAi fly has been adequately described in the life span and toxicology studies: It primarily consists of an accelerated degeneration of dopaminergic neurons, as well as shortened lifespans and increased sensitivity to exogenous toxins. Thus, after the establishment of the *Drosophila DJ-1A* loss of function model of PD, the model is now eligible for use in pre-clinical treatment studies.

In the drug study the *in vivo* neuroprotective effects of the four drugs celastrol, minocycline, CoQ10 and NBQX, were studied using the *Drosophila DJ-1A* RNAi model of PD. The pathological hallmark of PD in humans is the degeneration of dopaminergic neurons in the substantia nigra of the brain, accompanied by a depletion of dopamine within the striatum. Therefore, direct dopamine measurement of fly heads via HPLC and the quantification of DA neurons within the DMC, which was previously shown to degenerate preferentially in other *Drosophila* models of PD [133, 150], have been chosen as parameters to evaluate any potential neuroprotective effects. The results shown here have significant implications for the introduction of novel neuroprotective drugs into clinical practice as well as for a better understanding of the role of *DJ-1* in normal physiology and PD pathogenesis.

The combination of antioxidant and anti-inflammatory properties provides excellent neuroprotection in the *DJ-1A* RNAi model of PD: Celastrol and Minocycline

As outlined earlier, neuronal degeneration in PD is accompanied by features of oxidative damage and inflammation. Oxidative damage, likely generated by the metabolism of DA, manifests itself through mitochondrial dysfunction and elevated levels of metal-ions and neuromelanin. Inflammation in PD is mediated by activated microglia and their release of inflammatory cytokines [62, 50, 53, 63] and nitric oxide (NO) [16, 65, 48, 74]. A drug with both antioxidant and anti-inflammatory activity may prevent neuronal degeneration in PD. Minocycline and celastrol both fulfil this unique combination of properties. Both are potent antioxidants that can suppress the activation of microglia and inhibit the release of inflammatory cytokines and inducible nitric oxide synthesis (iNOS), thus decreasing peroxynitrite-mediated damage. In accordance with their comparable profile of action, similar effects on dopamine neuronal content and dopamine levels were observed with the two drugs in our study. Both drugs were able to completely halt the accelerated loss of DA neurons in *DJ-1A* RNAi flies as compared with age matched wild type levels at corresponding doses (at 50 mg minocycline/ml and 5µg celastrol/ml) and to replenish dopamine content to near to normal levels (85% of wild type levels for 50 mg/ml minocycline and 92% for 5µg/ml celastrol). As shown in figs. 35 and 36, a certain, though lesser, extent of DA neuronal loss and dopamine depletion occurs in wild type flies with age. This degeneration is considered physiological. It also occurs in healthy humans [42, 43]. Both drugs failed to influence the physiological age-related DA degeneration, suggesting their mechanism of action may be disease-specific.

Our results are consistent with previous studies in the MPTP mouse model of Parkinson by Du Y et al. [241] and Cleren C et al. [229], where minocycline and celastrol were shown to protect against MPTP induced neurotoxicity. Toxin induced models of a disease, however, always bear a confounder of the drug reacting directly with the toxin. Thus, a seemingly neuroprotective effect might be conferred by chemical attenuation of the toxin's pathogenicity. Here we show, in an independent and genetic model of PD, that the neuroprotective potential of the two drugs is not restricted to pharmacologically induced models, such as the MPTP model. To further increase the likelihood of the drugs' efficacy in idiopathic disease, it might be interesting to test the two drugs in other

models of PD as well, such as *α-synuclein* (*SCNA*) and *Parkin*, which may operate through different pathogenic mechanisms.

Our results suggest that both the chemically modified tetracycline minocycline and the Chinese herb celastrol may prove effective in preventing and/or delaying the progression of PD. Yet, importantly, comparable results were achieved at very different doses, indicating a difference in potency of the two drugs. To produce the same neuroprotective effect as celastrol, minocycline required a more than 10,000-fold concentration. This finding is relevant especially when developing protective or preventative medicine. After all, an augmentation of the drug concentration required to reach a desired therapeutic effect is generally accompanied by an increase of the drug's toxicity. The idea behind neuroprotective therapy in PD includes an employment of the drug very early in the course of the disease, i.e. with the very first observation of symptoms, when the use of L-DOPA is not yet justified [242], or even before the onset of any clinical manifestations (e.g. in high risk patients with a familial occurrence of PD). Neuroprotective therapy requires long-term administration of the drug in otherwise insignificantly handicapped individuals. In the MPTP mouse model of PD, an oral concentration of 120 mg/kg day minocycline was required to produce a protective effect [241]. The high dose of minocycline given to animals (significantly more than is typically given to humans) is a potential problem. (The dose applied in the drug study would translate into a daily oral dose of around 10 g in humans.) The dose that we found to be effective in the fly (50 mg/ml) must also be considered relatively high. Lower doses (e.g. 10 mg/ml) were tried in previous dosing studies by our group, but were insufficient to produce any neuroprotective effect (data not shown).

