Evaluation of Dopaminergic p25-overexpression in *C. elegans* as a Model for Parkinson's Disease and Dementia with Lewy bodies

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2. Abstract

Patients suffering from Parkinson's Disease or Dementia with Lewy bodies display a wide range of symptoms including motor deficits, such as bradykinesia, or cognitive impairments, such as impaired decision making and/or learning deficits. These impairments are primarily related the degeneration of dopaminergic neurons of the midbrain, especially of the substantia nigra where the formation of intraneuronal inclusions called Lewy bodies occur. A main constituent of Lewy bodies is the protein alpha-synuclein (αSyn), and both diseases are therefore termed neuronal synucleinopathies. Despite many years of great effort put into the investigation of αSyn, it is still not clear how pathology emerges in the first case, whereas a need for other possible pathogenic mechanism is warranted. The protein, Tubulin Polymerization Promoting Protein/p25α (p25), has been found in Lewy bodies from tissue samples of patients with Parkinson's Disease or Dementia with Lewy bodies, where it appears to co-localize with αSyn. The protein is able to induce aggregation of αSyn as well as the formation of aberrant microtubule structures, which are vital cytoskeleton structures whose dysfunction is associated with neuronal synucleinopathies.

To investigate the potential pathological properties of the p25-protein, a *C. elegans* transgenic mutant overexpressing this protein in the dopaminergic neurons was utilized. Behavioral dysfunction caused by p25-overexpression was examined using the salt aversion assay, where the worms are required to avoid salt after being conditioned with NaCl in the absence of food. Additionally, a strain expressing αSyn pan-neuronally was also utilized to investigate an association between p25 and αSyn. Both strains overexpression p25 was interestingly found to demonstrate some form of impairment in associative learning following the salt aversion assay.

To investigate pathological intraneuronal consequences of p25-overexpression in the worm's dopaminergic neurons, an immunostaining experiment was conducted to investigate the co-localization between microtubules and p25, in addition to co-localization of p25 and αSyn. Substantial co-localization between microtubules and p25 was detected, however, results of the staining for p25 and αSyn was inconclusive. These results suggests that the interaction of p25 and microtubules are pathological in these strains.

All in all, these observations suggests that the transgenic *C. elegans* model overexpressing p25 in the dopaminergic neurons is a valuable model for studying neuropsychological and intraneuronal impairment observed in neuronal synucleinopathies

3. Table of contents

4. Introduction

After Alzheimer's Disease, the second most common neurodegenerative disease is Parkinson's Disease. This disease has been estimated to affect around 2-3 % of the population above 65 years of age (Poewe et al., 2017), which is expected to increase (Ben-Shlomo et al., 2024; Bloem et al., 2021). The next most common form of dementia after Alzheimer's Disease is Dementia with Lewy bodies (Outeiro et al., 2019). Parkinson's Disease patients present motor disorder of varying nature (Sveinbjornsdottir, 2016). However, different forms of cognitive impairment, such as in decision making and learning, are increasingly being recognized as a substantial part of the symptomatology in Parkinson's Disease (Perugini et al., 2018). As the name suggests, Dementia with Lewy body patients suffers from continual cognitive decline (McKeith et al., 2017). In addition, fluctuating cognition and motor symptoms, as seen in Parkinson's Disease, are also demonstrated by these patients (Outeiro et al., 2019). Thus, both diseases are associated with neuropsychological impairment.

The neurodegeneration associated with these diseases is mainly observed as loss of dopaminergic neurons in the midbrain area called the substantia nigra (Outeiro et al., 2019; Simuni et al., 2024). The various behavioral deficits observed in these disorders are therefore associated with the dysfunction of the neurotransmitter, dopamine (DA). The degeneration of these dopaminergic neurons is clearly associated with abnormal protein homeostasis (Poewe et al., 2017). In neurons, proteins play decisive structural and functional roles such as providing fundamental mechanisms for neuron-to-neuron communication. It is therefore no surprise that one of the main culprits of neurodegenerative diseases are thought to be aberrant aggregates and/or dysfunctional proteins (Cyske et al., 2023). As such the investigation of protein dysfunction is utmost relevant for understanding the neuropsychological impairment observed in these diseases.

The malformation of especially one protein, alpha-synuclein (αSyn), is associated with Parkinson's Disease and Dementia with Lewy Bodies. In these diseases, abnormal aggregates of αSyn is found inside round neuronal inclusions called Lewy bodies – these diseases are therefore commonly referred to as neuronal synucleinopathies (Jellinger & Korczyn, 2018; Morris et al., 2024).

Despite much research, the reason why the neuronal degeneration begin in the first place is still not fully understood (Ben-Shlomo et al., 2024). The lack of knowledge may in part be the many difficulties involved in investigating biomolecular functions of the proteins

composing human neuronal tissue *in vivo*. Thus, the study of proteins associated with αSyn, that possess properties relevant for the observed neuropathology, like causing protein aggregates, may therefore aid in the understanding of what underlies these neurodegenerative diseases (Morris et al., 2024). The small 25 kilo Dalton protein, Tubulin Polymerization Promoting Protein/p25α (here simply p25), is such a protein. It has been identified as a component of Lewy bodies found in tissue samples from patients who suffered from Parkinson's Disease and Dementia with Lewy bodies, wherein it, very interestingly, co-localizes with αSyn (Kovács et al., 2004; Lindersson et al., 2005). In addition, it has been shown to have a pro-aggregatory effect on αSyn – the main pathological finding of the neuronal synucleinopathies (Oláh et al., 2024), as well as being able to cause microtubule malformations (Hlavanda et al., 2002). Microtubules are intracellular structures vital for the health and normal function of neurons, as these structures provide the neurons with a way of transporting cargo, like necessary proteins, from one end to the other (Waites et al., 2021). In addition, the dysregulation of microtubules have also been associated with the neuronal synucleinopathies (Mazzetti et al., 2024; Power et al., 2017). Thus, this p25 protein may play a role in the intracellular dysfunctions associated with αSyn, and potentially microtubules, as seen in Parkinson's Disease and Dementia with Lewy bodies.

The small roundworm, *Caenorhabditis elegans,* is a frequently applied model organism for the study of neurodegenerative disease, as provides a way to investigate the neuropathological as well as behavioral consequences of protein abnormalities in the nervous systems (Kaletta & Hengartner, 2006). Interestingly, earlier work in the Anders Olsen laboratory (AO-lab) utilizing a transgenic *C. elegans* mutant have found overexpression of the human p25 protein in the nematode's dopaminergic neurons to induce degeneration in a subset of these neurons (Christensen, 2013; Stenz, 2016). This *C. elegans* strain will therefore be applied as a model to examine the putative relationship between the p25 protein and intracellular dysregulation, like protein or microtubule abnormalities, observed in the neuronal synucleinopathies. Thus, the purpose of this thesis is to evaluate the overexpression of the human p25 protein in *C. elegans* as a model for dopaminergic and neuropsychological impairment related to Parkinson's Disease and Dementia with Lewy bodies.

