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„ Investigating the Cellular Fate of the Dopamine Transporter in Parkinson's Disease "

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„Künstler sein heißt:
nicht rechnen und zählen; reifen wie der Baum, der seine Säfte nicht drängt und getrost in den Stürmen des Frühlings steht ohne die Angst, daß dahinter kein Sommer kommen könnte. Er kommt doch“

- *Rainer Maria Rilke „Briefe an einen jungen Dichter“*

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Abstract

The dopamine transporter (DAT) is a vital part of the dopaminergic system in that it regulates the dopamine (DA) homeostasis by re-up taking excess DA back into the neuron. Parkinson's Disease (PD) is an age-related neurodegenerative disease which mainly affects the motor functions and cognitive functions related to the dopaminergic system. In this study the effects of PD related genetic mutations on the cellular DAT distribution are being investigated in a Drosophila experimental model. Changes of DAT levels and localization in the PD associated genes PINK1, PARK and α-Synuclein was determined. The in-vivo DAT expression in the Drosophila brain was quantified with an endogenously GFP-tagged transgenic DAT line in an age-dependent manner. In 2 of the 3 Drosophila genetic PD conditions, a significant and mutant specific difference in DAT levels were observed. In the PINK1-null mutant background DAT expression increased with age progression. In contrast, the knock-down Parkin showed a more variable DAT expression in dopaminergic neurons with an overall decrease over time. Similarly, the overexpression of mutant α-Synuclein, which forms toxic Lewy-bodies and result in decrease protein interaction affinity, the DAT expression is lower throughout the life span of the organism. These findings indicate PD gene-specific effects on DAT activity prior to neurodegeneration within the DA system.

Zusammenfassung

Der Dopamin Transporter (DAT) ist ein wichtiger Teil des Dopaminergen Systems, und für die Dopamin Homöostaste verantwortlich. Parkinson's Krankheit (PK) ist eine neurodegenerative Erkrankung, welches das Dopaminerge System befällt. Charakterisiert durch Neuronen Verlust, Ansammlung von Lewy-Bodys und gradueller Verlust der Motorfunktionen ist PK die zweithäufigste neurodegenerative Erkrankung der Welt. In dieser Arbeit werden die Auswirkungen von PK auf den DAT untersucht. Das verwendete Model für diese Studie ist *Drosophila melanogaster.* Dieser Model Organismus bietet die Möglichkeit die Veränderungen von DAT durch einen endogenen Green-Fluorescent-Protein (GFP) Marker zu charakterisieren und analysieren. Es wurden 3 verschiedene Methoden verwendet. Eine PINK1 null Mutation, welche zum Verlust des PINK1 Enzyms führt, eine PARK [2] knock-down Mutation welche zu reduzierten Expression des Proteins *Parkin* führt und eine durch das Gal4/UAS System überexprimierte SCNA mit einer A30P Mutation welches das Protein α-synuclein codiert. Es wurde eine altersabhängige Charakterisierung durchgeführt und die GFP-Intensität gemessen und quantifiziert. Es wurde festgestellt das die Transporter Expression in der PINK1 null Mutante signifikant steigt. Für die *Parkin* knock-down Mutante konnte eine variierende Expression festgestellt werden aber tendenziell sinkend. Die α-synuclein Überexpression führt zu einer fluktuierenden Expression wie in den *Parkin* Mutanten allerdings ist hier die allgemeine Expression verringert. In allen bis auf die PINK1 Mutante wurde die Expression verringert. Dies kann auf den eingeschränkten Abbau durch verringerte *Parkin* Expression und toxische Eigenschaften von α-synuclein. Im Allgemeinen kann der Effekt von PK assoziierten Genmutanten auf DAT deutlich festgestellt werden.

1. Introduction

1.1 The Dopamine Transporter

The dopamine transporter (DAT) is a transmembrane protein, which sits at the synaptic and extrasynaptical sites, and moves dopamine (DA) across the membrane to regulate dopamine homeostasis (Miller et al., 1999). The transporter is part of the solute carrier (SLC) 6 family of transporters and uptake of dopamine is driven by symport of one sodium (Pramod et al., 2013). The DAT protein comprises 12 transmembrane domains, connected by intra- and extracellular loops and cytoplasmic C- and N- termini (Figure 1). DAT is a target of various pharmacological compounds as well as illicit substances (Shizgal & Hyman, 2013).

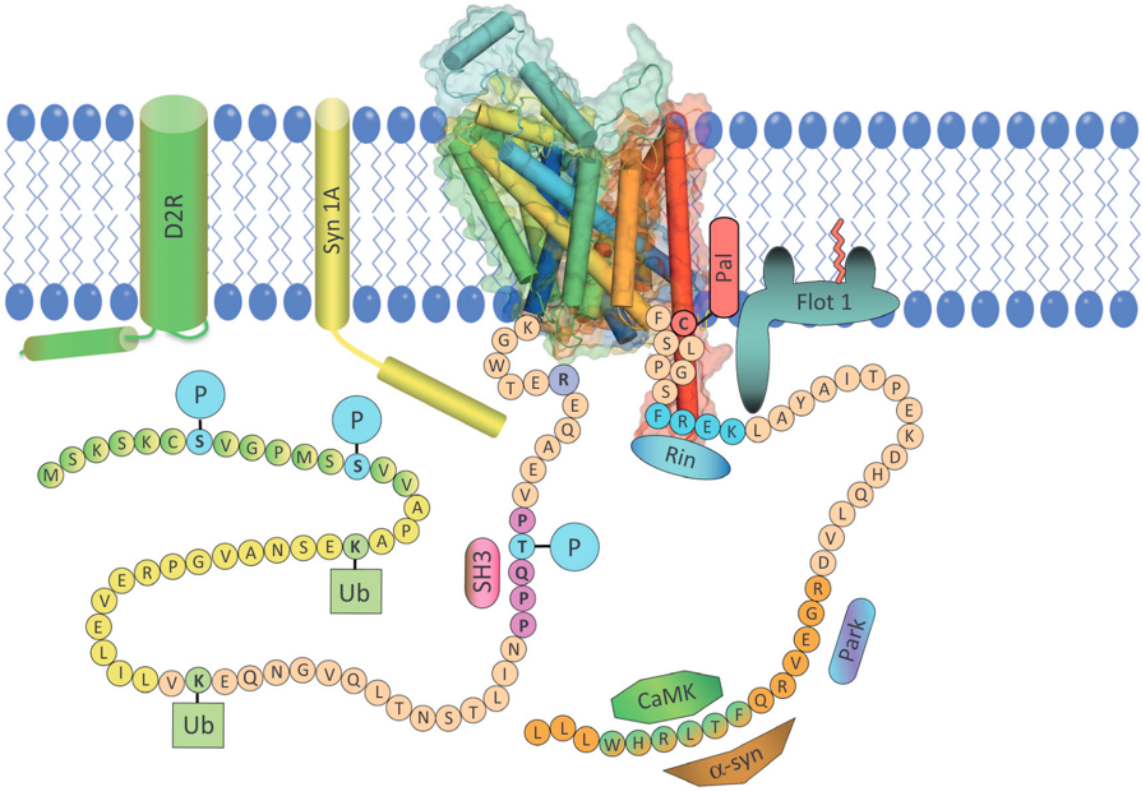

Figure 1 ***The dopamine transporter depicted in a schematic****(Vaughan, 2013)The transporter depicted is from a rodent. The main portion of the transporter is inside the plasma membrane. These are called the membrane spanning domains. The extracellular loop domain (EL domain) is important for post translational modification(Foster & Vaughan, 2011). The C and N termini are inside the cell and responsible for regulatory processes(Gorentla et al., 2009).*

DAT maintains functional homeostasis of dopaminergic synapses. Following dopamine release, presynaptic transmitter transporter “reuptakes” dopamine back in the dopaminergic neuron via the alternating access model (Andersen et al., 2009). DAT is a Na^+/Cl^- dependent symporter and undergoes a conformation change (Figure 2). The first stage is an outward facing open state, in which DAT is accessible to the extracellular Na^+ and Cl^- along with substrate dopamine (Andersen et al., 2009). Here the substrate along with two Na^+ and one Cl^- bind the transporter. The second outward facing stage closes the pore and no more substrate can enter the transporter. This outward occluded state changes into the inward

facing state and after opening the gates the substrate can flow into the cell. The inward open conformation is accessible to the potassium ion binding and as soon as potassium binds to the transporter, the transporter shuttles from inward open to outward open state.

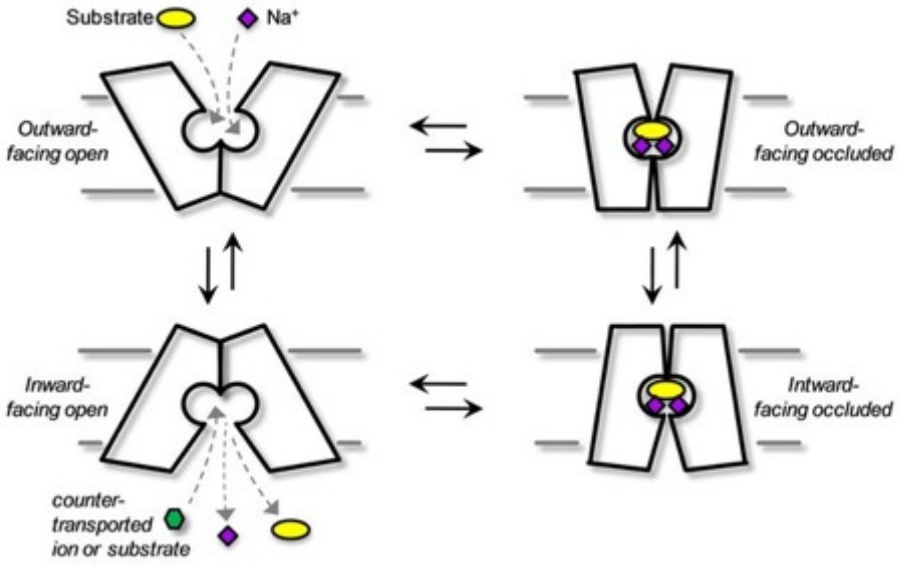

Figure 2 ***Alternating access model for the SLC6 transporters*** *(Andersen et al., 2009). Changing from an outward facing state, meaning the transporter is open to the outer side of the membrane, over to a transitional stage the occluded stage to the inward facing position. The Channel phase where both sides of the transporter are open for substrate flow. These stages can switch along the arrow direction shown in schematic.*

The human and the *Drosophila melanogaster* DAT-genes (hDAT and dDAT) show a 57.97% sequence similarity (Jumper et al., 2021; Varadi et al., 2022). AlphaFold, an A.I. developed to estimate protein structures through the protein sequence database Uniport, shows the crystal structure of the dDAT (Fig 3 left) and in humans (Fig 3 right). The certainty interval is color-coded ranging from dark blue (very high likelihood) to orange (low likelihood). This means, that if an area is dark blue the likelihood of the structure being correct is very high.

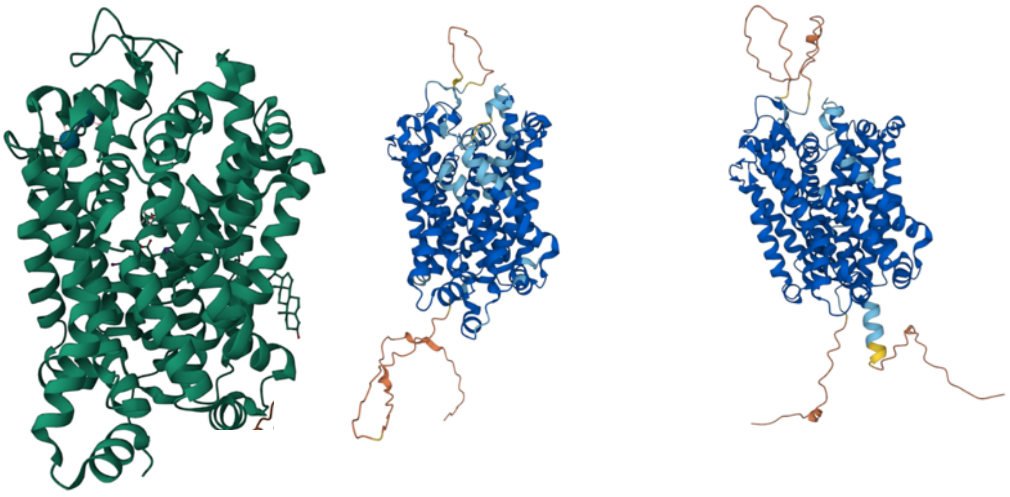

Figure ***3 Crystal structure and predicted structures of the Dopamine transporter****. On the left D DAT (PDB ID 4XP3), middle h-DAT (Homo sapiens) and on the right d-DAT (Drosophila melanogaster). Middle and right, the*

proteins were visualized with the Deep learning machine AlphaFold. The colour scheme represents the confidence modality with which the conformation is shown. Dark blue has a likelihood Very high (pLDDT > 90, light blue Confident (90 > pLDDT > 70), yellow Low (70 > pLDDT > 50) and orange Very low (pLDDT < 50). pLDDT is a pre-residue confidence score, scoring between 0 and 100. Values below 50 may be unstructured in isolation(Jumper et al., 2021; Varadi et al., 2022)

DAT is responsible for dopamine homeostasis by re-up taking the extracellular dopamine. This mechanism is vital to the recycling and the overall balance of the system. There are different neurological diseases which manifest if the DA system suffers in synaptic homeostasis. ADHD, OCD and addiction fall in this category (Cinque et al., 2018; Cook et al., n.d.; Li et al., 2004). After disassociating from the receptors the leftover DA is reuptaken by DAT and either stored, recycled or degraded in the presynaptic compartment.