Minocycline has been used for many years as an antibiotic and is considered relatively safe. However, the recommended dose of minocycline is 100 mg to 200 mg/ day [177]. High doses of minocycline produce the same side effects as other tetracycline antibiotics. Furthermore, minocycline has a tendency to accumulate in various body tissues [184]. Thus a high dose regimen over longer periods of time is not recommended. Studies using minocycline should therefore be cautiously evaluated before being used as a basis for human trials. Nevertheless, after testing in two toxin induced models of PD, (which may not translate well into clinical trials, as shown above [241, 243]), minocycline has already been incorporated into a presently still ongoing clinical investigation by the National Institute of Neurological and Communicative

Disorders, National Institutes of Health, termed “Neuroprotection Exploratory trials in Parkinson’s Disease”, or NET-PD. In this trial, minocycline is administered in twice daily doses of 100 mg, in combination with creatine (5g, twice daily) to otherwise untreated PD patients [244]. From the present animal studies, it might be expected that the administered dose of minocycline in this trial is too low to provide neuroprotective effects. The neuroprotective effect of minocycline in a drug-combining trial might also be hard to judge. Thus, minocycline may or may not play a therapeutic role in future treatment regimes of the disease. Our results show, however, that a drug with both antioxidant and anti-inflammatory effects provides excellent neuroprotection and might encourage the search for agents with a therapeutic spectrum similar to that of minocycline, but fewer side effects in the long term.

Celastrol shows more promising potential as a candidate to be further tested in preclinical Parkinson models. Celastrol has been described previously as a potent antioxidant and anti-inflammatory agent in models of rheumatoid arthritis (RA), Crohn’s disease and Alzheimer’s disease (AD). Here it is shown that the administration of celastrol in low nanomolar doses provided excellent neuroprotection in a fly model of PD. 5 µg/ml celastrol treatment resulted in an increase of DA neuronal numbers in the *DJ-1A* deficient fly from 63% to 83% of wild type counts. 5 µg/ml celastrol feeds elevated DA concentrations from 45% to 86% of wild type levels. The observed effects were similar to those with minocycline (when treated with 50mg/ml minocycline, TH-positive neuronal counts were restored completely to 105% of wild type levels, DA levels reached 85% of wild type levels). However, celastrol must be considered superior to minocycline with regard to tolerance in humans. Celastrol has a centuries-long history of use in traditional Chinese medicine [187, 188], without record of signs of intolerance or carcinogenicity. In the present study, celastrol exhibited approximately 10,000-fold higher neuroprotective potency than minocycline. In nanomolar doses (7 µg/kgxd) celastrol was shown to improve memory and learning in a mouse model of AD. Higher doses (3 mg/kgxd) were administered to mice, e.g. in the MPTP model of PD, without any observed side effects [189, 190, 187, 229, 245]. Based on celastrol’s unique properties, it could prove a very useful therapy in humans, once early safety and toxicology studies are carried out. Celastrol is a causative treatment of PD, as it targets at least two main pathogenetic factors. It is thought to prevent or delay the use of L-DOPA. Celastrol should be administered as single treatment early in the course of the

disease and later in combination with L-DOPA or dopamine agonists, where it might help reduce required doses. Since celastrol is readily available and comparatively inexpensive, its use, by delaying the administration of more costly symptomatic therapies, may also bear economic benefits.