5. Parkinson's Disease and Dementia with Lewy Bodies

The neuronal synucleinopathies associated with the p25 protein, Parkinson's Disease and Dementia with Lewy bodies, are characterized by continual loss of neuronal tissue. The characteristic symptoms of the respective diseases, including those that are associated with cognitive impairment and learning of interest in this thesis, are thought to reflect the underlying affected brain areas. There is, however, considerable overlap in the presented symptomatology of these neurodegenerative diseases.

5.1 Parkinson's Disease Patients Also Demonstrate Neuropsychological Impairment

The long-standing view of Parkinson's Disease as a disease of movement, stems from to more overt physical symptoms that patients present. In 1817, James Parkinson shed light on these characteristic motor disabilities with his essay, *An Essay on the Shaking Palsy* (Parkinson, 2002). Motor symptoms often displayed by Parkinson's Disease patients include, but are not limited to, bradykinesia, gait abnormalities, muscle rigidity and resting tremor (Morris et al., 2024). Bradykinesia, termed palsy by Parkinson, refers to slowed as well as impaired voluntary initiation of movement (Bologna et al., 2020). Presentation of bradykinesia is necessary for clinical diagnosis (Sveinbjornsdottir, 2016). In addition to bradykinesia, the demonstration of either rigidity or resting tremor is also a necessity for the diagnosis of Parkinson's Disease (Bloem et al., 2021). Muscle rigidity may manifest as discrete 'cogwheel'-like movement of the limbs and is present in around 20% of cases (Sveinbjornsdottir, 2016). Resting tremor refers to the shaking of limbs when in resting position. As many as 20% of Parkinson's Disease patients do not present this symptom (Bloem et al., 2021). Other motor symptoms, such as muscle weakness and altered/problematic gait may develop during the course of Parkinson's Disease (Bologna et al., 2020). A diagnosis of Parkinson's Disease is currently based on clinical judgment, however, the disease is ultimately confirmed post-mortem if presence of the neurobiological hallmarks, like neuronal loss of the substantia nigra and presence of Lewy bodies associated with the αSyn protein is demonstrated (Bloem et al., 2021).

However, non-motor symptoms are also frequently demonstrated by Parkinson's Disease patients (Fernandes et al., 2021). Despite the fact that some non-motor symptoms were recognized by Parkinson over 200 years ago, and that they may be more debilitating than motor symptoms, they have been somewhat neglected (Chaudhuri et al., 2006; Fernandes et al., 2021; Parkinson, 2002; Pfeiffer, 2016). The most frequently reported non-motor symptoms include

sleep disturbances, olfactory dysfunction and constipation (Chaudhuri et al., 2006). Although non-motor symptoms are prevalent among the general aging population, the non-motor symptoms demonstrated by patients tend to be more frequent and/or have a bigger detrimental effect on their life (Pfeiffer, 2016).

Interestingly, the cognitive and psychological impairment associated with the underlying neuronal pathology of Parkinson's Disease (Aarsland et al., 2009; Weintraub, 2020), potentially allows the condition to also be seen as a neuropsychological disease. Cognitive impairment generally correlates with the progression of the disease, and some Parkinson's Disease patients display mild cognitive impairments in some domains early in the disease (Stefanova et al., 2015). Should the patients live long enough, many of them will develop dementia. In a longitudinal study of 123 Parkinson's Disease patients it was found that 83% of the 33 still alive after 20 years had developed some form of dementia (Hely et al., 2008). The cognitive symptoms are typically associated with attentional deficits, executive dysfunction, decision making or even learning (Perugini et al., 2018) In an interesting study, amnesic patients were able to increase the level correct choice based on feedback in a test of probable outcome despite having no memory of performing the test. Conversely, Parkinson's Disease patients could remember engaging in the test, but did poorer on the learning task (Foerde & Shohamy, 2011). The association between Parkinson's Disease and learning deficits is nothing new. An older study by Taylor and colleagues (1990) demonstrated significant deficits of associative learning on the Conditional Associative Learning Test in early Parkinson's Disease patients. Here, patients were asked to associate identical plastic casters with identical cards, such that physical features could not be used to guide the learning of associations (Taylor et al., 1990). Patients were more erroneous and required more trials to the reach the stop criteria (12 consecutive correct associations) – only 34% of the Parkinson's Disease group reached this cut-off, while 80% of the control group did (Taylor et al., 1990).

Neuropsychiatric symptoms are some of the most frequently recorded non-motor symptoms (Fernandes et al., 2021), and the disease has even been suggested to be better described as a neuropsychiatric disorder instead of a movement disorder (Weintraub, 2020). The psychiatric symptoms include anxiety, depression, apathy and more (Aarsland et al., 2009; Pfeiffer, 2016). Some of these symptoms could be viewed as symptoms of receiving a Parkinson's Disease diagnosis, however, depression and anxiety are seen to predate the eventual diagnosis suggesting that other causes like neurobiological changes play a role

(Aarsland et al., 2009; Weintraub, 2020). Indeed, many of these non-motor symptoms tend to be present in the often long prodromal phase before diagnosis is made (Weintraub, 2020). Paradoxically, the motor symptoms used to define and diagnose Parkinson's Disease may not be the first symptoms patients and their family notices.

In summary, the motor symptoms demonstrated by Parkinson's Disease patients may be the most overt of the Parkinson's Disease symptoms, however, the broad spectrum of symptoms, including the high frequency of neuropsychiatric and cognitive disorders, prohibits the view of Parkinson's Disease to solely be a motor disorder (Hussein et al., 2023).

5.2 Dementia with Lewy Bodies Patients Display Parkinsonism and Fluctuating Cognition

As with Parkinson's Disease, Dementia with Lewy bodies is also diagnosed on the basis of clinical symptomatology (Simuni et al., 2024). According to the revised criteria for diagnosis of Dementia with Lewy bodies, the 'essential' symptom of the disease is progressive and debilitating cognitive decline (McKeith et al., 2017). For the diagnosis of probable Dementia with Lewy bodies, two or more 'core clinical symptoms', which include disturbances of REM sleep, fluctuating cognition, visual hallucinations and/or parkinsonism, should be presented – even without any present biomarkers (Outeiro et al., 2019). Probable Dementia with Lewy bodies can also be diagnosed if only one core clinical symptom is present together with any biomarker (McKeith et al., 2017). If only one core clinical symptom is present without any biomarkers, or no symptoms but presence of any biomarker is demonstrated, the diagnosis of possible Dementia with Lewy bodies is used (McKeith et al., 2017). To detect the essential symptom, dementia or cognitive decline, in potential Dementia with Lewy body patients, clinical assessment and tests like the classical Mini Mental State Examination has been applied (Vann Jones & O'Brien, 2014). Dementia with Lewy body patients have also demonstrated learning and memory deficits using the California Verbal Learning Test (Filoteo et al., 2009). Interestingly, in contrast to Alzheimer's Disease, the cognitive decline in Dementia with Lewy Bodies has been described to mostly affect attentional and executive functioning in contrast to memory – although memory loss tend to worsen in the later stages of the disease (Outeiro et al., 2019).