1.2 The Dopaminergic System

The dopaminergic system is involved in the sleep-regulation, mood-regulation, the reward system, memory, attention, addiction and stress-responses in humans (Alcaro et al., 2007; Cabib & Puglisi-Allegra, 1996; Gorwood et al., 2012).

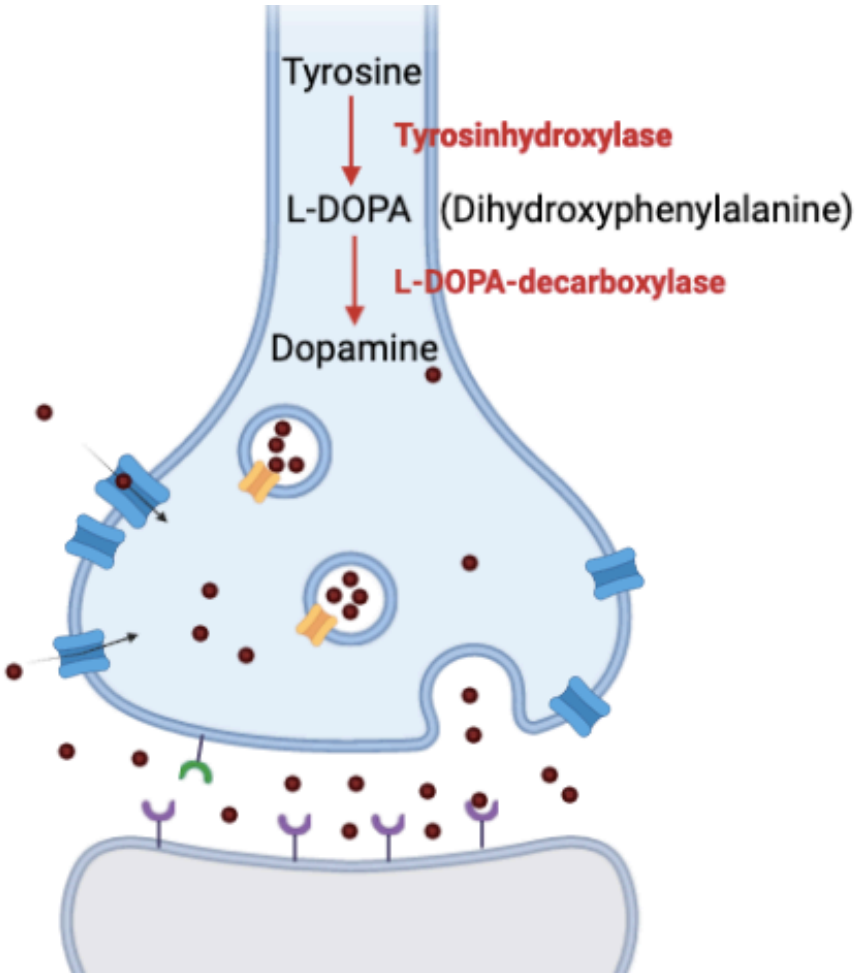

Figure 4 ***Schematic of a dopaminergic synapse.*** *In light blue the pre-synapse and in grey the post-synapse are shown. DA receptors are indicated in green (presynaptic receptors) and purple (postsynaptic receptors). DAT is coloured in dark blue and VMAT in yellow. The amino acid Tyrosine will be hydroxylated into L-DOPA via the tyrosine hydroxylase. L-DOPA will then be decarboxylated into Dopamine via the L-DOPA-decarboxylase. The produced DA then will be stored in vesicles and transported to the synaptic cleft. There the DA will be released and can bind to the DA receptors at the post-synaptic side. or presynaptic cleft. If enough DA binds to a receptor on the post-synapse an action potential can be produced. After binding to the receptor, dopamine disassociates and will be reabsorbed into the presynapse and stored or recycled.*

At the synaptic level two types of receptor families can be found, excitatory receptors D1 and D5 , 3 inhibitory receptors D2, 3 and 4, and a transmembrane protein DAT (Tab.1). The synthesis (Fig.4) of dopamine starts with the aminoacid tyrosine which either is readily available or itself synthesized by hydroxylation of phenylalanine. Tyrosine is also hydroxylated into L-Dihydroxyphenylalanine (L-DOPA). L-DOPA is being used in pharmacological treatment in various neurological conditions and will be discussed later. L-DOPA then decarboxylates into the neurotransmitter dopamine. After synthesis dopamine is packaged into vesicles via the vesicular monoamine transporter (Fig.4) (VMAT) (Fig. 4). These vesicles function as storage units (Caudle et al., 2008) or release dopamine into the synaptic cleft via exocytosis (Fig. 4). The release is triggered via a presynaptic action potential which opens voltage gated Ca^{2+} channels and the influx of Ca^{2+} causes the vesicles to fuse with the presynaptic membrane (Siegelbaum & Kandel, 2013). After release the dopamine will bind to the receptors either on the post or presynaptic membrane. Through binding to the post synaptic receptors second messenger proteins phosphorylate ion channels and thereby opening them (Siegelbaum & Kandel, 2013). If enough ion channels are opened the depolarization is big enough and an action potential is triggered. After depolarization the dopamine quickly disassociates from the receptors and are reabsorbed into the presynapse either through the DAT or the plasma membrane protein transporter. Back in the presynapse the dopamine can be put in storage or broken down and recycled.

1.2.1 The dopaminergic system in humans

There are 3 major DA projection pathways in the human brain, the mesolimbic, nigrostriatal and mesocortical pathway. Their projection pathways are shown in Figure 4. The mesolimbic pathway originates in the ventral tegmental area in the midbrain and projects into the ventral stratium (Reneman et al., 2021). It is involved in reward/aversion associated functions like motivation (Hauser et al., 2017). The mesocortical pathway originates in the ventral tegmental area into the prefrontal cortex and is involved in executive functions (Hauser et al., 2017). Together they make up the mesocorticolimbic system (Hauser et al., 2017). The nigrostriatal pathway originates in the zona compacta of the substancia nigra and projects to the putamen and the caudate nucleus (Reneman et al., 2021). Its associated functions include the motor function, associative learning, and reward cognition (Cox & Witten, 2019) and in a recent study

Chen et al. (2022) postulate that the nigrostriatal pathway is involved in auditory frequency discrimination.

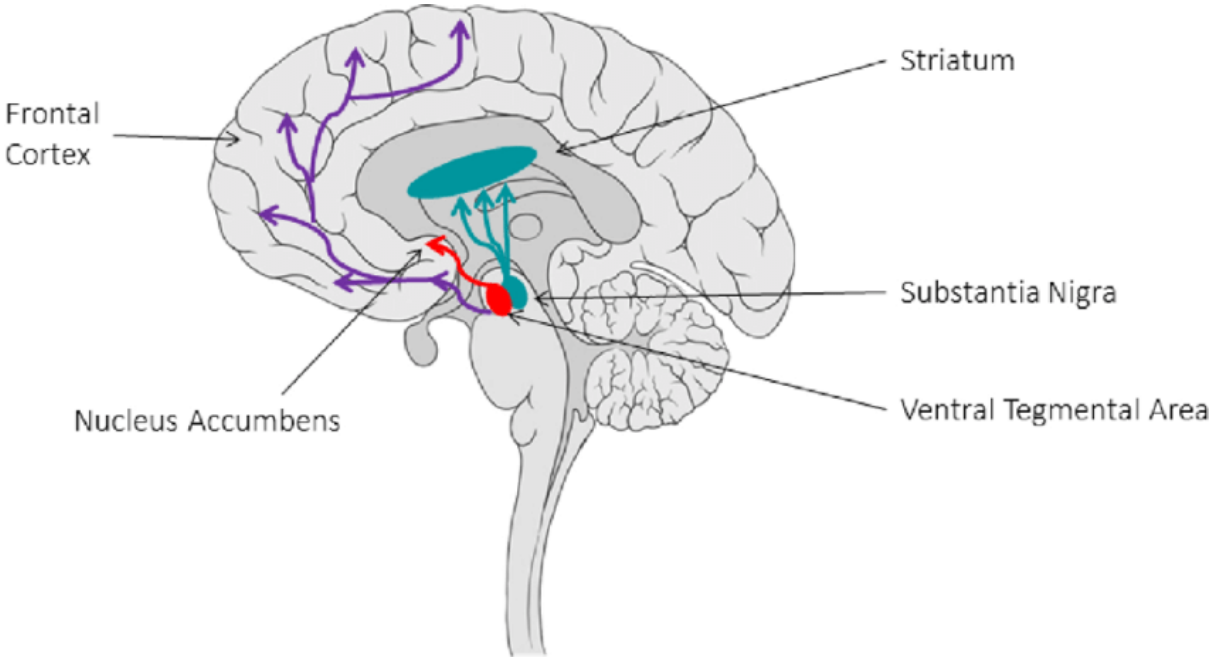

Figure 5 ***The dopaminergic pathway in the human brain*** *(Reneman et al., 2021) It consists of multiple pathways, but depicted are the 3 major ones: The nigrostriatal (blue), mesolimbic (red) and mesocortical (purple).*

Proteins associated with the dopaminergic system in humans include two types of receptors , Type 1 and Type 2 (Table 1) and the transmembrane protein DAT: Type 1 (D1-like-receptors) are excitatory G-protein coupled receptor types, which are mostly found on the postsynapse. Most abundantly found in the hippocampus, striatum, nucleus accumbens and olfactory tubercle, hypothalamus and in the limbic system(Jaber et al., 1996).

Type 2 (D2-like-receptors) are inhibitory G-protein coupled receptor types, which are involved in regulator functions such as locomotor activity, cognition and motivation (Björklund & Dunnett, 2007). A subtype of the D2-receptors are autoreceptors located mainly on the dendrites and soma of midbrain neurons in the ventral tegmental are and the substantia nigra pars compacta, as well as the axonal terminals of their projection areas (Beaulieu & Gainetdinov, 2011; Björklund & Dunnett, 2007). It directly regulates the potassium conductance and indirectly regulates tyrosine hydroxylase expression and the DAT for dopamine reuptake. (Anzalone et al., 2012)

Table 1 ***Associated proteins of the dopaminergic system in humans consists of two receptor subtypes Type1 and Type 2 and the transmembrane protein DAT.*** *Type 1 is excitatory and G-Protein couples, whereas Type 2 is inhibitory and G-Protein coupled.*

Protein	Signaling/mechanism
TYPE 1 (D1 and D5)	G-coupled, excitatory to cAMP
TYPE 2 (D2, D3 and D4)	G-coupled, inhibitory to cAMP
DAT	Na+, Cl- coupled

1.2.2 The dopaminergic system of *Drosophila melanogaster*

In *Drosophila* the dopaminergic system in the adult brain is comprised of 8 neuronal clusters consisting of 127 neurons (Figure 6) (Kasture et al., 2018). The associated receptors (Table 2) consist of 4 receptor types and one transmembrane transporter.

Table 2 ***Associated proteins of the dopaminergic system in Drosophila melanogaster.*** *Dop1R1/2 are excitatory G-coupled receptors which increase the intercellular levels of cAMP. DopEcR is involved in two signalling cascades: Dopamine activated has an excitatory effect and increases cAMP levels, Ecdoysone activates the MAPKinase pathway in the cell. D2R is inhibitory in nature and reduces if activated by dopamine the cAMP levels.*

Protein	Signaling/mechanism
Dop1R1	Gs-coupled, excitatory
Dop1R2	Gs-coupled, excitatory
DopEcR	Dopamine: Gs-coupled, excitatory Ecdysone: MAPKinase pathway
D2R	G_i-coupled, inhibitory
DAT	Na+, Cl- coupled

Like in humans these are of excitatory and inhibitory in nature (Karam et al., 2020) and G-protein coupled. Dop1R1/R2 are responsible for excitatory signaling cascades involving the increase of intercellular cAMP. They are also called D1-like receptors due to their similar function and overlapping sequence identity to the human D1 receptor (Karam et al., 2020). DopEcR is also responsible for excitatory signaling through elevating the cAMP levels intercellularly. This receptor also binds with the hormone ecdysone and activates the MAPK pathway (Srivastava et al., 2005). D2R falls under the D2-like receptor family and is highly homologous with the human D2 receptor. It is inhibitory in nature and reduces the intercellular cAMP levels. The DAT is a transmembrane protein which is responsible for the recycling and reuptake of extracellular dopamine.

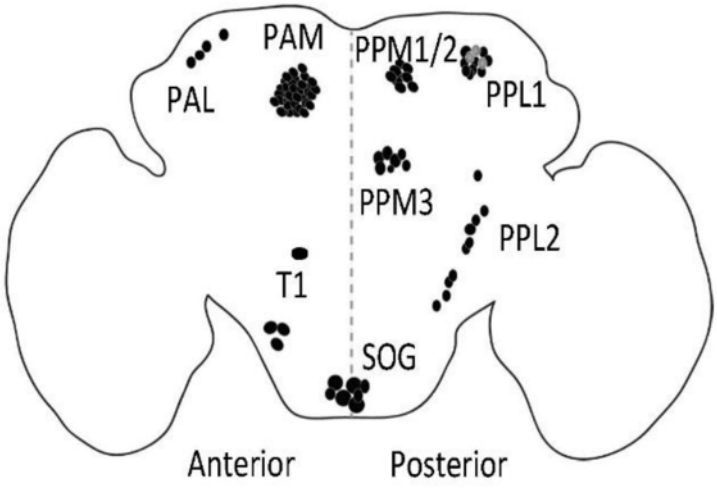

Figure 6 ***Dopaminergic Neuronal Clusters of the Drosophila melanogaster brain****(Kasture et al., 2018) Left side depicts the anterior view and the right side the posterior view. There are 8 distinct clusters and 127 neurons associated with the dopaminergic system in the Drosophila melanogaster.*

The main focus of this study is the Mushroom Body (MB)-area (Fig 7) which is responsible for olfactory memory and learning, decision making, aversive memory formation, sleep modulation and other functions associated with learning (Kasture et al., 2018). It is mainly composed of Kenyon cells (KCs), MB output neurons (MBONs) and dopaminergic neurons (DANs) (Guo et al., 2017). The dopaminergic neurons innervate the MB by 3 neuronal clusters: PAM, PPL1 and PPL2 (Fig 6). In Table 3 the innervating area and the function of these neuron clusters are summarized. PAM neurons are part of reward signalling, memory formation, aversive memory formation, negative geotaxis, promote wakefulness and foraging behavior. They innervate the β- and γ- lobes of the MBs. PPL1 is involved in modulating sleep, aversive memory formation and negative geotaxis. They innervate the γ- and α- lobes of the MBs. PPL2 neurons enhance behavioral memory strength when activated during olfactory classical conditioning and increase odor evoked calcium response to a paired odor. They innervate unlike the other groups the calyx. The central complex (CX) is another area innervated by the dopaminergic neurons.