The biogenic amine CoQ10 fails to protect in the *DJ-1A* RNAi model of PD

The reduction in complex I in the mitochondrial electron transport chain in PD patients prompted the therapeutic use of CoQ10, the electron acceptor for mitochondrial complex I and an antioxidant itself. Administration of CoQ10 to the MPTP mouse model (200 mg/kgxd, [202]) and monkey model (15-22 mg/kgxd, [199]) attenuated the effects of low dose MPTP. Unlike these previous studies, our present results do not suggest any neuroprotective effects of CoQ10. Mean number of TH^+ neurons was not significantly different from untreated controls (9.5 ± 1.5 vs. with 10.1 ± 1.3), nor were brain dopamine levels (16.9 ± 2.0 vs. 16.1 ± 2.0 ng/ml). There are several possibilities how lack of efficacy in the fly could be explained. Due to its large molecular weight and hydrophobicity, bioavailability of CoQ10 is low in humans [201, 246, 247] and might also be limited in the fly. CoQ10 might not be stable in yeast paste or be metabolized rapidly before diffusing into brain tissue. Secondly, the administered dose of 100 mg/ ml might have been too low (CoQ10's limited solubility in DMSO set an upper limit to increasing doses.). Thus, it cannot be ruled out that higher doses of CoQ10 may confer neuroprotection in the fly. Alternatively, CoQ10 may simply be inefficacious in the *DJ-1A* RNAi model of PD. All previous animal testing of CoQ10 was done in a MPTP model of PD. MPTP acts through inhibition of complex I of the mitochondrial respiratory chain, and CoQ10 is the very electron acceptor of complex I. Based on this linkage, the therapeutic effect of CoQ10 might be restricted to the MPTP model solely and thus not applicable to other PD models or idiopathic disease. *DJ-1* acts to protect against oxidative stress and is also present in mitochondria [138, 141], however, its role in PD pathogenesis may not be restricted to mitochondrial dysfunction. CoQ10 has already been used in clinical trials on humans with inconclusive results so far [196, 204-208]. These studies differ greatly in their chosen outcome measures, and only part of them are in patients with early disease not yet requiring medication, others are in patients with advanced disease already requiring medication, which might not be suited for

neuroprotective trials. Based on our present findings, CoQ10 might not be neuroprotective in idiopathic PD.

NBQX exhibits differing effects on neurodegeneration and dopamine depletion in *DJ-1A* RNAi flies and is toxic to wild type animals.

Now that it has been established that excitotoxicity conferred by excess glutamate in the basal ganglia circuit contributes to neuronal degeneration in PD, AMPA selective glutamate receptor (GluR) antagonists have been pursued as potential neuroprotective agents.

The use of the AMPA receptor antagonist NBQX in the *DJ-1A* RNAi fly model of PD yielded divergent results with regard to neuroprotection and the attenuation of neurotransmitter depletion. NBQX conferred excellent protection to DA neurons from *DJ-1A* RNAi-induced degeneration. In fact, when compared with all other drugs applied in this study, NBQX administration (30 mg/ml) offered the best preservation of DA neurons in the DMC, restoring DA neuronal number completely back to wild type levels. However, NBQX administration had no effect on DA level reduction. This divergence might be explained if DA depletion occurs before neuronal degeneration. NBQX might thus have successfully protected from secondary glutamate induced excitotoxicity and the subsequent apoptotic DA cell death, but not from diminishing DA levels, for which mechanisms other than excitotoxicity ones are causative. Apparently, DA depletion in the fly brain has the logical consequence of transmitter imbalances with increased glutamate levels, not unlike that in mammalian brains. The dopamine metabolising enzyme tyrosine hydroxylase (TH) clearly was still present in DA neurons, since it was detected by immunostaining. Its activity, however, could already have been reduced.

Several neuropathological studies suggest that neuronal death in PD results from a so called "dying back" process, in which striatal DA nerve terminals are the primary target in the degenerative process. The degree of terminal loss in the striatum was described as more pronounced than the degree of SNpc dopaminergic somata loss [46]. In addition, studies of the 6-OHDA [248-253] and MPTP [254] models describe retrograde degenerative changes in the DA neurons of the SNpc. In MPTP-treated mice, protection of striatal terminals prevented the loss of DA neurons in the SNpc [255]. Our findings