According to the diagnostic criteria, it is possible to be diagnosed with Dementia with Lewy bodies without having any motor deficits. However, in many cases, Dementia with Lewy

bodies patients will develop Parkinson's symptoms on par with Parkinson's Disease patients (Berg et al., 2015). The presented movement deficits may vary but bradykinesia, rigidity and/or resting tremor, as described above, are commonly demonstrated (McKeith et al., 2017). Another diagnosis that covers the cognitive decline and movement symptoms seen in Dementia with Lewy bodies is Parkinson's Disease Dementia (Jellinger & Korczyn, 2018). Yet, Dementia with Lewy bodies is diagnosed if dementia and motor symptoms occur within one year of each, whereas Parkinson's Disease Dementia is used if dementia develops after a longer period with Parkinson's symptoms (Outeiro et al., 2019). Thus, the difference between diagnoses is the arbitrary timing of events (Jellinger & Korczyn, 2018; Menšíková et al., 2022). The useability and validity of distinguishing between Dementia with Lewy bodies and Parkinson's Disease Dementia have thereby been questioned (Jellinger & Korczyn, 2018). A recent paper has even suggested a biologically based approach, where all diseases associated with neuronal αSyn pathology should be encapsulated by the common term: neuronal α-synuclein diseases (Simuni et al., 2024). Here, both Dementia with Lewy bodies and Parkinson's Disease will therefore be referred to as neuronal synucleinopathies in this thesis.

5.3 Dementia with Lewy Bodies and Parkinson's Disease are Associated with Alpha-Synuclein and Dopaminergic Pathology

The motor and neuropsychological symptoms demonstrated by Parkinson's Disease and Dementia with Lewy bodies patients are associated with the degeneration of dopaminergic neurons in the substantia nigra (Perugini et al., 2018). Thus, a better understanding of what causes the neuronal pathology in these neuronal synucleinopathies will not only aid in the development of treatment, but also bring about a new and enriched understanding of how the biology of the human brain constitutes behavior.

As a rule of thumb, the degeneration is fairly localized to the substantia nigra in Parkinson's Disease, while a main part of the Dementia with Lewy body disease is additional changes in the neocortex and limbic structures (Outeiro et al., 2019). However, neuronal loss in other areas than the substantia nigra is also observed in Parkinson's Disease patients (Esteves & Cardoso, 2020).

The neurons of the substantia nigra project to the striatum, which, like the substantia nigra is part of the basal ganglia (Lanciego et al., 2012). Thus, the consequence of dopaminergic neuronal loss is reduced dopaminergic signaling to the striatum, which, as a

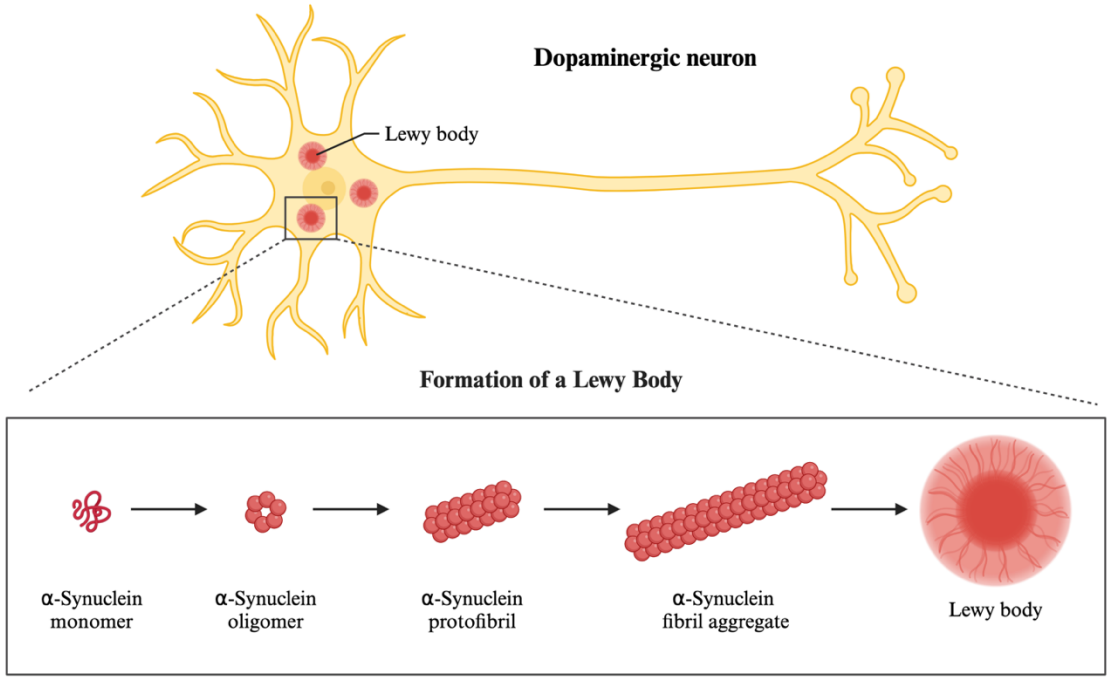
structure, is interconnected with many other brain areas, including the cortex (Bloem et al., 2021; Weintraub, 2020). For these reasons, a ligand, that binds to dopamine transporters (proteins expressed by dopaminergic neurons) in SPECT or PET scans, is used to reveal dopamine-related pathology in the striatum of both diseases (Outeiro et al., 2019).

The intracellular alterations underlying degeneration is of focus in this thesis. The major neuropathological hallmark in the neuronal synucleinopathies, Lewy bodies, are generally spread out in the substantia nigra (Morris et al., 2024). The spreading pattern has been used to differentiate Parkinson's Disease and Dementia with Lewy bodies, as Lewy bodies in Dementia with Lewy bodies appear more often in parietal and temporal lobes (Jellinger & Korczyn, 2018). However, Lewy bodies have also been detected in other brain areas, like the cortex, in Parkinson's Disease patients (Panicker et al., 2021). Another potential difference could be the occurrence of Alzheimer's-like pathology tau and amyloid beta deposits) that is relatively frequent in Dementia with Lewy bodies, though not a ubiquitous finding (Jellinger & Korczyn, 2018). However, Alzheimer's like pathology has also been found in samples from Parkinson's Disease patients (Esteves & Cardoso, 2020).

As αSyn has been uncovered as a main constituent of Lewy bodies (Spillantini et al., 1997), it is thought to be the main toxic protein that underlies the tissue degeneration in the neuronal synucleinopathies (Morris et al., 2024). The physiological function of αSyn has not yet been confidently determined, but it appears to locate to synaptic terminals, where it plays a role in release of neurotransmitters and vesicle transport (Morris et al., 2024). In the pathological condition, it is believed, that αSyn starts out in a basic unit monomeric form, that band together with other monomers and forms a bigger αSyn oligomer in the process. These αSyn oligomers in turn accumulate and form ordered arrangements termed protofibrils (Calabresi et al., 2023). These protofibrils form larger fibrils, that form aggregates and together with other protein-aggregates development into Lewy bodies (Calabresi et al., 2023).