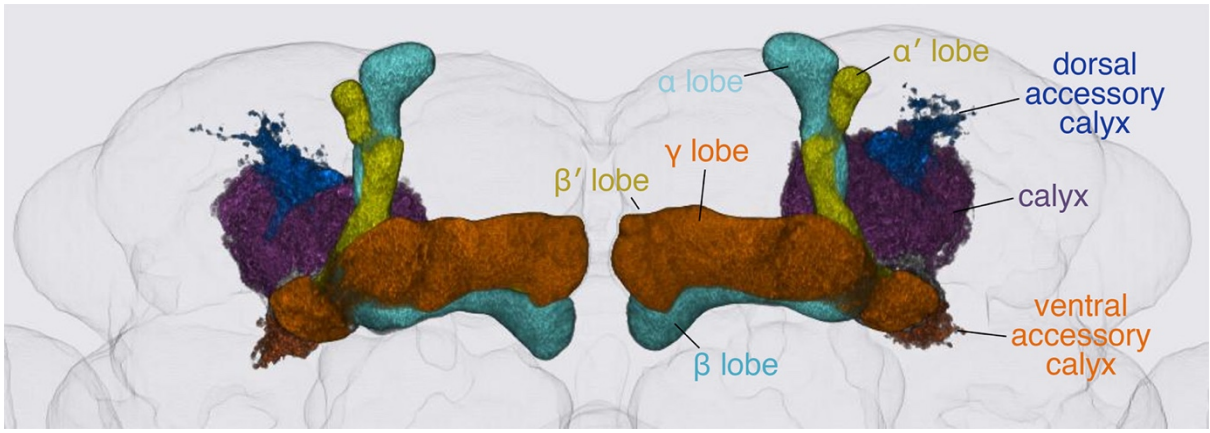

Figure 7 ***Mushroom Body anatomy. Depicted are the α-, β-and γ-lobes.*** *The calyx, the ventral and dorsal accessory calyx (Aso et al., 2014)*

Table 3 ***Dopaminergic neurons which innervate the mushroombody include the PAM, PPL1, and PPL2 clusters.*** *PAM neuronal cluster innervates the β- and γ- lobes of the MB and are involved in reward signalling, memory formation, aversive memory formation, negative geotaxis, promote wakefulness and foraging behavior. PPL1 neuronal cluster innervates the γ- and α- lobes of the MB and are involved in modulating sleep, aversive memory formation and negative geotaxis. PPL2 innervates the calyx of the MB and is involved in enhancing behavioral memory strength and increase odor evoked calcium response to a paired odor.*

Neuronal cluster	Innervation area	Function	References
PAM	β- and γ- lobes of the MB	Reward signalling, memory formation, aversive memory formation, negative geotaxis, promote wakefulness, foraging behavior	(Kasture et al., 2018; Tanaka et al., 2008)
PPL1	*innervate in the γ-and α-lobes of the mushroombody*	Modulate sleep, aversive memory formation, negative geotaxis	(Kasture et al., 2018; Tanaka et al., 2008)
PPL2	*innervate in the calyx of the mushroombody*	enhance behavioral memory strength, increase odor evoked calcium response to a paired odor	(Boto et al., 2019)

The dopaminergic system is highly conserved throughout evolution (Kaun et al., 2012; Neckameyer et al., 2000). Furthermore, *Drosophila* carry over 75% of human disease causing genes orthologues (Reiter et al., 2001). *Drosophila* is an ideal model organism to study the mechanisms underlying human neurodegenerative diseases, due to their fast generation cycles and shorter life spans. The model has a broad genetic tool kit which can be useful studying the underlying genetic causes and implication in neurodegenerative diseases like Parkinson's Disease

1.3 Parkinson's Disease

A human neurodegenerative disease which specifically effects the dopaminergic system is Parkinson's Disease (PD). It is an age-related disease in which the symptom onset is around the age of 50-59 (Bloem et al., 2021). Early onset PD variants are mostly genetically caused and can be diagnosed around 35+ years of age (Balestrino & Schapira, 2020). The disease progression involves the formation of Lewy-bodies which are misfolded protein aggregations

inside the cells of the dopaminergic system. The main region affected is the substantia nigra in the basal ganglia of the human brain. PD is most commonly diagnosed via the motor disfunction. There are early symptoms which are non-motor related (non-motor symptoms, NMS). These symptoms include depression, anxiety, apathy constipation, sleep disorders (ranging from insomnia to sleep-disordered breathing), olfactory disturbances and pain either primary or secondary (Balestrino & Schapira, 2020; Bloem et al., 2021; Noyce et al., 2012). PD is not the primary cause of death but facilitates diseases like aspiration pneumonia or complications via fractures.

1.3.1 Diagnosis

The clinical diagnosis of PD is dependent on multiple symptoms, most importantly bradykinesia (slowness of movement and speed). The difficulty of diagnosing PD relates to the symptoms which are associated with the disease. Most of the time a valid diagnosis in the early stages is very difficult even for experts and the term used for PD like symptomatic diseases is parkinsonism. Only after onset of motor-defects (bradykinesia, rest tremor and rigidity), a positive response to levodopa treatment and the exclusion of certain symptoms like early falls and ataxia can lead to a Parkinson diagnosis (Bloem et al., 2021). It is speculated that the actual disease onset starts up to 5 years before the appearance of motor-defect symptoms. In general, the diagnosis follows a 3-step progress:

1. Distinguishing between Parkinson syndrome and its lookalikes (pyramidal slowing, depression, dystonic tremors)
2. The actual PD diagnosis or the diagnosis for Parkinsonism
3. Differentiation between genetic and sporadic PD

Another way of diagnosing patients with PD is the use of positron emission tomography (PET) and Single Photon emission computed tomography (SPECT). Here a radiotracer fluid is injected into the patient which labels a specific protein, in this instance the dopamine transporter. The reduction of the transporter in the PD affected regions such as the caudate nucleus, the anterior and posterior putamen(Kong et al., 2020; Pogarell et al., 2006) can be measured and used for early diagnosis, prior to the motor defects

1.3.1.1 Genetic causes for PD

Nine gene-families are known to be linked to a specific type of PD, the genetic early onset PD (Balestrino & Schapira, 2020). Name them! These genes have garnered attention in research recently. For this project three of these five genes were chosen for further investigation of the role of dopamine transporter and will be presented below.

The PTEN-induced putative kinase 1 (PINK1) is part of the of the autophagy of the mitochondria, also called mitophagy, and the cycle which controls the amount of mitochondria

(fission-fusion-cycle) (Deas et al., 2009) in the cell. In a healthy state the kinase is steadily degraded in the mitochondria, more specifically between the outer and inner membrane. If the mitochondria are damaged and lose their membrane potential this degradation process cannot occur and PINK1 accumulates at the surface. This recruits the E3 ubiquitin ligase *parkin* and ubiquitylates the mitochondria. Afterwards the mitochondria will be isolated and engulfed by the lysosomes. If this degradation process is inhibited through mutations of PINK1 or *parkin* , the quality control which keeps up mitochondrial health cannot occur in the cell and this produces Radical Oxygen Species (ROS) which are toxic to the cell (Deas et al., 2009; Springer & Kahle, 2011; Youle & Narendra, 2011). This loss of quality control has been linked to early onset PD (Valente et al., 2004). The question why dopaminergic neurons specifically suffer under this mutation type is still unclear.

The *parkin*-mediated ubiquitination process consists of three steps: E1-ligase activates ubiquitin, then gets passed to the E2 and finally interacts with E3 to transfer the ubiquitin to the target protein (Seirafi et al., 2015). In early onset PD it is most often associated with PINK1 in the mitophagy process. However, the loss of *parkin* through missense mutations have been established to cause specific type of early onset PD which is not associated with PINK1 (von Coelln et al., 2004). *Parkin* has also been reported to promote cell survival, preventing cell death through proteasomal degradation (Ekholm-Reed et al., 2013). In non-neuronal cells it is also implicated in tumour suppression (Seirafi et al., 2015). The predominant theory regarding the disease progression with the loss of *parkin* is, that without the proper degradation of specific proteins, which are targeted by parkin the accumulation leads to cell stress and ends in neuronal death (Seirafi et al., 2015)

SNCA encodes the membrane associated protein α-Synuclein. α-Synuclein is only found in vertebrates (Oaks & Sidhu, 2011) and is part of the vesicle transport of neurotransmitter and release (Burré et al., 2010; Nemani et al., 2010). It is also found to modulate the expression and distribution of several cell compounds (Scott et al., 2010; Sousa et al., 2009).The A30P mutant, which is located at the N-terminus of the protein, leads reduced affinity for biological membranes and with 2 other mutations (A53T and E46K) are the causes for α-synuclein associated familial PD (Bellucci et al., 2012). These mutations lead to several different effects in the neurons. In PD the most important effect is the aggregation of α-synuclein and formation Lewy Bodies. α-synuclein is also implicated in other neurodegenerative diseases such as Alzheimer's Disease and Dementia. Specifically PD has been modelled in Drosophila by Feany & Bender (2000). They showed, via the introduction of the α-synuclein mutant A30P, punctate formation which were suggested to be aggregation. Through electron microscopy they found cytoplasmic inclusions in the brain. Furthermore, the locomotive abilities were reduced and with age they lost the ability to climb. There have also been studies which show that mouse models are suboptimal for PD research (Vingill et al., 2018).

1.3.1.2 Environmental

The main factors in environmental causes for PD are the influence of pesticides and physical Trauma. There is a high correlation between the PD onset and the patient's pesticide contact rate (Balestrino & Schapira, 2020). There is also an impression that dietic factors such as smoking and drinking alcohol play a role in risk reduction (Bloem et al., 2021).

1.3.2 Treatment

Treatment of PD most prevalently targets the production, release and reuptake of DA (Bloem et al., 2021). The most important treatment is the administration of levodopa (L-DOPA). L-DOPA is a precursor of DA and if administered orally the DA levels will increase and counteract motor-function related symptoms. The second type of treatment is DA agonists. These drugs bind to the DA receptors and mimic the lacking DA, for example DA agonists like pramipexole or ropinirole. The third group are monoamine-oxidase-B inhibitors. These oxidases break down monoamines in neurons. By inhibiting, the DA levels can be increased and motor-symptoms slightly reduced. All these drugs are not meant for curing the disease and are only used for symptomatic treatment and disease diagnosis.

1.4 Aims

In this thesis the influence of the neurodegenerative Parkinson's disease on the dopamine transporter in the model organism *drosophila melanogaster* is being studied. Since Parkinson's Disease targets specifically the dopaminergic system it is a perfect disease model to study the effect on the DAT.

To achieve these 3 aims have been phrased:

- First the characterization of the DAT expression in the mushroombody area, an area where the most dopaminergic neurons are clustered and project into. This expression will be compared to a control group, which is the w1118 wildtype fly.
- Second, since PD is an age-related disease the degradation of the cellular dopamine neurons will progress over time. To account for this an analysis of the DAT expression in a time frame of 3-20 days, or in the case of the PARK[2] mutant 3-10 days will be conducted.
- Third, the impact of the degradation of the dopaminergic neurons in the model organism has a direct effect on the locomotion and climbing activity of the organism, since dopamine neurons are responsible for motor activity in the brain, it will be analyzed via a locomotion assay and the RING assay.

These aspects will be studied via 3 model systems:

- In mutant background via PINK1 null-mutants and PARK[2] knock-down mutant

- Via α-synuclein mutant overexpression through the Gal4/UAS system

2. Results

2.1 DAT-GFP Characterization and Quantification

In the present work, a transgenic Drosophila lines expressing carboxyl-terminus GFP-tagged DAT was used to study effect of neurodegenerative processes on the expression of the DAT. Figure 8 shows the schematic of a fly brain, which are used for the characterizations.