would be consistent with this course of events, in which the DA depletion, caused by oxidative and inflammatory events, is primarily due to DA neuronal death. These results suggest that excitotoxicity makes certain contribution to neuronal death in PD, but it is not causative. Treatment with glutamate receptor antagonists could therefore never constitute a single treatment for PD. In combination with other drugs, however, they might bestow neuronal survival to a certain extent, which is confined to the effects of excitotoxicity. Interestingly, NBXQ treatment (30 mg/ml) appears to be toxic for wild type flies. Reduced neuronal numbers and decreased DA levels were registered. Furthermore, it was noted that NBQX-fed wild type flies exhibited premature mortality and markedly reduced life spans by approximately 40% (data not shown). In contrast, NBQX-fed *DJ-1A* RNAi flies did not show any life span reduction when compared to untreated flies, nor did any fly of either genotype, treated with any of the other drugs used in this study in their administered doses. It is not quite clear how NBQX confers toxicity to wild type flies, but not to the genetically altered ones. It can be hypothesized that toxicity is conferred by its competitive mechanism of action. In the wild type fly with normal glutamate concentrations, AMPA inhibition might reduce the intrinsic activity of glutamate to an extent that is not compatible with life, leading to neuronal death by lack of physiological stimulation. In the *DJ-1A* RNAi fly with presumed excess glutamate, competitive inhibition might lead to near to normal glutamate binding. Despite NBQX's receptor selectivity and potency in animal models, it has to be eliminated as a drug candidate for humans because of poor pharmacokinetics. Its low water solubility at physiological pH, combined with a fast renal excretion, would cause crystallization in the kidney already at therapeutic doses [215]. The fast excretion also prevents any prolonged systemic activity in humans following oral administration. Therefore newer compounds have been and are being designed by computational mapping of the AMPA recognition site, in combination with x-ray crystallographic analysis of cocrystallized complexes at the receptor binding domain to produce better physicochemical properties (especially higher water solubility) in humans. For example, PNQX and YM872 were designed with NBQX as a template [216-218]. Here the easily available NBQX was administered to the fly PD model and still produced observable neuroprotective effects. This should encourage the design and subsequent clinical testing of newer quinoxalindiones with better pharmacokinetic properties.

This study presented a new animal model for neuroprotective drug testing for PD. None of the drugs discussed above would provide a cure for PD. Some of them, however, appear to be able to delay or decelerate disease progression by directly influencing established pathogenic factors. The direct manipulation of disease progression in PD constitutes a major breakthrough in terms of symptomatic treatment.

Preventive/protective medication will be most efficacious on the condition of an early initiation of treatment. In the *DJ-1A* fly model, treatment was offered as early as from the beginning of adulthood (day 1). Of the four drugs tested in this study, celastrol may bear the greatest neuroprotective potential for use in idiopathic PD, taking into account its described neuroprotective effect and its side effect profile. The findings of the drug study favour the hypothesis that oxidative stress is the first and foremost pathogenic factor underlying PD. Oxidative stress is thought to arise from the metabolism of dopamine itself, which produces DOPA-quinones through auto-oxidative processes and leads to the accumulation of neuromelanin and iron and to mitochondrial dysfunction. All other players thought to precipitate PD, like protein aggregation, defects in the UPS, inflammation and excitotoxicity, can easily be considered as consequences caused by oxidative damage. In turn, they all give rise to a cycle of degenerative events through the generation of secondary oxidative stress (fig. 13). Of these secondary disease-propagating factors, inflammation must be considered the cellular mechanism with the greatest and fasted destructive potential. Logically, suppression of the prime executor of injury and death, in addition to the primary insult, namely oxidative stress, would confer the most effective delay of the neurodegenerative progress. The present results of minocycline and celastrol treatment in the *DJ-1A* deficient fly strongly corroborate this hypothesis.