Figure 1

αSyn Aggregates and Form Lewy Bodies



The proposed step-like process of Lewy body formation from αSyn in the neuronal synucleinopathies is shown. Adapted from "Formation of Lewy Body from α-Synuclein", by BioRender.com (2024). Retrieved from https://app.biorender.com/biorender-templates.

Whether the Lewy bodies themselves are the main pathological component of the underlying neuronal death in these diseases is an outstanding question. The amount of detected Lewy bodies seems to correlate poorly with severity of symptoms (Outeiro et al., 2019). It has therefore been speculated whether these inclusion actually offer some neuroprotection against the aggregated proteins (Outeiro et al., 2019; Panicker et al., 2021). Instead, the smaller αSyn oligomers (figure 1) have been suggested to be the more toxic αSyn-specie (Alam et al., 2019). These oligomers have also been found able to disrupt the cell membrane of induced pluripotent stem cells-derived dopaminergic neurons, underscoring them as toxic (Cascella et al., 2021). It might therefore be the case, that more than one form of αSyn is involved in the pathogenesis (Alam et al., 2019). Further demonstrating the potential toxicity of αSyn, Luk and colleagues (2012) showed that engineered αSyn protein, when injected into the striatum of mice, was able

to spread to the substantia nigra and result in parkinsonian-like loss of DA-neurons as well as the formation of Lewy body-like inclusions.

The potential molecular mechanism that causes the degeneration observed in the neuronal synucleinopathies are not straightforward. However, a strong link to αSyn has been established. Thus, a focus on this protein and its potential pathological interaction partners, such as those found inside Lewy bodies together with αSyn, like the p25 protein, could be a valuable starting point.

6. Tubulin Polymerization Promoting Protein/p25α (p25)

The p25 protein is of special interest for the neuronal synucleinopathies because of its relationship with αSyn (Oláh et al., 2020; Oláh & Ovádi, 2019). Investigating its pathological properties may, in part, shed light on the mechanisms, that can contribute to neurodegeneration. As such, it may be involved in the loss of dopaminergic neurons that cause the neuropsychological dysfunctions presented by Parkinson's Disease and Dementia with Lewy bodies patients.

p25 is made up of a 219 long amino acid chain and is normally only found in oligodendrocytes (Oláh et al., 2020). Conversely, the clue to its potential pathological nature it the localization of p25 to Lewy bodies in tissue samples from Parkinson's Disease and Dementia with Lewy bodies patients (Kovács et al., 2004). This finding was subsequently confirmed by Lindersson and colleagues (2005). In these studies, p25 was also shown to co-localize with αSyn (Kovács et al., 2004; Lindersson et al., 2005). Additionally, various cell studies have found the p25 protein able to induce a major hallmark of the neuronal synucleinopathies – the aggregation of αSyn (Lehotzky et al., 2021). In contrast, the normal physiological function of the p25 protein is to induce the assembly, or polymerization, of microtubules (Schofield & Bernard, 2013). Microtubules make up essential cytoskeleton structures in neurons, where they are much more stable than they are in many other cell types (Rolls et al., 2021). Thus, loss of microtubule structural integrity can be detrimental to the neuron's health (Mazzetti et al., 2024). The p25 protein has also been shown to cause formation of abnormal or aberrant microtubule structures *in vitro* (Hlavanda et al., 2002). This is very interesting, as microtubule structural changes are implicated in many neurodegenerative diseases, including neuronal synucleinopathies (Mazzetti et al., 2024; Power et al., 2017). For these reasons, the ectopic (out of place) localization of p25 to neurons instead of

oligodendrocytes to neurons is of interest as its interaction with αSyn and/or microtubules could provide insight into what could cause dysfunction and death of neurons underlying neuropsychological dysfunction observed in neuronal synucleinopathies.

6.1 p25 is an Intrinsically Disordered Protein

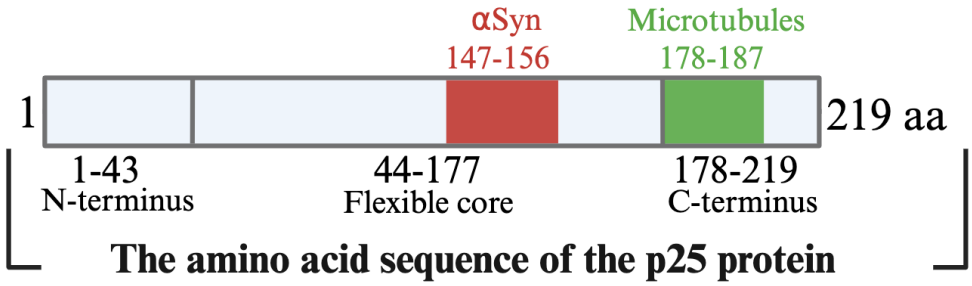
The structure of a protein largely determines its function, why investigating the physical aspects of the p25 protein could reveal insights into its assumed pathological effects. p25 is an example of a protein possessing intrinsically disordered regions (Oláh et al., 2020; Kovács et al., 2004). Intrinsically disordered regions refer to the fact, that the amino acid chain, that makes up the protein, does not take up a clearly defined structure, and may instead switch between structures (Holehouse & Kragelund, 2023). This has functional consequences as proteins make use of binding sites to interact and physically bind with regions of amino acid sequences of other proteins. Thus, proteins with intrinsically disordered regions may therefore bind to more targets (Turoverov et al., 2010).

p25 follows the trend of intrinsically disordered proteins having multiple functions and interaction partners depending on the given context – termed moonlighting properties (Jeffery, 1999; Singh & Bhalla, 2020). In the case of p25, being located to neurons instead of oligodendrocytes, as seen in the synucleinopathies, provides a change in scenery, that may induce p25's moonlighting abilities and the binding to microtubules and/or αSyn (Jeffery, 1999; Oláh et al., 2024).

A region of the p25 protein termed the C-terminus is especially responsible for the binding to microtubules – as a truncated version of this protein now only making up this region (178-187 amino acids – see figure 2), displayed similar binding affinity to the full length p25 (Tőkési et al., 2014). The 147-156 amino acid region was originally thought to be the main binding site of αSyn. However, mutant forms of p25 e.g., lacking the 147-156 amino acid region can still bind αSyn (Szénási et al., 2017). Therefore, additional regions may also contribute to the binding of αSyn – which has been ascribed to the protein's intrinsically disordered regions (Oláh et al., 2020; Oláh & Ovádi, 2019).