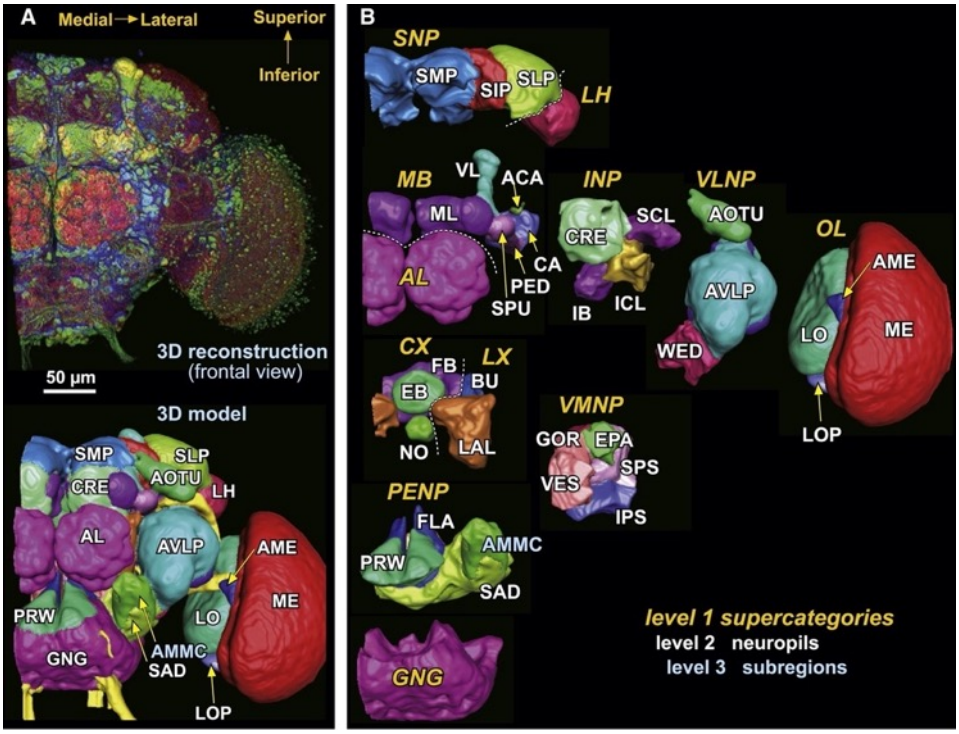

Figure 8 ***Nomenclature used for the Drosophila melanogaster brain*** *(Ito et al. 2014)*

Figure 9 shows the DAT-GFP expression across the adult fly brain. The main DAT expression can be found in the mushroom bodies (MBs) (Fig 9d1) in which the ventral and medial lobes are clearly visible. This area was indicated via the dotes outline in Figure 9 A. There is faint expression in the Antennal Lobes (AL) (Fig 9D). In addition, a noticeable GFP signal comes from the crepine area (CRE) (Fig 9d1) which lies between the mushroombody lobes.

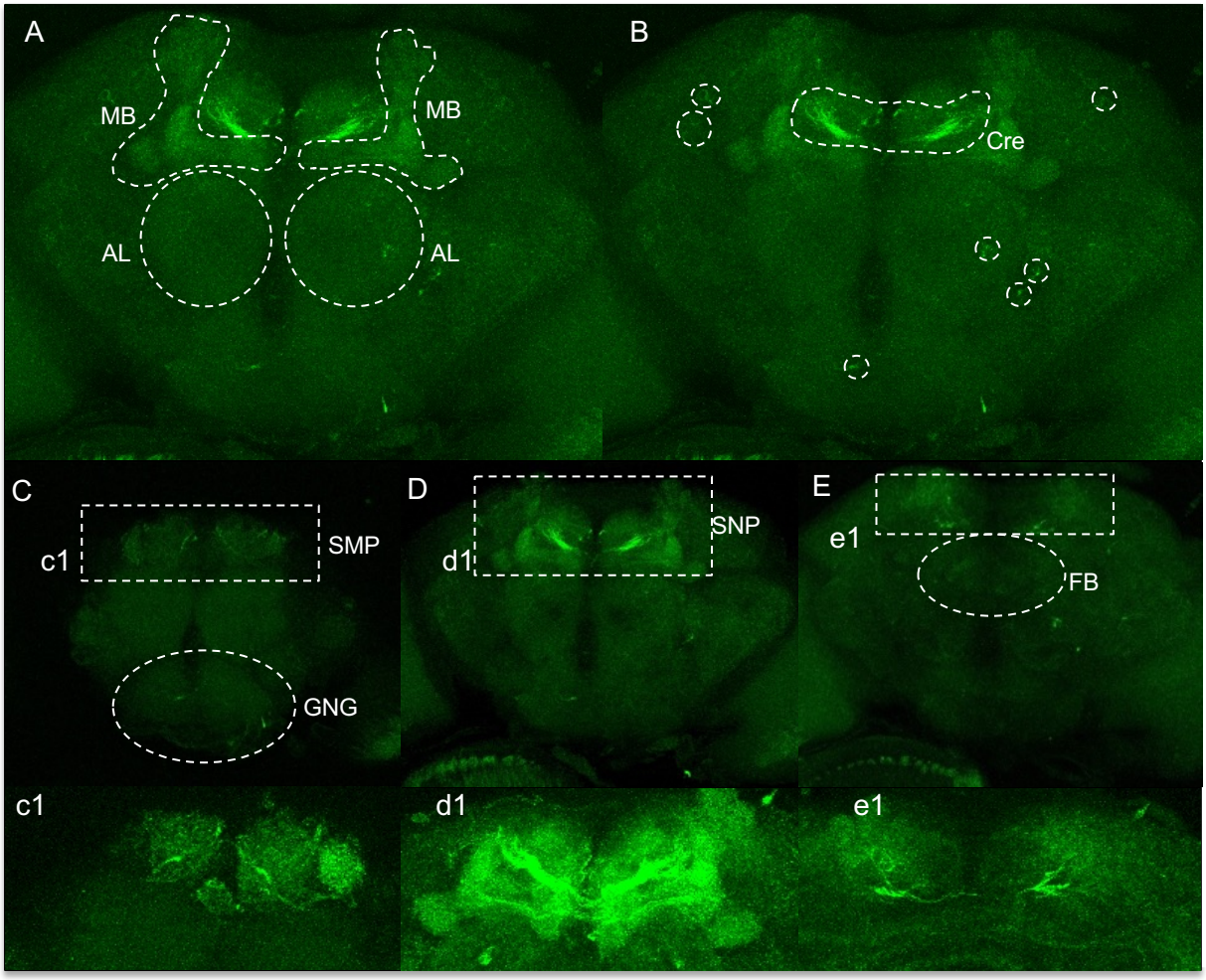

Figure 9 ***DAT-GFP expression in the central*** *brain of a 5-day old male D. melanogaster.(A to B) Merged Image showing a frontal view. The green signal represents the endogenously GFP-tagged DAT, which shows high expression levels in the mushroom bodies (dotted line in A) and in the cell bodies which are indicated with dotted circles(B). Depicted from anterior position(C) to the central (D)and posterior (E) part of the brain. Anterior: Visible expression in the SMP area(c1). and two puncta in the gnathal ganglia (GNG) area(C). Centre: Higher Cre and SNP area expression. The mushroom body shows increased expression in the ventral and medial lobes, as well as the calyx and the pedunculus. Some cell bodies display high expression (d1). Posterior: Expression in the fan-shaped-body (FB).*

In Figure 9 E the posterior of the brain the expression can be seen in the central complex (CX) and especially in the fan shaped body (FB)(Fig 9E) Furthermore, in the posterior part(Fig 9e1) the superior lateral protocerebrum (SLP) shows DAT-GFP expression. Apart from general area expression, singular puncta-like expressions were observed in the brain. Some are associated with the cell bodies (Fig 9B), which were visualized via anti-body staining targeting tyrosine hydroxylase.

Since the 3 mutants used in the experiments, namely null-PINK1 mutation, PARK[2] knock-down mutant and α-synuclein overexpression with a A30P mutation, are known to show age dependent changes of what, the DAT-sfGFP expression in the MB area over a range of 20

days has been quantified and analyzed (Figure 10). There is a noticeable fluctuation in the 5 different timepoints, Day 7 (fTRG D7) being the highest day of expression and Day 20 (fTRG D20) being the lowest.

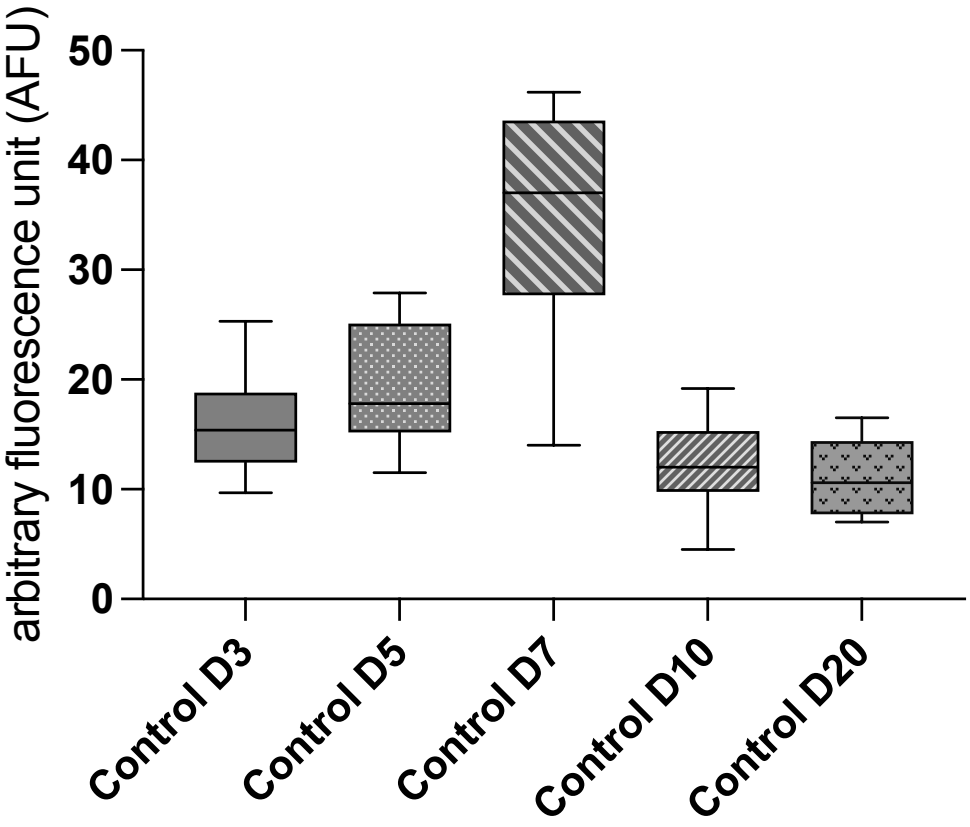

Figure 10 ***Quantification of GFP-intensity measured in the arbitrary fluorescence unit (AFU) over the course of 20 days.*** *Normal expression around 10 to 20 units. Fluctuations in-between days could be observed. Day 7 shows the highest expression levels and day 20 being the lowest.*

2.2 PINK1 mutant Drosophila show significant difference in DAT-GFP expression

In the null-PINK1 mutation (PINK1;;;) first the DAT-GFP signal was characterized in age dependent manner and quantified to study age associated changes.

2.2.1 Age dependent characterization of PINK1 mutant flies

The characterization of the age dependent DAT-GFP signal change through PINK1 mutant influence has been broken down to 5 time points: 3, 5-, 7-, 10- and 20-day old flies. Here the phenotypical differences were analyzed.

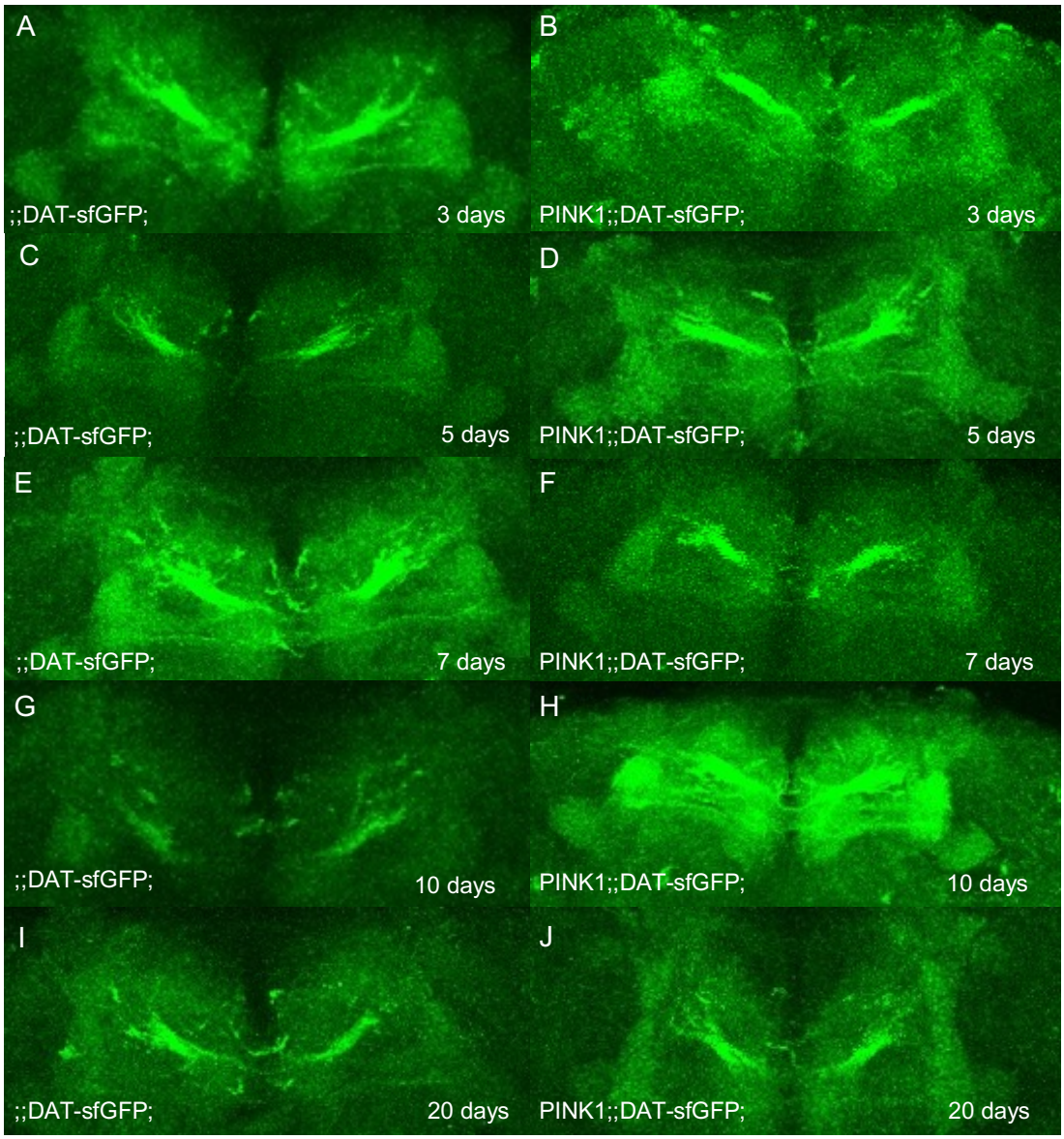

Figure 11 ***Characterization of Age dependent DAT-GFP expression in PINK1 mutants.*** *Mutant condition (right) compared to control group (left).* ***Day 3*** *(B) condition shows a higher DAT-GFP intensity. There are puncta like signal in the mutant condition compared to the control (A). The control has these puncta as well but with lower intensity. The expression pattern in the MB area is similar.* ***Day 5*** *(D) condition is significantly higher in intensity and not only the MB area is clearly distinguishable from the control (C) but also the antennal lobes and the AVLP. Reduction in puncta formation in mutant condition as well as control. Cell bodies associated with the PAL DA neuronal clusters are visible (this was confirmed via TH-staining).* ***Day 7*** *(F) Here the mutant condition DAT-GFP signal is significantly lower than the control group (E). Puncta formation is present although not as prevalent.* ***Day 10*** *(H) In mutant condition the intensity is higher again compared to the control (G) the lobes of the MB are clearly visible. Puncta are also clearly visible. The AVLP shows a signal increase.* ***Day 20*** *(J) In the mutant condition the lobes of the MB are distinct compared to the control (I) where the lobes seem to have the same intensity as the parts behind it. Puncta formation reduced compared to the control group.*