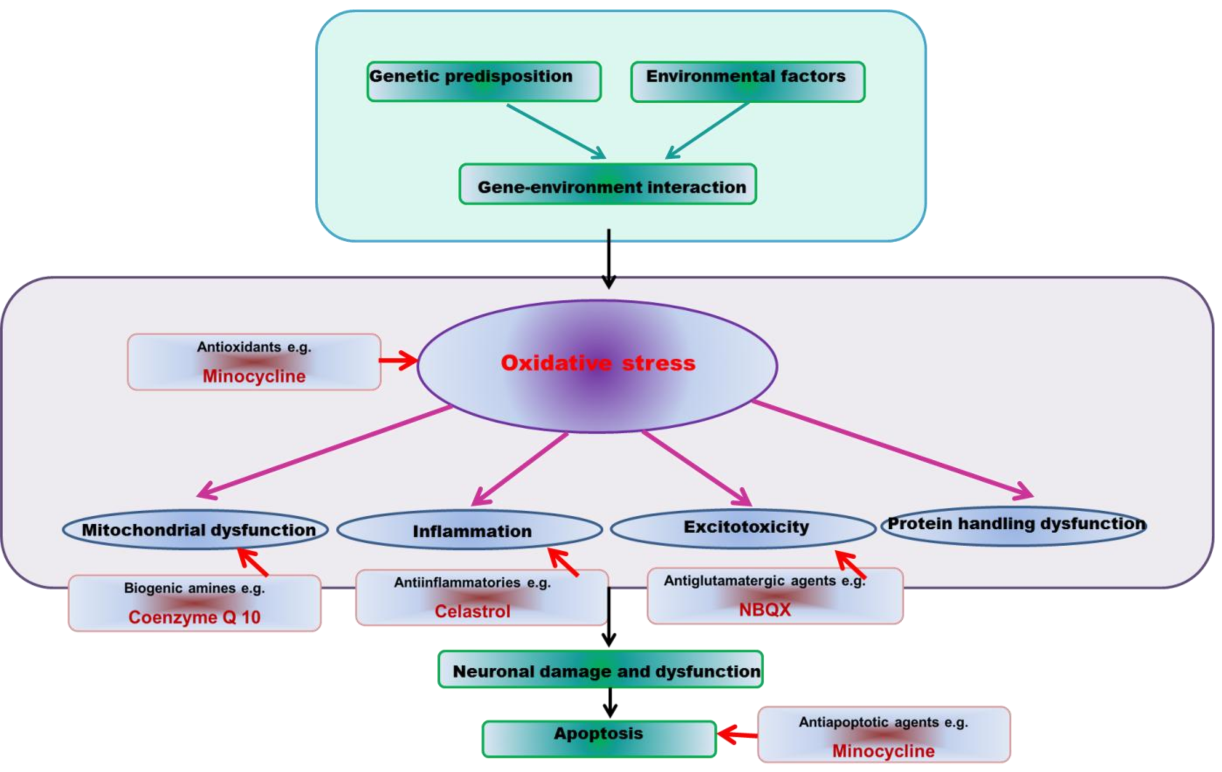


Fig. 47: Hypothesis on the pathogenesis of PD favouring the oxidative stress theory, with employed drug targets

Drosophila: a valuable new model for preclinical pharmacological studies

A major finding of this study is the suitability of *Drosophila* for pre-clinical pharmacological studies. To date, a very limited number of studies have been carried out, in which flies respond to drugs added to their food. Drug feeding to flies has been described for reserpine [256] and several biogenic amines, such as octopamine [225], L-DOPA, dopamine, tyramine, tyrosine [257], 3, 5-diiodotyrosine [227]. In the *Drosophila α-synuclein* model of PD oral administration of the Hsp90 inhibitor Geldanamycin was shown to have efficacy [228].

A few publications exist, which describe the administration of volatile drugs, such as cocaine, alcohol, and nicotine in aerosols to *Drosophila* [222-224, 258, 259]. At present, preclinical *in vivo* trials are performed predominantly in mammals, such as mice or rats, which are closer relatives to humans and in which dosage finding is generally easier. However, drug treatment in mammals is usually expensive and lengthy. In addition, results that were considered promising in cell culture studies often cannot be reproduced *in vivo*. Therefore suitable invertebrate systems in which potential drug candidates can be screened before they are administered to mammals would be time

and cost effective. Those drugs that prove inefficacious in the invertebrate might not necessarily have to be tested in mammals.

Our present studies support the notion that *Drosophila* represents an excellent preclinical tool in which to study human neurological disease. Staining of DMC dopaminergic neurons and direct dopamine measurement represent easily measurable and reliable methods for drug screening in *Drosophila*. In addition, the administration of drug preparations in yeast paste was accepted by the fly and proved to be a feasible approach. Ingestion of the drug by the fly could easily be monitored, since most drugs bear chromophores that could be detected in the digestive system through the fly's transparent chitin and also in their faeces. It goes without saying that the experimental procedures used in this pilot trial of drug feeding to flies might be improved and refined. Dose finding in the present study was based on previous empirical values. It might be helpful in the future to measure the effective concentrations of at least a certain number of drug prototypes within the fly's brain tissue after oral administration to generate conversion factors, which would facilitate dosage estimation in future studies. The major limitations on which drugs will be effective in flies are their *in vivo* stability, and the stability of the drug in yeast (or other food). For this reason, drugs were prepared freshly every second day. Previous substance feeding studies prepared the respective drug in standard fly food, which requires warming up of the instant fly food. This may affect the effective drug concentration since many drugs are not heat stable. In this study the drugs were directly mixed into yeast paste without heating. Although yeast feeding constitutes a change in the diet of *Drosophila* that can affect fly body functions including life span [260] and oxidative stress response, by including a yeast-fed control, the potential influence of change of diet on PD phenotypes is eliminated.