Figure 2

The p25 Binding Sites of αSyn and Microtubules



The 219 long amino-acid sequence making up the p25 protein is demonstrated. Although additional sites may allow for the binding of αSyn (red), it was originally proposed to bind to the 147-156 sequence of the flexible core, as shown. Microtubules (green) bind to the 178-187 sequence of the C-terminus of the p25 protein. Created with BioRender.com.

p25 therefore possess the necessary binding sites to interact with both microtubules and αSyn (figure 2), which, if occurring inside neurons, may be pathological in the neuronal synucleinopathies because of the moonlighting abilities of the protein.

6. 2 Microtubules are Involved in Vital Neuronal Activity

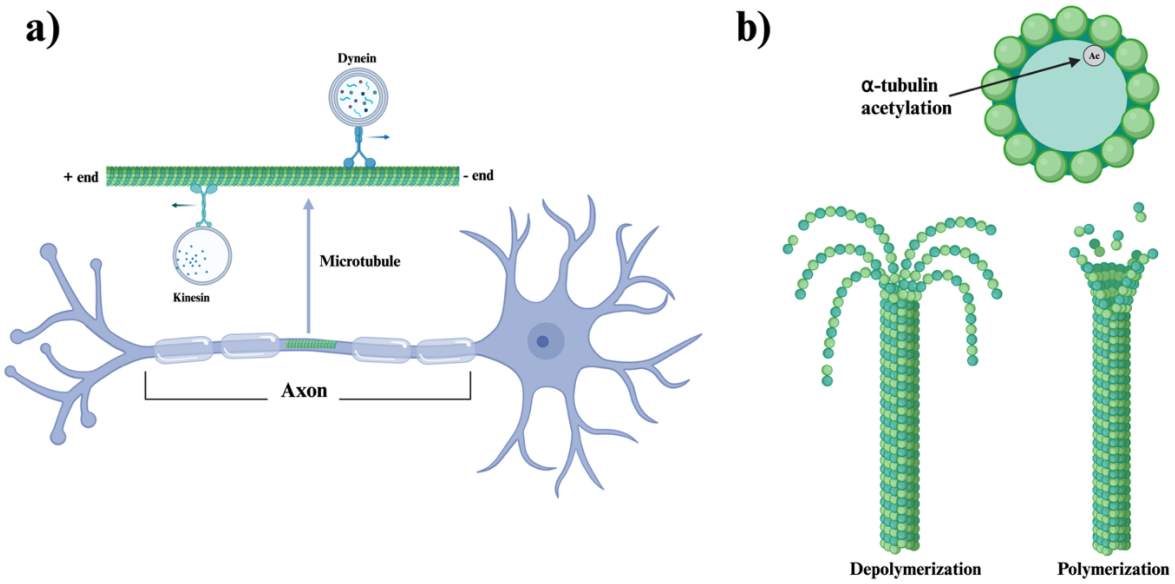
The microtubule structures are essential for the intraneuronal transport e.g., from the neuronal soma to the axon terminals. This includes the transport of synaptic vesicles, that contain neurotransmitters, to the presynaptic active zone, where the neurotransmitter can be released e.g., following an action potential (Waites et al., 2021). Microtubules are of interest because of their direct relationship with the p25 protein (Oláh & Ovádi, 2019), which could be pathological when present in neurons. As such, their dysregulation could lead to neuronal death and thereby play a role in the varied symptoms of the neuronal synucleinopathies.

Microtubules are cylindrical hollow structures made up of subunits called α-tubulin and β-tubulin monomers that band together in a ring-like structure (Baas et al., 2016). The polymerization of tubulin, which the p25 protein is proposed to induce, refers to the addition of subunits to the microtubule structure, making it grow, on the other hand, depolymerization refers to the loss of subunits, or shrinkage (Waites et al., 2021 - see figure 3, b). In neurons, there are additional free tubulin subunits available to allow continual polymerization and depolymerization processes (Baas et al., 2016). When assembled, one end form a minus end,

generally oriented towards the soma, whereas the other form the plus end typically oriented towards the periphery of the neuron (Chakraborti et al., 2016). This polarization is important for the transport of cargo e.g., alongside axons (figure 3, a), as the motor protein dynein carry cargo towards the soma and the minus-end, e.g., for degradation of proteins, whereas kinesin transport cargo towards the axon terminal and the plus-end e.g., for vesicle release (Baas et al., 2016). While axons are typically uniformly polarized as plus-end out, dendrites show a mixed polarization (Rolls et al., 2021; Waites et al., 2021).

Figure 3

Microtubules in Neurons



a) A schematic of a microtubule structure inside an axon is shown. Dynein and kinesin motor proteins are shown to move towards the minus-end and plus-end, respectively. b) The depolymerization and polymerization processes where tubulin monomers are added or removed, respectively. A schematic of the post-translational modification, acetylation, of lysine 40 inside the microtubule structure is also shown (exaggerated). Created with BioRender.com.

Although microtubules are more stable in neurons, they are generally dynamic in nature, meaning that they undergo assembly and disassembly (Baas et al., 2016). Microtubules dynamics have also been more directly associated with learning and memory, as administration of the drug paclitaxel (that stabilizes microtubules) after conditioning, caused an increase in learning and memory in mice – on the contrary treatment with nocodazole (that destabilizes

microtubules) caused a decrease in learning and memory (Uchida et al., 2014). Indicating regulation of microtubule dynamics in behavior possible related to regulation of cargo transport (Waites et al., 2021).

Microtubules are also subject to post-translational modifications (Roll-Mecak, 2020). These modifications refer to the addition of small molecules, called functional groups, that can alter the properties of microtubules. One such post-translational modification is acetylation (Roll-Mecak, 2020). Here, the addition of an acetyl group is added to the 40th amino acid of the α-tubulin subunit, which is the amino acid lysine, on the inside of the microtubule structure (figure 3, b). This post-translational modification, although not fully understood, is linked to the stabilization of microtubule-structures (Chakraborti et al., 2016), suggesting that they do not undergo as much dynamic change as unstable microtubules (Baas et al., 2016). Post-translational modifications are also associated with the p25 protein, as the p25 protein has been shown to inhibit the function of an enzyme called Histone Deacetylation 6 (HDAC6). This enzyme removes the acetylation of lysine 40. The p25 protein is therefore associated with higher levels of acetylated α-tubulin (Tőkési et al., 2010). Increased levels of acetylated α-tubulin has also been associated with Parkinson's Disease (Mazzetti et al., 2024). Finally, humans possess variants of the α-tubulin and β-tubulin subunits, termed isomers, that have slightly different amino acid sequences, that are expressed differently depending on the cell type (Roll-Mecak, 2020). As these isomers are essentially slightly different proteins, they are thought to impact the function and structure of microtubules (Roll-Mecak, 2020). Microtubules are therefore vital for the health of a neuron, and investigating proteins associated with them and their dynamics, like the p25 protein, could provide interesting insights into the neuronal death in neurodegenerative diseases like Parkinson's Disease.