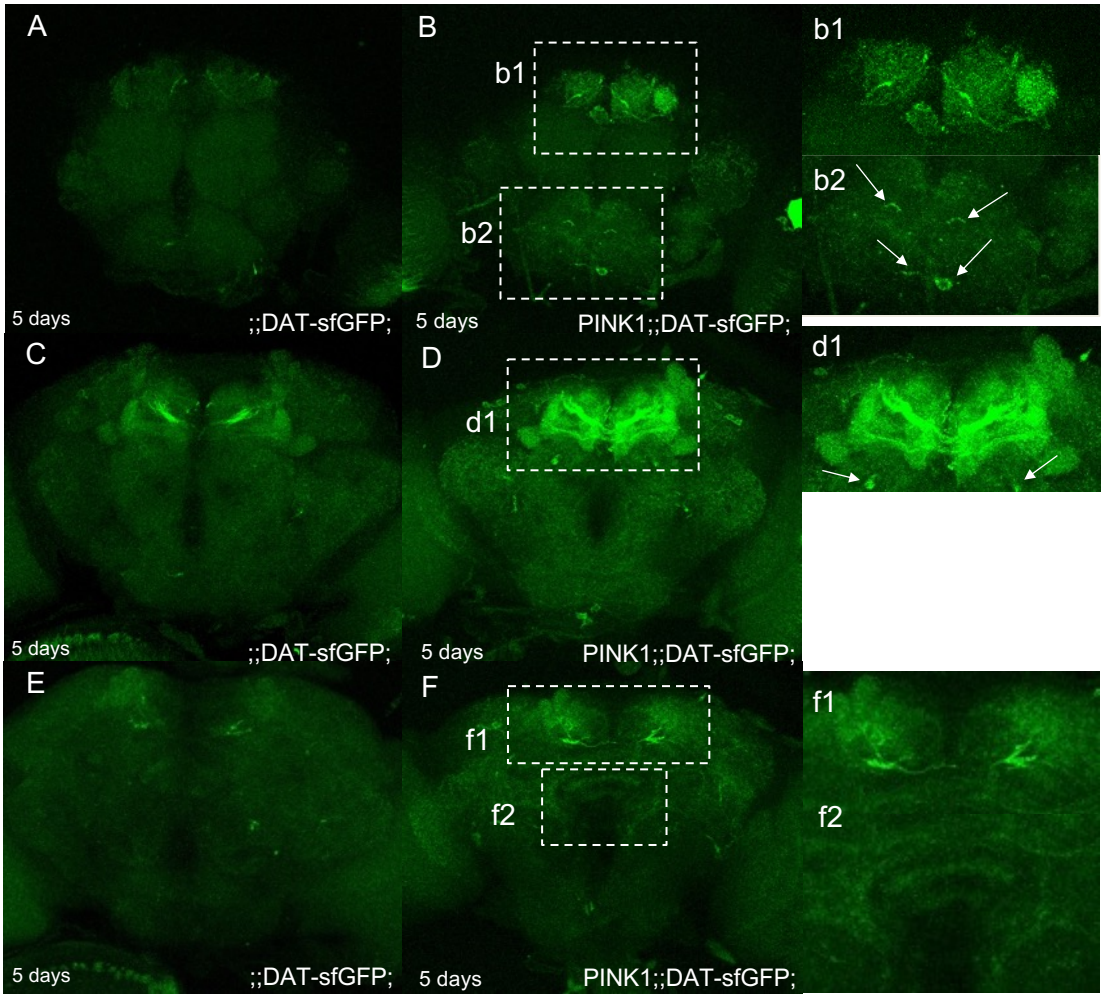

Figure 12 ***Detailed depiction of the 5-day old mutant (middle) and the control (left)*** *more specific!. On the right side of the figure area of interest of the mutant brain are depicted. Anterior(B), Middle (D), posterior (F). The* ***anterior*** *plane shows higher expression in the superior medial protocerebrum (SMP) area(b1) and the gnathal ganglia (GNG) area (b2). Higher expression in the cell bodies in the GNG area compared to the control (A). The* ***middle*** *plane shows higher expression in the MB area(d1). The lobes, pedunculus and calyx are clearly visible compared to control(C).* ***Posterior*** *plane shows higher expression In the MB area (f1) and the CX especially the FB (f2). Puncta formation higher in the posterior plane compared to the control (E).*

Overall, the mutant flies show a similar GFP expression pattern to the control group (Fig. 11). The mushroom body and crepine area show similar expression pattern compared to control group as well (Fig. 12). Additionally depending on the age group more cell body unrelated puncta are exhibited. 3-day old flies show more diffused and cell body unassociated puncta (Fig. 11B). 5-day old flies (Fig. 11D) show an increase in signal intensity compared to the control group (Fig. 11C). At 7 days the control group (Fig.11E) shows a higher intensity in expression than the experimental group (Fig. 11F) and the expression pattern stays the same. 10-day old mutant flies (Fig.11H) have higher signal intensity especially in the MB compared to the control (Fig.11G). Here the lobes are clearly distinct and visible. There are more puncta visible as well. In 20-day old flies we can see a decrease in random puncta throughout the

brain(Fig.11J) compared to the control(Fig.11I), which shows significantly lower expression in the MB area.

To further illustrate the GFP expression differences in expression patterns the 5-day old brain is analyzed from the anterior to the posterior plane in figure 12. On the anterior plane the PINK1 mutant (Fig. 12B) shows two distinct differences in expression. First the superior medial protocerebrum (SMP) area shows higher signal intensity of DAT-GFP(Fig.12b1) and the neurons are more visible than in the control (Fig.12A) Second the gnathal ganglia (GNG) area shows higher GFP signal(Fig.12b2), especially in the cell bodies show higher expression. The middle plane(Fig.12D) has a distinct MB area. The lobes the pedunculus and the calyx are clearly visible(Fig12D). The control does not show this clear expression pattern (Fig.12C). Also, the cell bodies around the MB show higher expression as well (Fig.12d1). The posterior plane (Fig.12F) in the mutant shows higher expression in the MB area (Fig.12f1) and the CX, the fan shaped body(Fig.12f2) is clearly visible compared to the control (Fig.12E). In this plane the puncta similar signal is better visible.

In general, the DAT-GFP signal intensity in the PINK1 mutant varies over the course of 20 days (Fig 13). The intensity hits a low point at day 7 and picks back up again at day 10 and stays at that level till day 20. The quantification shows a significant difference in every age group. At Day 5 and day 20 the PINK1 mutant shows less GFP intensity than the control. Day 7 PINK1 the mutants show lower GFP levels than the control group. The sample sizes range from 7-11 flies per condition and the area which was quantified was exclusively the MB area.

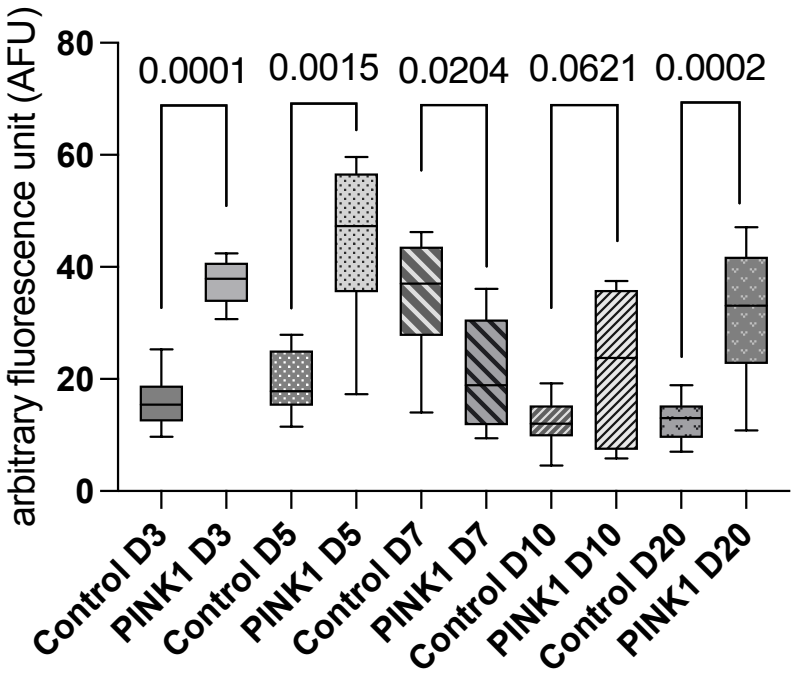

Figure 13 **Quantification of *PINK1 DAT-sfGFP expression.*** The DAT-sfGFP-levels was quantified with the arbitrary fluorescence unit (AFU) Two conditions are presented next to each other, control and the PINK1 mutant. The age progression going from left to right ascending. Comparing the groups with an unpaired t-student test, there are significant differences between the control and the condition group. Sample sizes PINK1 D3=9, PINK1 D5=7, PINK1 D7=9, PINK1 D10=8, PINK1 D20=11.

2.2.2 Reduction in climbing ability and activity pattern

To understand and analyze the functionality of the dopaminergic system the locomotive behavior was tested via RING and locomotion activity assay.

In figure 14 the RING assay results are shown. The experimental group shows steady decline in climbing success, while the control group shows an increase over time. In condition day 10 has only 10% of flies which were able to pass the threshold, this was the only time point which shows a significant decrease.

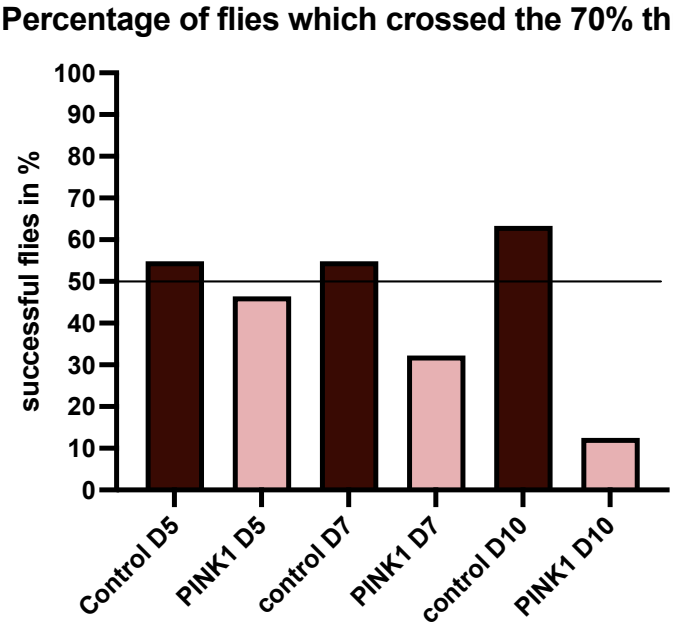

Figure 14 ***RING assay of the control flies and the PINK1 mutants****.The control group consistet of w1118 flies and the mutant condition were PINK1;;; flies. The climbing success in percent of control and mutant flies. Control and condition were compared via unpaired student t-test. Age progression from left (youngest) to right (oldest). In all the control groups over 50% of the flies were successful. None of the mutant fly groups could reach this barrier, also there was a decline of success rate observed with increasing age of the flies. Sample size ranged from 15-20 flies per condition.*

For the general locomotion a significant decrease in activity has been measured from day 1 onward as seen in figure 15. Compared to the wild type control all other experimental groups reveal a massive decrease in movement. The activity increases over time and 10-day old flies have an increased locomotion activity compared to the 5-day old flies.