Drosophila possesses a highly complex nervous system of approximately 300,000 neurons, composed of numerous specialized cell types that utilize all the major classes of neurotransmitters, receptors, ion channels and signalling pathways found in humans. The level of complexity in *Drosophila* brain makes the fly capable of learning and memory and other higher cognitive functions. Moreover, *Drosophila* exhibits numerous sophisticated behaviors [142-144], which can be assessed to describe disease phenotypes. Climbing and flight can be used to a certain extent to assess motor function, however clear extrapyramidal symptoms cannot be reproduced in the fly. However, all of these qualities can be employed to sufficiently qualify and quantify the

effect of neuroactive drugs, making *Drosophila* a valuable model in preclinical drug testing, especially in the context of neurodegenerative diseases. After all, more than 70% of disease related genes in humans have a clear orthologue in the fly [261]. Based on its short lifespan and high reproduction rate, *Drosophila* will be especially valuable for large-scale and first-step screening. Only those drugs that show promise in *Drosophila* might proceed to mammals, resulting in faster and more economical animal testing.

5. Conclusions

Genetic *Drosophila* models of PD offer tremendous potential for elucidating disease mechanisms because of their amenability to systematic genetic analysis, including genetic screening for novel players in the disease process. Our results presented here prove that genetic *Drosophila* models are excellent tools for testing the therapeutic potential of drugs. Our analysis showed that Minocycline and celastrol, two compounds with antioxidant and anti-inflammatory properties, are effective in attenuating the loss of dopaminergic neurons and reduction of brain dopamine levels in the *Drosophila DJ-1A* model of PD. This result emphasizes oxidative stress and inflammation in *DJ-1* pathogenesis consistent with previous findings. Further testing of Minocycline and celastrol in other fly PD models will test the generality of involvement of oxidative stress and inflammation in the disease process. Second-generation compounds based on modification of Minocycline or celastrol could offer more effective drugs for PD intervention. High throughput compound screening with the fly PD models could also facilitate the discovery of novel PD therapeutics.

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LIST OF ABBREVIATIONS

AB	Antibody
AD	Alzheimer’s disease
ATP	Adenosine triphosphate
BBB	Blood brain barrier
ch	Chapter
CNS	Central Nervous System
COMT	Catechol-O-methyltransferase
d	day
DA	Dopamine
DLB	Dementia with Lewy bodies
DNA	Deoxyribonucleic acid
Dm	*Drosophila melanogaster*
DM	dorsomedial
DMC	dorsomedial cluster
DMSO	Dimethylsulfoxid
ER	Endoplasmatic reticulum
Fig.	Figure
HD	Huntington’s disease
IL- 1ß	Interleucin- 1 beta
IPS	Idiopathic Parkinson's syndrome
LBs	Lewy bodies
LNs	Lewy neurites
MAO-B	Monoamine oxidase-B
min	Minute(s)

MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP+	1-methyl-4-phenylpyridium
MS	Multiple sclerosis
MSA	Multi-sytem-atrophy
o.n.	overnight
Pael-R	Parkin-associated endothelin-receptor like receptor
Paraquat	1-1’-dimethyl-4,4’-bipyridium
PCR	Polymerase chain reaction
PD	Parkinson’s disease
pI	Isoelectric point
PINK-1	PTEN-induced kinase
PTEN	Protein tyrosine phosphatase with homology to tensin
RNA	Ribonucleic acid
RNAi	RNA-interference
ROS	Reactive Oxygen Species
R.T.	room temperature
s.g.	see glossary
SNCA	α- Synuclein / alpha- Synuclein
SN	Substantia nigra
SNpc	Substancia nigra pars compacta
SNPR	Substancia nigra pars reticulata
SOD1	Superoxide dismutase
SUMO	small ubiquitin-like modifier
Tbl.	Table
TH	Tyrosine Hydroxylase

TNF-α	Tumor necrosis factor- alpha
Tris	Trishydroxymethyl-aminomethan
UAS	Upstream Activating Sequence
UCH-L1	Ubiquitin carboxy-terminal hydrolase
UPS	Ubiquitin proteasom sytem

GLOSSARY

Akt-signalling:

Akt is a protein kinase, also known as protein kinase B, that promotes cell survival and opposes → apoptosis.

For example, phosphorylation of Ikappa-B-kinase by Akt leads to activation of the anti-apoptotic transcription factor NF-κB. Akt also blocks the activation of forkhead transcription factors; it phosphorylates caspase 9 and Bad, both proteins involved in the apoptotic pathway.

Akt in turn is activated by the 3-phosphoinositide-dependent protein kinase-1 (PDK1).

Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder. It is a form of dementia, characterized by progressive deterioration of cognitive function (-most strikingly: loss of short term memory), accompanied by psychiatric and behavioural changes.