6.3 p25's Potential Role in Neuronal Synucleinopathies

The ability of p25 to bind to both microtubules and αSyn could potentially play a pathological role when occurring in neurons, as aggregation of αSyn and microtubule dysregulation are thought to occur in Parkinson's Disease and Dementia with Lewy bodies (Calabresi et al., 2023; Pellegrini et al., 2017; Power et al., 2017).

p25's ability to induce aggregation of αSyn is supported by evidence from tissue samples and *in vitro* cell studies. A study demonstrated the interaction of p25 and αSyn by using Bimolecular Fluorescent Complementation technology. This technology permits the

visualization of the direct interaction of two proteins, as both proteins contain a fragment of a fluorescent protein, that together lights up if and when the proteins are close (Miller et al., 2015). Although not in neurons, but in tumor HeLa cell line, the hetero complex of p25 and αSyn were demonstrated to form on what seems to be the microtubule network – potentially demonstrating an interaction of all three interaction partners of interest in this thesis (Szénási et al., 2017). Additionally, p25 may also impair the degradation of αSyn, which could also play a role in the formation of αSyn aggregates e.g., found in Lewy bodies of patients with neuronal synucleinopathies (Lehotzky et al., 2021).

The pathological association of p25 with αSyn has also implicated the HDAC6 enzyme, as the impairment of this enzyme by the p25 protein has been associated with increased leakage of αSyn (Borland et al., 2022; Ejlerskov et al., 2013).

The ability of p25 to cause microtubule dysfunction has also been demonstrated in a cell study showed that high expression levels of p25 can alter the cytoskeleton in an aberrant manner (Lehotzky et al., 2004). Although Parkinson's Disease has been associated with decreased levels of acetylated α-tubulin (Esteves & Cardoso, 2020), a recent study by Mazzetti and colleagues (2024) also demonstrate presence of acetylated α-tubulin in neuronal cell bodies of subcortical regions including the substantia nigra of Parkinson's Disease patients, whereas acetylated α-tubulin was absent in controls in corresponding areas. In addition, they find acetylated α-tubulin to play a role in the aggregation of αSyn-oligomers as these proteins appear to co-localize and drive the formation of Lewy bodies (Mazzetti et al., 2024). This further underlies the involvement of microtubules in the neuronal pathology in Parkinson's Disease.

Taken together, the p25 protein has been shown to cause microtubule abnormalities and αSyn aggregation. This could suggest that the interaction of p25 with these proteins can be pathological and potentially cause neuronal death – especially when found in ectopically in neurons, as demonstrated to be the case in the neuronal synucleinopathies (Kovács et al., 2004; Lindersson et al., 2005). Thus, the observed neuropsychological impairments in the neuronal synucleinopathies can potentially be brought about by the p25 protein in various ways.

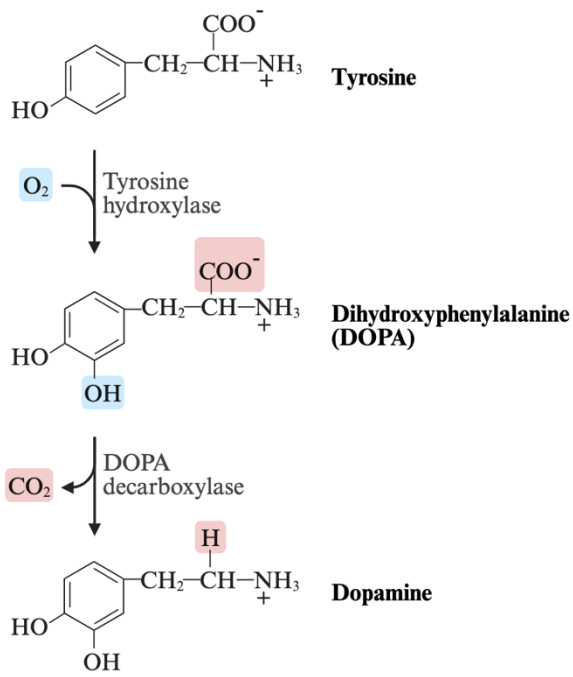
7. The Biochemistry of Dopamine Synthesis

Humans, as well as other species, utilize many different neurotransmitters for interneural communication. Of these neurotransmitters, DA could be said to be of a more elusive nature. This interesting molecule is associated with the p25-related neuronal synucleinopathies, where DA dysfunction may underlie the varied symptomatic presentation. DA is part of the catecholamine family, which includes norepinephrine, epinephrine and DA (Nakatsuka & Andrews, 2017). It is widely regarded as an important neurotransmitter, that impacts many distinct and seemingly unrelated functions in concert with having both neuro- inhibitory and excitatory properties. Some of the areas of DA-involvement could be neurodegenerative and psychiatric diseases in addition to functions like movement and learning. Although catecholamines can act as hormones, the function of DA as a neurotransmitter will be discussed in this thesis (Daubner et al., 2011).

DA is a monoamine primarily associated with the substantia nigra and the ventral tegmental area (Björklund & Dunnett, 2007). The synthesis of DA in these two midbrain structures depends on a range of enzymes. The starting point of the pathway can be the essential amino acid phenylalanine or, normally, its derived amino acid, tyrosine (Chagraoui et al., 2019). The catalytic effect of tyrosine hydroxylase is the rate-limiting step for DA biosynthesis (Daubner et al., 2011). Thus, this part of the pathway determines how fast the reaction can occur. To perform its function, tyrosine hydroxylase is phosphorylated by Protein Kinase A (PKA), that is activated by cAMP (Daubner et al., 2011). Tyrosine hydroxylase adds a hydroxyl group to tyrosine, which yields L-dihydroxyphenylalanine (or L-DOPA) (Chagraoui et al., 2019). Finally, by catalyzing the removal of a carboxyl group, L-DOPA is decarboxylated by aromatic L-amino acid decarboxylase (or DOPA decarboxylase), which produces the DA molecule (Chagraoui et al., 2019 - see figure 4).

Figure 4

The Biosynthesis of Dopamine



The biosynthesis of DA is shown. Tyrosine is hydroxylased into DOPA (or L-DOPA), which in turn is decarboxylased into DA. Adapted from "Catecholamine Neurotransmitters - Biosynthetic Pathway", by BioRender.com (2024). Retrieved from https://app.biorender.com/biorender-templates.

The activity of tyrosine hydroxylase can in turn be inhibited by DA itself (Daubner et al., 2011). After DA release, DA is transported back inside the neuron, which in turn inhibits the synthesis of additional DA. This 'backwards' transport of DA is performed by the dopamine transporter 1 (Bu et al., 2021; Daubner et al., 2011). The actions of dopamine transporter 1 thereby regulates the levels of DA (Jaber et al., 1997).

DA is, on the other hand, catabolized, or broken down, by enzymes such as Monoamine Oxidases (MAOs), whereas MAO-inhibitors can be used to increase the level of available DA (Jones & Raghanti, 2021; Stocchi et al., 2015). In general, the synthesis of DA occurs in the cytosol of the neurons. In here, the newly produced DA is packaged into vesicles by the protein Vesicular Monoaminergic Transporter 2, where it can be kept for storage until its release is warranted (Xu & Yang, 2022).