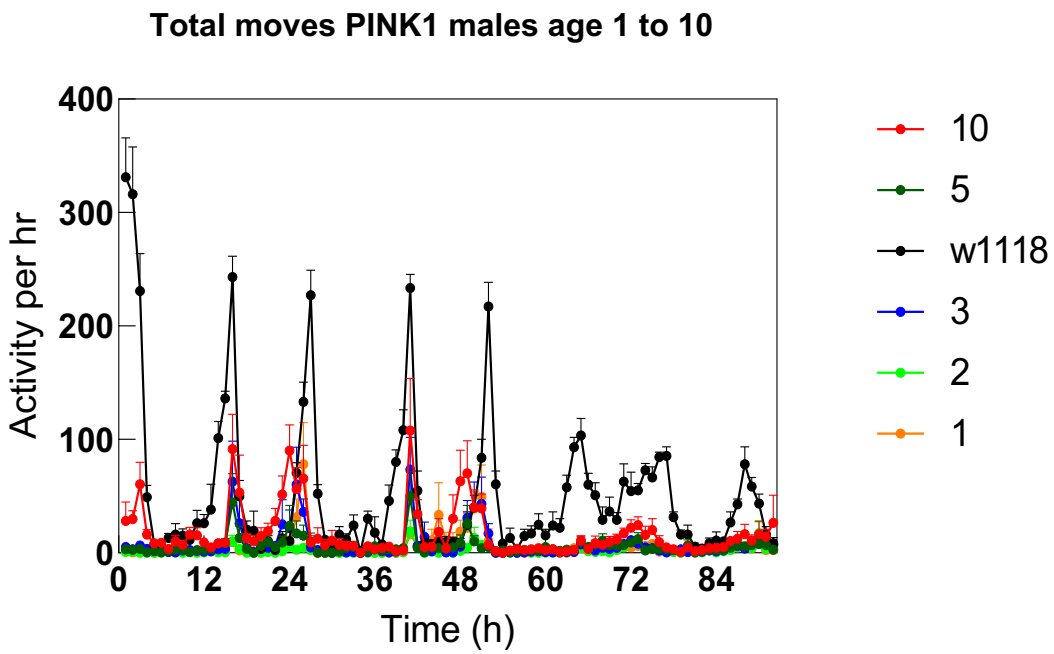

Figure 15 ***Locomotion activity assay with w1118 as control and PINK1;;; as mutant condition****. Total move count for 1 to10 day old flies compared to the control 5-day old wildtype w1118 fly. The wildtype (black) shows more activity than the experimental groups. Day 10 (red) has the highest activity from the experimental groups. Day 1(orange), 2 (teal), 3(blue and 5(green) show similar activity patterns and compared to the control group have significantly lower activity patterns. Sample size 3-5 flies per condition.*

2.3 PARK [2]mutants shows varying DAT-sfGFP expression differences.

2.3.1 Characterization of the DAT-sfGFP expression in PARK[2] mutants

For the PARK [2] mutant ;;PARK[2] /+; characterization 4 time points were chosen: 3-, 5-, 7- and 10-day old flies. In Figure 16 PARK [2] is compared to the control group of the same age. A locomotion assay was also performed on these flies to understand the effect of the mutant on the fly and the functioning of the dopaminergic system.

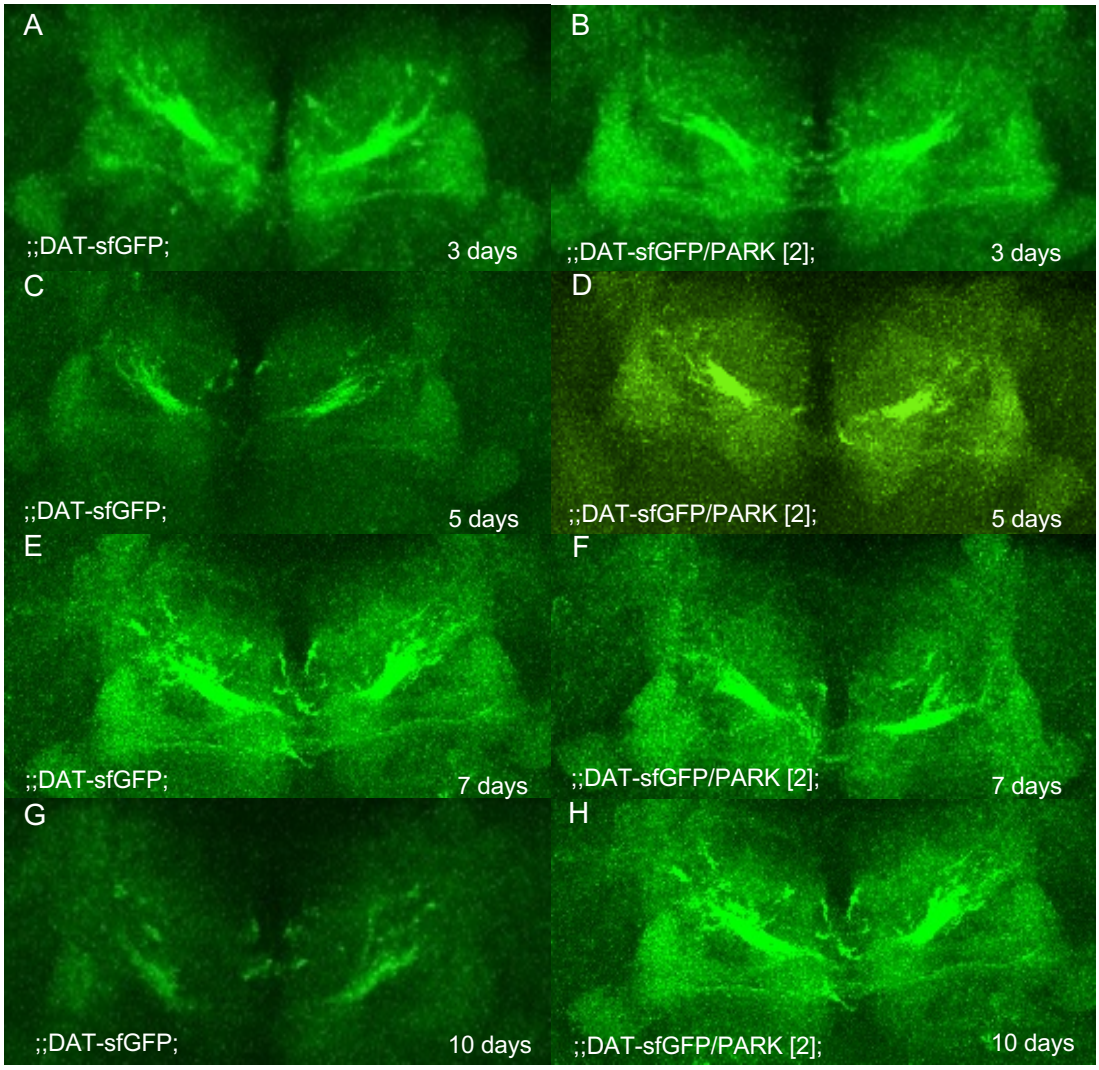

Figure 16 ***Characterization of the PARK[2] mutant****. GFP tagged DAT is visualized in green. The left column is the Park mutant crossed with the tagged DAT and on the right is the control group. The age progression goes from youngest (top) to the oldest (bottom).* ***Day 3*** *shows similar expression patterns and the same amount of puncta formation as the control.* ***Day 5*** *shows higher puncta formation crepine region clearly visible.* ***Day 7*** *similar expression patterns more puncta in the control than the mutant.* ***Day 10*** *lower intensity in the control group less puncta than the mutant.*

The PARK[2] mutants seem to have differences in the puncta formation(Fig 16), but overall MB area expression seems to stay the same. Day 3 old mutants (Fig 16B) have a similar expression pattern to the control group (Fig 16A). The intensity is similar, and the puncta seem to be at same intensity as well. Day 5 mutants (Fig 16D) seem to show a slightly higher DAT-GFP intensity than the control (16C). The 7-days old flies (Fig 16F) expression pattern is similar, but there are more puncta in the mutant than in the control group(Fig 16E). Day 10 (Fig 16H) shows higher GFP expression than the control(Fig 16G).

Comparing the 4 time points to the control group in Figure 17 reveals that two time points which are significantly different. On day 7 the experimental group with the PARK [2] mutation shows higher GFP intensity than the control group and on day 20 the mutant group shows less signal. No significant changes could be shown at day 5. Day10 has a bigger range in GFP signal than the control, but the median is nearly the same. The sample sizes range from 7-9 flies per condition and the area which was quantified was exclusively the MB area.

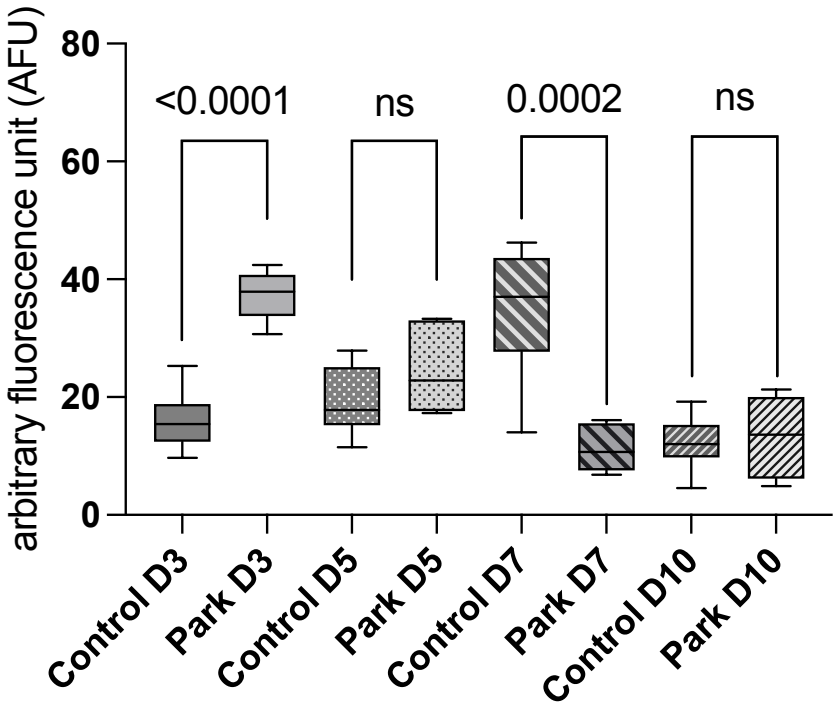

Figure 17 ***Quantification of the GFP tagged DAT in PARK [2] mutants.*** *Age range from 3 days to 10. Two significant differences in age group D3 and age group D7. 3-day old flies show significantly higher expression intensity than the control group. On day 7 the PARK [2] mutant shows less signal compared to the control group. Day 5 and 10 show no difference in intensity compared to the control. Sample sizes: Park D3=9, Park D5=7, Park D7=7, Park D10=7*

2.3.2 Overall increase in activity patterns, but sudden decline in climbing ability

The climbing assay (Fig. 18) showed a significant decrease in climbing capabilities with 10-days old flies. The two previous time points show no significant difference. On Day 7 the mutant group outperformed the control.

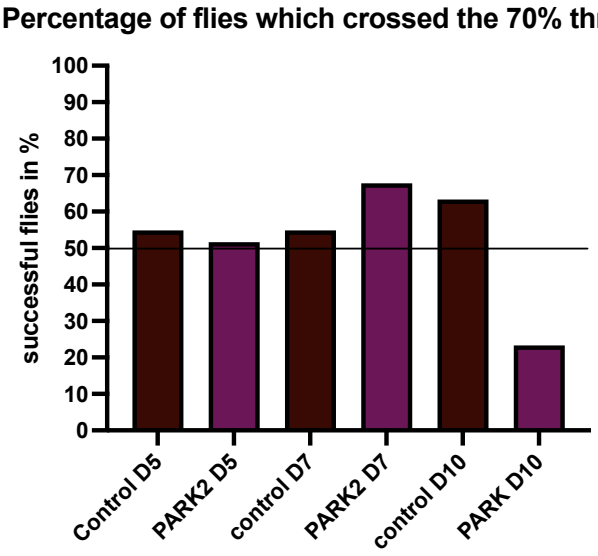

Figure 18 ***RING assay of the PARK [2] mutants compared to the control group w1118.*** *No differences in the first two age groups, but in 10-day old flies a significant reduction of climbing height was measured. Sample size ranged from 15-20 flies per condition.*

For the locomotion activity assay mutant flies were used without the GFP tagged DAT and as a control the wildtype w1118 was used as a control. The locomotion activity levels were measured (Fig. 19) and the activity of the flies show that the mutants have an increase of activity with double the levels compared to the wildtype w1118 flies.

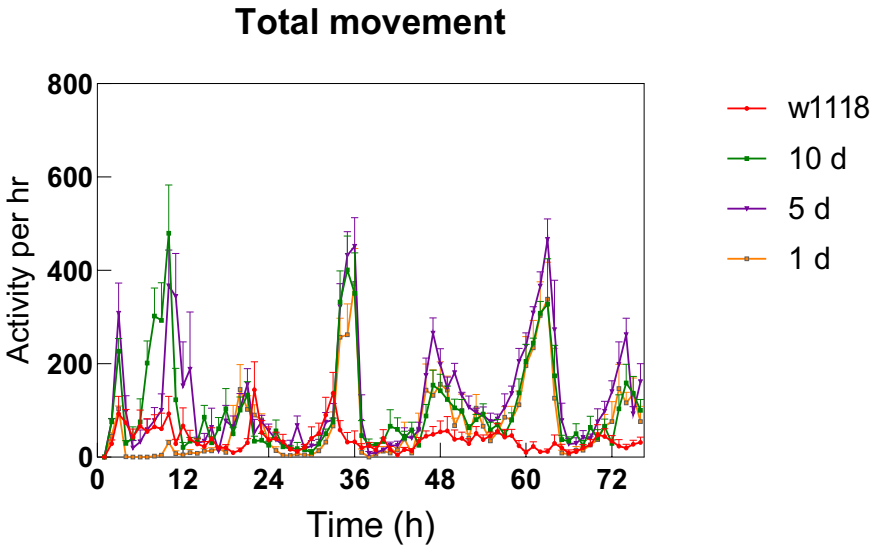

Figure 19 ***The total movement of the PARK mutant compared to the wildtype control w1118****. The experimental groups show a significant increase in activity which here is the full range of movement from 1 laser barrier the other. There are peaks observed every 12 hours and compared to the wildtype the movement is tripled. Sample size 3-5 flies per condition.*

2.4 Mutant α-synuclein overexpression leads to similar DAT-sfGFP expression patterns and higher DAT-sfGFP expression in early life stages

2.4.1 Characterization and quantification of the DAT-sfGFP expression

The age dependent characterization of the GFP expression patterns in the α-synuclein overexpression ;;UASSNCA/+; shows no difference in general patterns, but in GFP intensity. In Figure 20 the 4 different timepoints were compared to the control group and ranging from 3 to 20 days