The histopathological hallmark of AD is an atrophy of the frontal and temporoparietal cortex, accompanied by an inflammatory response on the cellular level, typical neuronal inclusions, called amyloid plaques and neurofibrillary tangles.

Apoptosis:

Apoptosis is a form of programmed cell death, which is executed by the cell in an active, deliberate and precisely controlled manner, in order to safely dispose of cell corpses without affecting adjacent tissue. Thus, it stands in contrast to necrosis, another form of cell death, which is passive and traumatic, usually resulting from acute cellular injury.

A certain type of proteolytic enzymes, the so-called caspases, play a central role in the apoptotic pathway.

Apoptosis is an integral part of normal tissue development and metabolism.

Astrocytes:

Astrocytes are the most abundant type of -->glial cells within the brain. They play a number of invaluable roles for the brain's cellular community: They are involved in

neuronal metabolism, extracellular ion homeostasis, recycling of neurotransmitters and modulation of synapitic plasticity. They form the --> BBB and regulate cerebral blood flow. In addition, the star-shaped astrocytes physically stabilize the brain's cellular network and form scars after brain injury.

Caspases:

Caspases are proteases; they are the main executors of → apoptosis.

Chaperones:

Chaperones are proteins that assist proper conformation of other proteins:
some chaperones are involved in repairing misfolding of damaged proteins(→ heat shock proteins); some help fold newly synthesized amino acid chains as they are extruded from the ribosome; some assist in transport across membranes; some act in protein degradation.

Excitotoxicity:

Excitotoxicity is a form of toxic damage to nerve cells that is inflicted by excess glutamate, an excitatory neurotransmitter, or by glutamate-like substances, such as NMDA or kainic acid. Overactivation of glutamate receptors leads to an influx of calcium into the cytosol, which activates a number of destructive endonucleases, phospholipases, and proteases that eventually lead to apoptotic cell death.

Fenton reaction:

Hydrogen peroxide can react non-enzymatically with ferrous iron, thereby being converted into reactive hydroxyl radicals.

$$H_2O_2 + Fe^{2+} \longrightarrow OH^- + OH^\cdot + Fe^{3+}$$

(hydrogen peroxide) (ferrous iron) (hydroxide ion) (hydroxyl radical) (ferric iron)

Glia:

Glial cells are non-neuronal cells in the brain. They outnumber neurons by 10-fold. There are various different types of glial cells with numerous functions. In summary, glia

protect and support neurons. Amongst other things they are involved in nutrition, homeostasis, transmitter metabolism, signal transmission, myelination and cellular defense against pathogens. (see also--> microglia and --> astrocytes).

Heat shock proteins:

Heat shock proteins are a subtype of → chaperones. Their expression is transcriptionally upregulated upon exposure to elevated temperatures or other cellular stressors such as toxins, inflammation/infection etc.

Huntington' s disease:

Huntington's disease(= Chorea Major) is a familial neurological disorder based on a mutation in the Huntington gene that causes trinucleotide expansion. It results in mitochondrial dysfunction and the degeneration of the caudate nucleus of the basal ganglia. Symptoms include uncontrollable movements, dystonia, cognitive impairments and various psychiatric pathologies.

JNK pathway:

C-Jun N-terminal kinase (JNK) is a serine-threonine kinase and belongs to the family of --> mitogen-activated protein kinases (MAPK). Another name for JNK is „stress-activated phosphokinase" (SAPK), since it gets activated by environmental stress, such as UV light or inflammatory cytokines. To date, more than 50 different substrates of JNK have been found. Most of them are nuclear and involve cell proliferation and transcriptional regulation, such as the transcription factor c-Jun (hence the name). Other, non-nuclear substrates of JNK are involved in signal transduction, protein degradation, inflammatory response, cell movement, and, most intriguingly, →apoptosis.

Lewy bodies:

Lewy bodies are cytoplasmic inclusion bodies that contain a heterogenous mixture of insoluble, filamentous proteins and lipids, including *α- synuclein*, ubiquitin, *PINK1*, neurofilaments and oxidized and nitrated forms of cellular proteins. They are the pathological hallmark of PD.

Longevity genes:

Genes that when overexpressed prolong life span.

MAPK:

Mitogen-activated protein-kinases are serine-threonine kinases that are involved in the regulation of numerous cellular processes, such as transcription, translation, cell division (mitosis), cell differentiation and → apoptosis. They are induced by various external stimuli, such as →mitogens.