7.1 Dopaminegic Neurons Are Volume Transmitters and Exhibit Differing Firing Patterns

Physiologically speaking, the actions of DA are neither straightforward nor easy to survey. One of the reasons could be the non-canonical nature of DA release. Instead of the standard synaptic transmission, DA is often released through volume transmission (Costa & Schoenbaum, 2022; Liu et al., 2021). Volume transmission refers to a sort of spill-over-like release of the neurotransmitter in question - DA is therefore not always released directly post-synaptically as seen in standard synaptic transmission. Thus, the DA can be released into the extracellular space (Liu et al., 2021), which can lead to activation of targets far away from the original release site – which in relation to DA is termed varicosities. These varicosities resemble pre-synaptic boutons and reside along axons, yet, only a subset (around 20%) release DA (Liu et al., 2018, 2021). DA is sometimes referred to as a neuromodulator, which, as an example, may affect (or modulate) the strengths of the synaptic relationship between other pre- and post-synapses (Liu et al., 2021; Nadim & Bucher, 2014). This physical change in the connection between synapses is termed synaptic plasticity, and neuromodulators are thought to play important roles in affecting this plasticity throughout the brain (Magee & Grienberger, 2020). DA therefore impacts the sensitivity or ease to which neurons can communicate – potentially far away from its release site.

Voltage-gated ion channels play a large role in the regulation of the action potentials underlying DA release (Gantz et al., 2018; Liu et al., 2021). Although different ion channels play a role in dopaminergic firing, as with many other neurons, the calcium ion channels heavily supports the formation of action potentials in these neurons – whereas activation of engineered calcium receptors, the so-called GCaMPs, are used as an indirect marker of DA release (Howe & Dombeck, 2016). There do, however, exist evidence for calcium-independent DA release (Gantz et al., 2018).

DA-neurons generally exhibit either a basal rhythmic (or tonic) firing pattern or a more frequent burst (or phasic) firing (Gantz et al., 2018; Martel & Gatti McArthur, 2020). The firing rates are thought to support different types of behavior, but cannot be said to directly correlate with DA release, as DA has been detected in the extracellular space without precursor action potential (Liu et al., 2021; Martel & Gatti McArthur, 2020). In addition to the release of DA, some dopaminergic neurons co-express either glutamate or GABA in addition to DA – further

adding to the modulatory effects of DA-neurons and complicating a straightforward understanding of DA-neurons (Costa & Schoenbaum, 2022; Gantz et al., 2018).

7. 2 Humans Possess Functionally Different Dopamine Receptors Distributed Widely Throughout the Brain

The target(s) of DA are the five DA receptors, D1-D5, in humans. These five receptor subtypes are divided into D1-like (D_1 and D_5) and D2-like (D_2, D_3 and D_4) receptors (Martel & Gatti McArthur, 2020). The five DA receptors are metabotropic, and more specifically, G-protein coupled receptors. Thus, their activation releases an intracellular G-protein, which in the case of D1-like receptors are the G-protein subtypes, G_s and G_{olf}, and the G_i and G_o subtypes in the case of D2-like receptors (Gurevich et al., 2016). The distinction between D1- and D2-like receptors have historically dependent on the subtype's effect on the secondary messenger called cyclic Adenosine Monophosphate (cAMP) (Martel & Gatti McArthur, 2020). D1-like receptor activation are understood to increase cAMP levels, whereas D2-like receptors have the opposite effect (Beaulieu & Gainetdinov, 2011). The DA receptors affect the level of cAMP, by releasing their associated G-protein subtypes, which are mainly thought to activate or inhibit adenylyl cyclase, a protein that induces the formation of cAMP (Gurevich et al., 2016). cAMP can activate various enzymes, such as protein kinase A, that in turn may upregulate the translation of mRNA and thereby the production of proteins (Martel & Gatti McArthur, 2020). This is therefore one pathway, where DA can affect neuronal plasticity, depending on the activated receptor subtypes.

DA receptors show both distinct functions and distribution throughout the brain, including structures in the basal ganglia, hippocampus, cortex and most prominently, the striatum, where a class of GABAergic neurons, termed medium spiny neurons are the target (Bu et al., 2021; Gurevich et al., 2016; Martel & Gatti McArthur, 2020). These types of neurons generate the main output from the striatum. Some medium spiny neurons are associated with excitation-related D_1 receptors and others, inhibitory-related D_2 receptors (Liu et al., 2021). Many DA receptors has been located to places outside of synapses, suggesting that released DA must travel further away to contact DA receptors (Liu et al., 2018), like the pyramidal neurons of the prefrontal cortex (Tritsch & Sabatini, 2012).

7.3 Dopaminergic Neurons Exhibit Characteristic Morphology and Make Up Several Neuronal Pathways

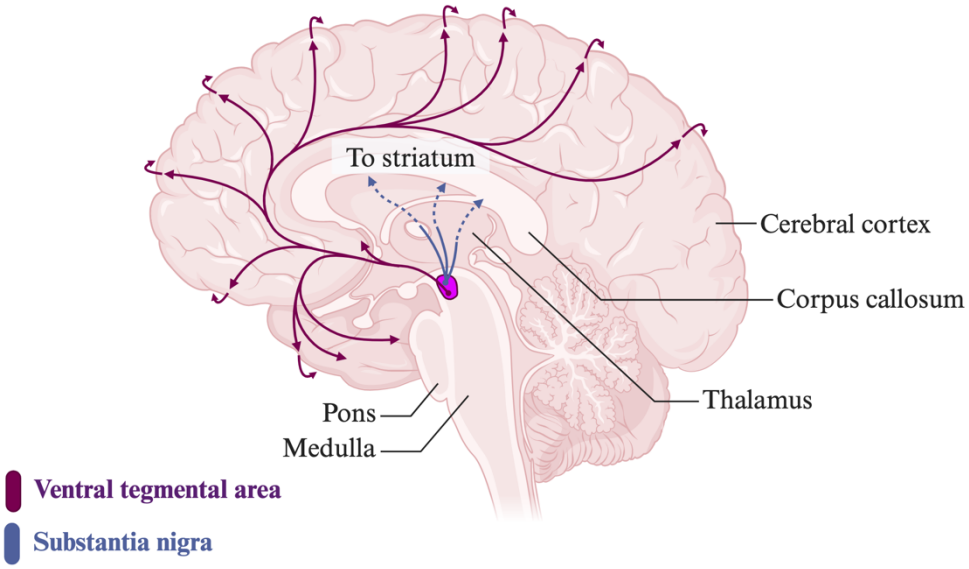
Although DA-neurons exist in heterogeneous forms (Gantz et al., 2018), the morphology of DA-neurons that allows for the distinct characteristics described above differs from that of other neurons. One example is the long axonal projections of one substantia nigra neuron that has been estimated to form 1 to 2.4 million synapses in the striatum (Bolam & Pissadaki, 2012). Thus, axonal projections from dopaminergic neurons have the potential to impact a vast number of other neurons. Other than the scale of some DA-neurons, another characteristic is their lack of myelination that, together with their size, leads to a great energy-demand, which has been suggested to create neurons more vulnerable to periods of homeostatic stress – such as the removal of build-up protein in the cell (Bolam & Pissadaki, 2012).