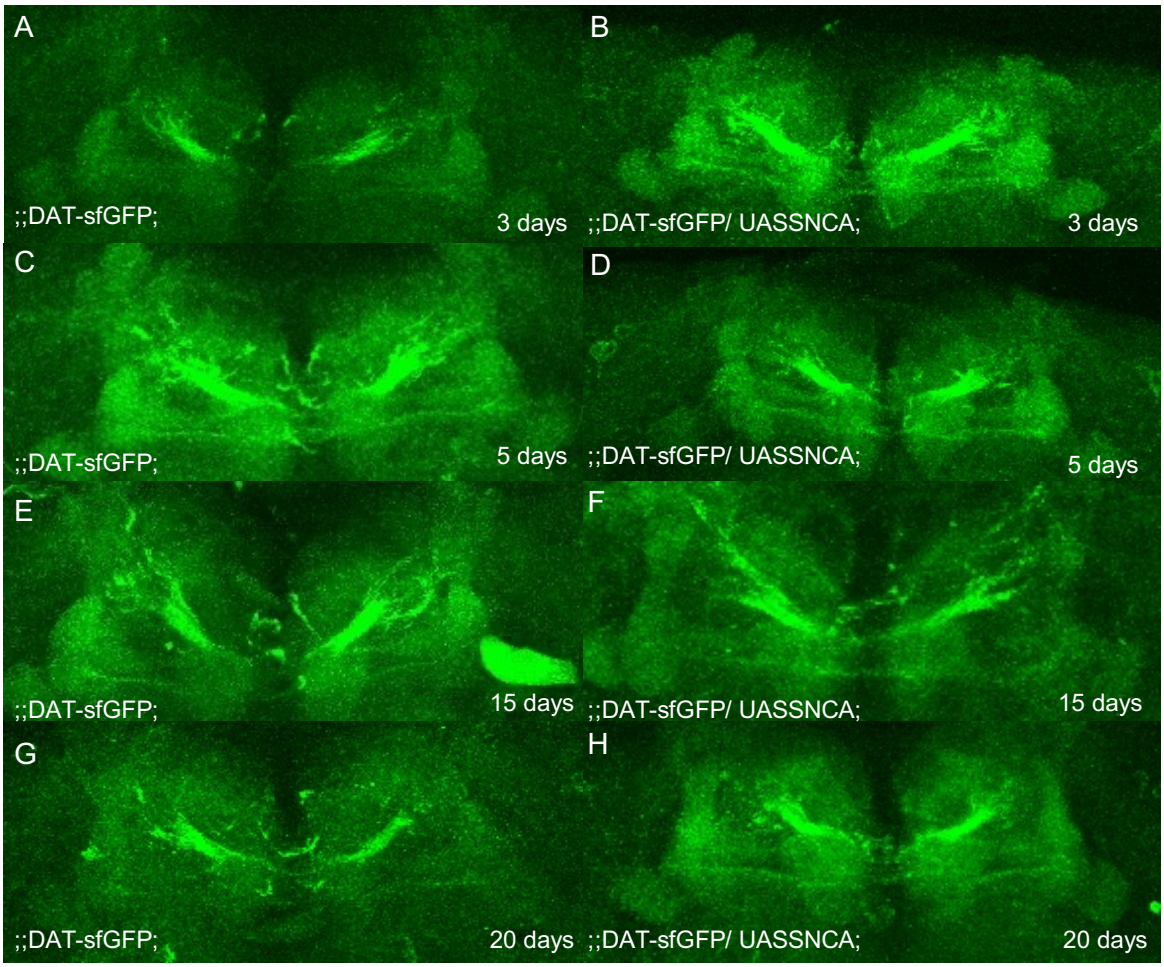

Figure 20 ***Comparison of Expression patterns of α-synuclein mutant overexpression from 3-day old (top right) to 25-day-old (bottom right) flies.*** *The corresponding control is on the left to the experimental group. Intensity differences in the mushroom body can be observed. General expression pattern stays the same throughout the time points.* ***Day 3*** *Expression(B) is higher than the control group(A) but no other significant changes could be observed.* ***Day 5*** *(D) shows similar pattering and similar signal intensity compared to the control group(C). On* ***day 15*** *(F) a switch between experimental condition and control group(E) could be seen, in which the control group shows higher GFP intensity.* ***Day 20*** *(H) shows similar pattering and intensity levels as the control group(G).*

The corresponding control is presented next to the experimental group (Fig 20). Day 3(Fig 20B) shows no difference in pattering but an increase in signal compared to the control group (Fig 20A). Quantification shows that the signal increase is significant (Fig 21). For Day 5 (Fig

20D) the UASSNCA flies have similar pattering and puncta formation compared to control (Fig 20C). The Quantification for this day also reveals little difference in signal intensity.
Day 15 has a lower GFP expression(Fig 20F), but the overall pattering is the same compared to the control. On day 20 the control group (Fig 20G) shows significantly higher expression levels than the experiment flies(Fig 20H). Characterization of the expression patterns in the Mutant α- synuclein overexpression shows no difference in general pattering but in GFP intensity. In Figure 21 the 4 different timepoints were compared to the control group and ranging from 3 to 20 days. Overall does the GFP quantification (Fig 21) show an increased GFP intensity in the first 2 timepoints (day3 and 5). The intensity is reduced at day 15 to a lower level than the control group and on day 20 control and experimental group show nearly the same intensity levels. The sample sizes range from 8-10 flies per condition and the area which was quantified was exclusively the MB area.

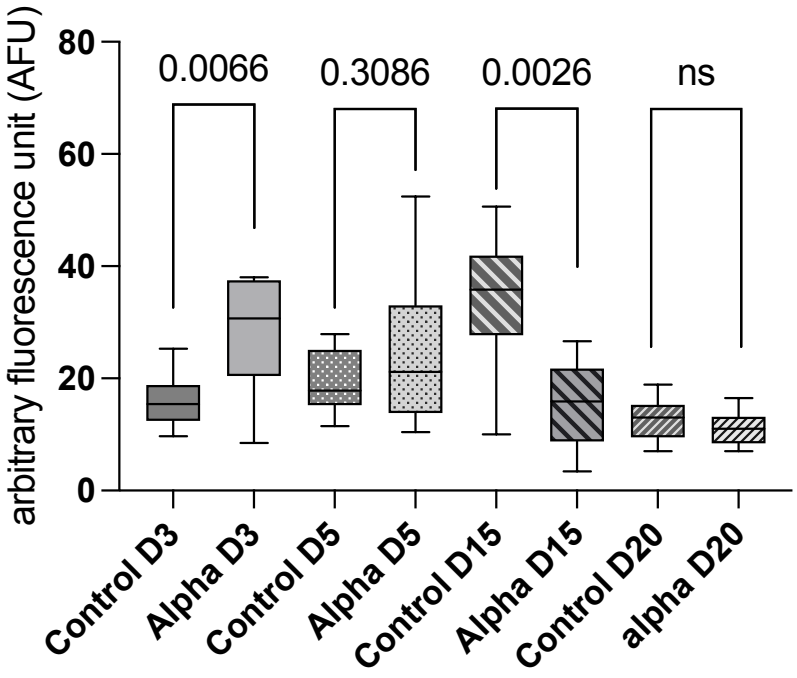

Figure 21 ***Quantification of GFP intensity with 4 different time points.*** *Day 3 and 5 show significantly higher expression levels in the experimental group. This finding vanishes with increased age of the flies. Day 15 and 20 are no longer significantly different and show a slight trend towards lower GFP intensity. Sample sizes: Alpha D3=9, Alpha D5=10, Alpha D15=,9 Alpha D20=8*

3. Discussion

In this project I investigated how dopamine transporter expression gets impacted by the molecular processes which eventually leads to neurodegeneration. PD is the most relevant neurodegenerative disease for the dopaminergic system. It directly affects the DA neurons and the most treatments are focused on DA supplementation. Since DAT is the main regulator at the presynaptic site investigating how PD causing mutations and genetical defects affect DAT is of upmost importance. Furthermore, the loss of DAT in PD progression is used as a diagnostic tool, whether the loss of DAT is critical in the disease progression is unclear, but assumed (Bellucci et al., 2012). Therefore, taking a closer look at this protein is a logical step in PD research.

The first mutant used in this study was the null-PINK1 mutation. The PINK1 mutant affects the regulatory function of the mitochondria. The expressed protein, however, does not directly interact with DAT and its functioning. The mutant was selected due to its severe impact on the dopaminergic system and therefore it is reasonable to assume the regulatory functioning of DAT is impacted as well. Clark et al., 2006 and Park et al., 2006 show that if PINK1 is knocked-out the mitochondria is severely affected. It swells and increases in size. A consequence of this malfunction is the motor functions of the flies suffer severely, flies are unable to fly, and their climbing abilities are limited. These findings were replicated here as well (Fig. 14 and Fig. 15). Furthermore, more dopamine levels decreased in the fly brain overall. Similar findings show Kitada et al., 2007 and Zhi et al., 2019 in a rodent model of PD. In the mouse model of PD the effect of the PINK1 null mutant had no effect on the DAT levels in the brain. However, in fly model studies this could not be replicated (Clark et al., 2006 and Park et al., 2006) . In this study an increase was observed in DAT-GFP levels in the mutant flies except for 7-day old flies but the overall expression pattern is not changed, which means that the synthesis and transportation of DAT is not affected.
The fact that DAT is still being transported to the membrane means, that the reduction of ATP, following the malfunctioning mitochondria, is not severe enough to have an influence on transportation. Furthermore, PINK1 is not implicated in autophagy therefore the degradation cannot be the problem either. Park et al., 2006 showed that the loss of PINK1 can be compensated through Parkin overexpression.

One theory for the increase in DAT in the mutant flies is (Fig 22), that the *parkin* used for degradation is recruited by the mitochondria to compensate the loss of PINK1 functioning. Therefore, degradation cannot occur, however, this was not tested. Since the tagged DAT-GFP does not indicate its functioning, one cannot know whether this increase in DAT expression are functioning transporters or malfunctioning non degraded transporters stuck in

the membrane. Also, whether DAT is even in the membrane and not just held in a storage pool or anchored to the scaffold intracellularly, cannot be differentiated.

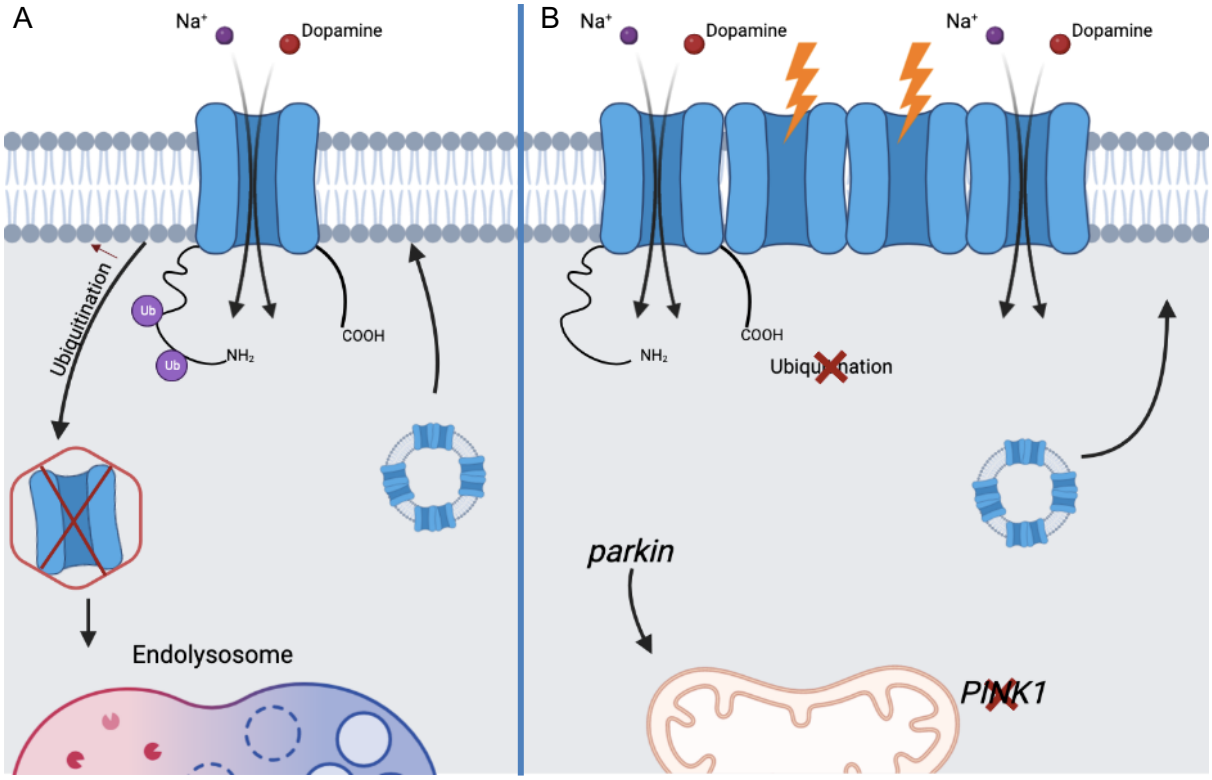

Figure 22 ***Schematic showing the regular DAT degradation (right) and the compromised mutant form (left)*** *(A) In regular functioning neurons the degradation is mediated via ubiquitination. Transporter get transported to the cell membrane via vesicle transport. There it functions as a DA regulator. If damaged or malfunctioning the transporter will be ubiquitinated and degraded or recycled (not depicted here). (B) On the right side the mutant condition is depicted. If PINK1 is not available for mitochondria health parkin is therefore the ubiquitination process is inhibited and not degrading proteins. Figure made in BioRender*

The second mutation caused a downregulation of *parkin*. *Parkin* is involved in the degradation of the mitochondria as well as the autophagy of other proteins. DAT has a direct binding site for *parkin* and it has been shown that the overexpression parkin in drosophila melanogaster leads to an increase in degradation and subsequently an increase in surface level expression (Jiang et al., 2004) Inhibiting this ubiquitination could interfere with DAT homeostasis and the overall DA neuronal health. Further, Goldberg et al., 2003 showed that the extracellular dopamine levels were increased in *parkin* knock-out mice and the dopamine reuptake reduced. This could be caused by malfunctioning of the DAT, however, this was not looked into. To investigate the role of *parkin* in DAT regulation and ensuing disease onset is vital for the understanding of the protein in the disease itself. This study showed a varying DAT level change, but the tendency was a reduction of DAT levels over time in the mutant condition (Fig 17). A significant difference could be observed on day 3 with a higher signal intensity and day 7 with a lower signal intensity than the control group. Jiang et al., 2004 showed that this

increase in surface level expression, which was previously mentioned. This does not occur in the PD mutation of *parkin*. In this study the DAT expression did not only not increase, but it reduces over time. This leads to the assumption that the PD causing mutation inhibits the degradation process which impacts the recruitment of DAT to the cell surface. This would be an indirect effect of the mutant which impacts the DA homeostasis of the cell.