Microglia:

Microglia are small, phagocytosing cells that clean up cell debris and protect neurons from pathogens. Microglia are immune cells; they derive from the bone marrow and are close relatives of macrophages.

Mitogen:

Mitogens are various molecules that stimulate a cell to begin cell division= mitosis.

Monoamine:

Neurotransmitter that contains one amino group connected to an aromatic ring. Examples: Epinephrine, Norepinephrine, Dopamine, Serotonin, Melanin

MSA

Multiple system atrophy (MSA) is another progressive neurodegenerative disease. It comprises the formally individually defined disease entities Shy-Drager-Syndrome and Olivopontocerebellar atrophy- also known as Parkinson-plus syndromes. It is diagnosed clinically by a combination of: -autonomic nervous symptoms (hypotension, sleep apnoe, urinary retention etc.), - parkinsonism (rigor, tremor, bradykinesia), - and ataxia (poor coordination, unsteady walk etc.). Histopathological findings include nerve cell atrophy and glial inclusion bodies.

Oxidative stress:

Oxidative stress is the result of an imbalance between pro-oxidant and antioxidant forces within a cell, leading to a change in the redox state of the cell towards a more

oxidizing environment. The main mediators of oxidative stress are reactive oxygen species (ROS) and reactive nitrogen species (RNS).

p53:

The transcription factor p53 acts as a tumor supressor: after DNA damage it initiates DNA repair mechanisms, regulates cell cycle and --> apoptosis. Loss of p53 function is critical for tumorigenesis, since p53 is mutated in approximately 50% of all human carcinomas.

Pick's disease

Pick's disease (PiD) is a sporadic subtype of frontotemporal dementia (FTD) and thus a → tauopathy. In fact, it overlaps with other syndromes of this disease complex and is not clear cut. It's pathological hallmark are so-called Pick bodies that contain tau-protein. Affected brain areas are the neocortex and the limbic system. Symptoms include apathy, neglect and memory loss.

SUMO:

SUMO (small ubiquitin like modifier) belongs to the superfamily of ubiquitin-like proteins. Like → ubiquitin, SUMO tags numerous intracellular proteins so as to post-translationally modify them in order to alter their destined location, function or half life.
It is hypothesized that SUMO plays an important role in protecting the cell from various stressors, especially heat – as it is upregulated under these conditions.

Tau:

Tau is a neuron-specific, microtubule-associated protein. Tau gets phosphorylated by a number of kinases, and hyperphosphorylation of tau leads to the self-assembly of tau filaments, which then form inclusions implicated in the pathogenesis of so-called tauopathies, such as Alzheimer's disease (AD), corticobasal degeneration (CBD), frontotemporal dementia with Parkinson's disease linked to chromosome 17 (FTDP17), Pick's disease (PiD), progressive supranuclear palsy(PSP and Down's Syndrome (DS).

Ubiquitin:

Ubiquitin is a small regulatory protein that is highly conserved through evolution and

present in all eukaryotic cells. In a process called ubiquitylation, ubiquitin is covalently attached to a protein. Mono-ubiquitylation is associated with targeting proteins to another cellular compartment or with initializing protein-protein interactions. Poly-ubiquitylation generally leads to the degradation of the substrate via the proteasome.

Eidesstattliche Versicherung

„Ich, Katharina Angela Faust, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „The role of DJ-1 in neurodegeneration and aging in a Drosophila model of Parkinson's Disease and its implications for novel neuroprotective therapies" selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)" des ICMJE -*www.**icmje**.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.
Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Betreuer, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst."

Datum Unterschrift

Anteilserklärung an etwaigen erfolgten Publikationen

Katharina Faust hatte folgenden Anteil an der folgenden Publikation:

Publikation 1:

Faust K, Gehrke S, Yang Y, Yang L, Beal MF, Lu B. (2009)
Neuroprotective effects of compounds with antioxidant and anti-inflammatory properties in a Drosophila model of Parkinson's disease.
BMC Neurosci., 2009: 10:109.

Beitrag im Einzelnen:
Katharina Faust hat die in der obigen Publikation veröffentlichten Versuche konzipiert und die Medikamentenstudie und die immunhistologischen Färbungen durchgeführt. Die HPLC-Analyse der durch Katharina Faust bereitgestellten Lysate wurde durch Lichuan Yang an der Cornell Universität in New York ausgeführt. Katharina Faust hat alle Ergebnisse ausgewertet und das Manuskript für die Publikation geschrieben.

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

Unterschrift des Doktoranden/der Doktorandin

CV

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

PUBLICATIONS by May 2014:

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