For the last condition, the overexpression of human α-synuclein with the A30P mutation, the experiments showed a lower level of DAT-GFP than the control. α-synuclein in general modulates the trafficking of DAT to the surface membrane, by reducing the DAT on the cell surface (Wersinger & Sidhu, 2003). This happens through a similar mechanism to the parkin overexpression, trafficking the DAT away from the cell surface. However, they are not degraded like with the *parkin* overexpression. With the A30P mutation a similar response could be replicated. However, because of the mutation at the A30P loci the protein interaction with DAT is reduced (Bellucci et al., 2012). Hence, the trafficking to the surface is inhibited. There was no indication that the protein got stuck on the way to the cell surface, this would be visible in different expression patterns at the different timepoints. So, the more probable explanation would be a defect at the production at the ER or the packaging into vesicles at the Golgi-apparatus. A key aspect of PD is the formation LB's. In the drosophila model the formation of inclusion which resemble LBs was observed in drosophila (Feany & Bender, 2000). Bellucci et al., 2012 showed that in these inclusion DAT is present and faulty α-synuclein is responsible for redistribution of DAT in mice, which could be the case in drosophila as well. However, this was not tested. Moreover, through pH changes the GFP could be instable, and the signal could be lost if inside these inclusions.

An interesting aspect which this study did not touch on is whether the DA levels are also different in these conditioned states. It is known that DAT has an impact on DA levels in that if there is more DAT more DA will be transported back into the cell and vis versa. So, if in the PINK1 mutant the DA levels are severely lowered with the significant increase in DAT levels. Or in the parkin mutant whether the decrease of DAT leads to higher DA levels in the extracellular space. For α-synuclein it has already been established, that TH enzymatic activity is affected by the protein interaction (Perez et al., n.d.). This leads to the presumption that if α-synuclein is misfolded and non-functional that the DA levels are also affected. Future studies looking at the extracellular dopamine concentrations in these three models will enhance our understanding neurodegenerative changes affecting the dopaminergic homeostasis.

4. Material and Methods

4.1 Flies or experimental setup

4.1.1 Fly culture

Flies were kept at 23,5 degrees Celsius. If the experiment was temperature sensitive the flies were kept in an incubator at 25 degrees. Two different vials were used, small and big vials, depending upon the amplification need for the flies. The vials were filled with food for approximately two generations.

4.1.2 Fly stocks

Mutant flies were ordered form the Bloomington fly facility. This includes the PINK1 mutant #51649 and #34749 however due to fragile stock and bad viability the stock with the number #34749 was excluded, Park [2] mutant #34747 and the human α-synuclein overexpression under UAS control #8147. DAT-GFP fly was ordered from VDRC (#318840).

4.2 DAT-GFP Fly

For the experiments in which DAT-GFP was measured, a fly with an insertion on the third chromosome was used. This DAT insertion was tagged with a superfolded GFP (sfGFP). The inserted sequence is depicted in Figure 23.

Figure 23 ***Genomic sequence inserted in the 3rd Chromosome of the Drosophila melanogaster fly.*** *The DAT was tagged with a sfGFP.*

4.3 Behavior assays

To study the functionality of the dopamine system behavioral assays have been utilized. Depending on the studied function the appropriate assay was used.

4.3.1 Locomotion activity assay

The device used is seen in Figure 24. In a glass tube a fly was placed with an appropriate amount of food on one end and a cotton ball to seal the tube on the other end. A cotton ball is used to grant airflow through the tube and the flies have enough oxygen to stay alive in the tube. Then the glass tube is placed into a rack, in which infrared beams track the movement of the fly and whether it walks form one end of the tube to the other or stays on one side or does not move at all e.g., rest periods. The rack has 32 slots therefore 32 flies can be tested simultaneously. This apparatus is placed into an incubator which is set for 25 degrees and a 12h day-night cycle. After at least 72h the locomotion pattern is recorded and analyzed via Prism.

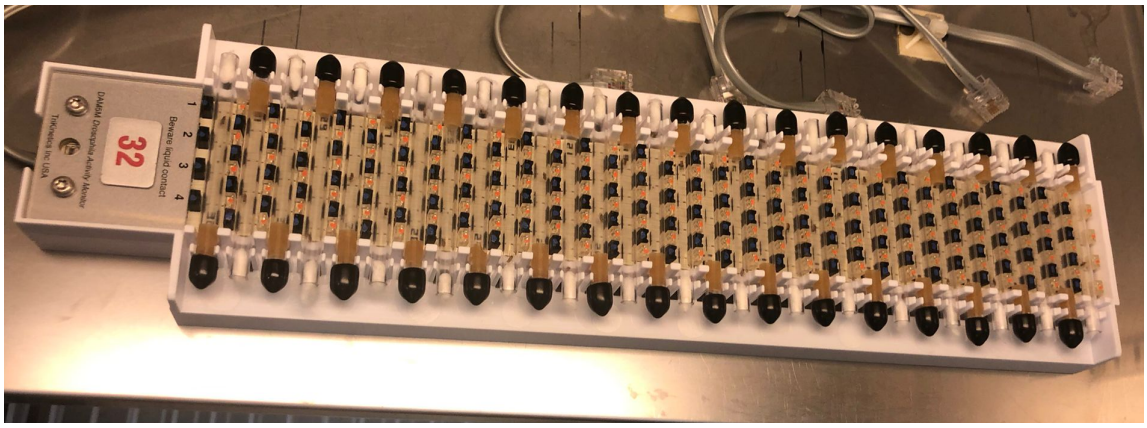

Figure 24 ***Setup for Locomotion activity assay****. The tubes are placed in the rack. One side carries the food and a black cap, on the other side is the cotton ball. Each fly has its own tube and different conditions are marked. The setup is placed in an incubator with a day night cycle of 12h.*

4.3.2 RING assay

The RING assay, also known as the Rapid-Iterative-Negative-Geotaxis assay (RING assay), analyzes the climbing activity of flies. 10-15 flies were placed in an empty vial and through tapping on a desk the flies start climbing up the vial walls. The climbing height was measured with a lined paper behind the tubes (Fig 25). The assay was recorded and the climbing height of flies at the end of a 30-second period was recorded. The climbing height was analyzed through the program Prism.

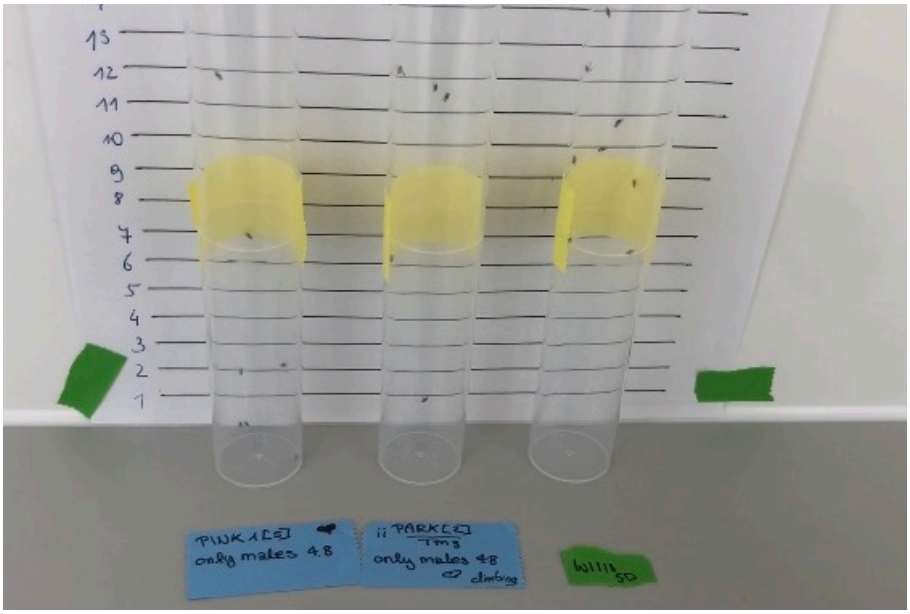

Figure 25 ***Setup for RING assay****. Depicted are 2 experimental groups on the left and middle. On the right the control group can be seen. In the back the measuring lines are seen. The video is taken at a 90-degree angle to the table.*

4.4 Immunohistochemistry

The protocol for immunochemistry was kept constant throughout the experiments. For dissection the flies were transferred into 96% ethanol and washed three times with PBS (Phosphate Buffer Saline). The dead flies were placed on a silicone pad with a PBS droplet on it and dissected. After dissection the freshly removed brains were fixed in 2% PFA (paraformaldehyde) for 1 hour.

4.4.1 Staining Protocol

Fixed brains were then washed with PBT 4 times every 15 minutes. The brains were blocked with the use of diluted goat-Serum. Afterwards the primary antibody (Table 3) was applied to the brains and left on overnight. Then the brains were washed again 4 times every 15 minutes with PBT. The second antibody (Table 3) was applied and left on for at least 3 hours but for high quality staining, it is recommended to leave it on overnight. Then the brains were washed a final time 4 times every 15 minutes with PBT and then mounted on a glass slide in Vectashield.

Table 4 Primary and secondary antibodies used with dilution

Primary Anti-body	Dilution with GOAT-Serum	Secondary antibody	Dilution with GOAT-Serum
Anti-TH rabbit (P21962, Invitrogen, Vienna, Austria)	1:1000	Rabbit, AlexaFluor 647 goat (Invitrogen, Vienna, Austria)	1:500
Anti-NCAD rat (MNCD2, (by M.Takeichi and H. Matsunami) Developmental Studies Hybridoma Bank, Iowa, USA)	1:100	Rat, AlexaFluor 647 goat (Invitrogen, Vienna, Austria)	1:500
Anti-FasII mouse (1D4, (by Goodman, C.) Developmental Studies Hybridoma Bank, Iowa, USA)	1:200	Mouse, AlexaFluor 647 goat (Invitrogen, Vienna, Austria)	1:500

Used antibodies included:

1. Anti-TH antibody (Tyrosine hydroxylase) recognized the enzyme which turns Tyrosine into Dopamine
2. Anti-NCad antibody (Neuronal Cadherin) labels the surface protein Cadherin in neurons used in morphology visualization
3. Anti-FasII labels the glycoprotein Fasciclin 2 and was used to delineate the mushroom body.

4.5 Imaging

The mounted brains were scanned via Leica SP5 II confocal laser scanning microscope. The settings were adjusted to the control and afterwards not changed to track intensity changes in the experimental brains.

4.6 Picture editing and Quantification

All images were edited with Fiji and the quantification of the GFP intensity was done with Imaris. The quantification was done with FasII stained brains. The mushroom-body was defined as the area of interest since there are the most dopaminergic innervations.

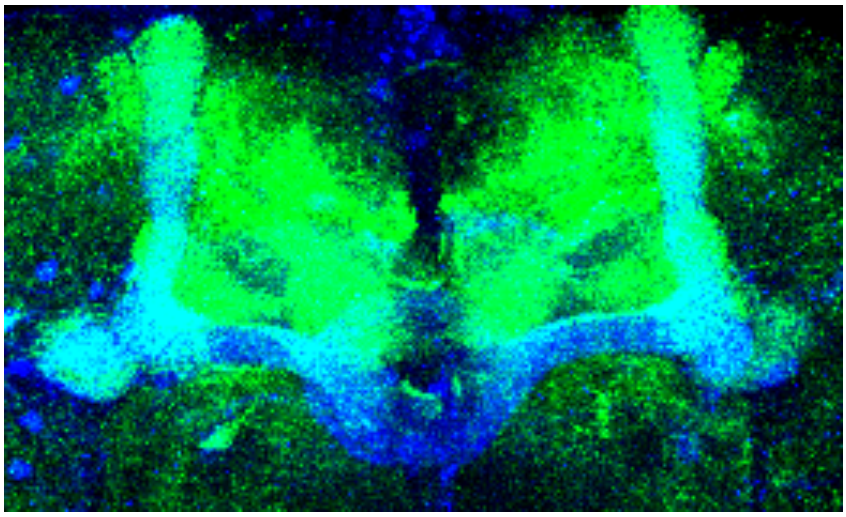

Figure 26 ***Example image of Imaris quantification of a 7-day old control group MB area.*** *The DAT-GFP Signal in green and the anti-FasII with a mouse-AlexaFluor-647-goat secondary antibody staining in blue. The area of interest is the FasII stained MB area. The GFP intensity in this specified area was compared across conditions and control groups.*

4.7 Statistical analysis

The statistical analysis was done using Microsoft Excel as a data cleaning program and Prism for statistical testing and analysis. The test used are unpaired t- student test and one way ANOVA

Figure legend

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