The substantia is part of a collection of nuclei termed the basal ganglia, which also include the striatum, that is made up of globus pallidus, the caudate and putamen nuclei, subthalamic nucleus and the pons (Lanciego et al., 2012). The ventral tegmental area is located near the substantia nigra but is not a part of the basal ganglia nuclei. Although the majority of DA-projections from these areas reaches the striatum, the modulatory effect of DA expands to areas far beyond due to bi-directional connections with cortical areas (Björklund & Dunnett, 2007; Costa & Schoenbaum, 2022).

The substantia nigra and ventral tegmental area are commonly described to form the basis of two distinct dopaminergic pathways in the human brain (figure 5). The nigrostriatal (or mesostriatal) pathway originates in the substantia nigra and projects to the dorsal striatum. The pathways from the ventral tegmental area are divided into the mesolimbic and mesocortical pathways, where projections end in limbic and cortical areas, respectively (Björklund & Dunnett, 2007; Gantz et al., 2018). However, as Gantz and colleagues (2018) mention, a distinct division between the pathways is not always applicable, as neuronal overlap between substantia nigra and ventral tegmental area do occur. As an example, ventral tegmental area neurons intermixes with the mesostriatal projections, while substantia nigra neurons intermixes with the mesolimbic and mesocortical pathways, at least in rats and primates (Björklund & Dunnett, 2007). Although being a long-standing view, the clear cut division of substantia nigra and ventral tegmental area into two distinct dopaminergic mesencephalic areas is therefore thought to be a too simplistic understanding of the DA-systems (Björklund & Dunnett, 2007).

Figure 5

Dopamine Pathways Originating From the Substantia Nigra and Ventral Tegmental Area



A simplified representative schematic of DA-pathways originating in the ventral tegmental area and the substantia nigra is shown. The substantia nigra primarily projects to the striatum, whereas the ventral tegmental area projects to the cortical areas. Adapted from "Distribution of Dopamine Neurotransmitters in the Human Brain", by BioRender.com (2024). Retrieved from https://app.biorender.com/biorender-templates.

Taken together, the different DA receptors as well as their functions, the diffuse nature of DA release and the architecture as well as the projections of DA-neurons support the vast functional involvement of this neuromodulator throughout the brain. Therefore, the loss of dopaminergic neurons, as seen in some neuronal synucleinopathies, have a wide variety of consequences.

7.4 Dopamine is Involved in Different Functions Associated with Neurodegeneration Diseases

Although the focus is on neuronal synucleinopathies and their association with dopaminergic neuron loss, these are not the only diseases associated with alterations of DA. Schizophrenia is frequently brought up, where DA receptors are of keen interest (Martel & Gatti McArthur, 2020). Depression is another severe neuropsychiatric disorder, which many Parkinson's Disease patients also suffers from, that has been linked to DA (alongside serotonergic) dysfunction for many decades (Opmeer et al., 2010).

As demonstrated by the various symptoms of motor dysfunction in Parkinson's Disease and Dementia with Lewy body patients, DA is pivotal for movement (Simuni et al., 2024). On the other hand, midbrain DA is also involved in other processes, as evident by the cognitive impairments also exhibited by these patients (Perugini et al., 2018). One such related function is learning, which is associated with DA activity.

7.5 Dopamine Modulates Movement

The motor symptoms exhibited by Parkinson's Disease patients, are one of the reasons that the dopaminergic midbrain areas are well studied (Tritsch & Sabatini, 2012). It was originally thought that the steady tonic firing rate exhibited by DA-neurons underlies movement, while phasic firing rates served a different purpose, such as responding to unexpected reward (Howe & Dombeck, 2016). But as a mouse optogenetic study by Howe and Dombeck showed (2016), the phasic firing rate of DA-neurons in the substantia nigra and ventral tegmental area is also associated with the mice's initiation of movement. Suggesting that not simply one firing rate supports movement.

A study by Birkmayer and Hornykiewicz cemented DA's role in movement, as they demonstrated the therapeutic effects of L-DOPA in Parkinson's Disease patients (Hornykiewicz, 2002). Compared to controls, the administration of L-DOPA significantly increases the level of DA in the striatum of Parkinson's Disease patients (Hornykiewicz, 2002). However, the exact mechanisms as to how L-DOPA administration causes an increase in available DA is not fully understood (Chagraoui et al., 2019). Some norepinephrine or 5-HT neurons may be able to metabolize L-DOPA and subsequently release it to its surroundings – although the evidence is not clear (Chagraoui et al., 2019). Possible related, the rate-limiting enzyme, tyrosine hydroxylase, is also expressed in non-dopaminergic neurons and found in increased quantities in the striatum of Parkinson's Disease patients (Björklund & Dunnett, 2007). It is also speculated whether L-DOPA itself possess neurotransmitter-like properties (Hornykiewicz, 2002). In either case, the effect of L-DOPA on Parkinsonian motor symptoms is immense, which underscores the role of DA in movement.

7.6 Substantia Nigra and Ventral Tegmental Area are both Involved in Learning

DA's role in learning has been apparent for many years. Both old and new evidence calls for the substantia nigra and the ventral tegmental area to be involved in learning (Costa &

Schoenbaum, 2022; Ott & Nieder, 2019; Schultz et al., 1997). Dating back to Schultz and colleagues' work (1997) in macaques, they demonstrated that dopaminergic neurons seem to fire in response to unexpected reward. If this reward was associated with a stimulus, i.e., a cue, the dopaminergic firing was detected at the cue-onset instead of the reward. Thus, the idea, that dopaminergic neurons encode how well a stimulus predicts a reward, or in other words, the reward-prediction error, was born (Schultz et al., 1997). Interestingly, they recorded from both the substantia nigra and ventral tegmental area, which implicates both these areas in learning (Schultz et al., 1997).

An example of the involvement of both areas in learning is a study by Ilango and colleagues (2014). To test the respective DA-areas' involvement in reward and aversive response, they injected viral vectors (that encoded opsins) into mice. This intervention allowed for optogenetic stimulation of the ventral tegmental area and/or the substantia nigra. Thus, using a self-activation paradigm (where the mice could activate their DA-neurons by lever-press), they found that activation of the substantia nigra neurons acted as a reward of similar level to the already established role of the ventral tegmental area in reward (Ilango et al., 2014). Interestingly, selective inhibition of the substantia nigra DA-neurons, when the mice were residing in specific area of their cage, induced aversion towards said area – implying that nigrostriatal DA-neurons can be involved in aversion in addition to reward (Ilango et al., 2014).

Projections to the substantia nigra from the cerebellum, which has been implicated in movement control as well, have been shown to be activated in response to reward, as an increased response of the dopaminergic somas in the substantia nigra was observed (Washburn et al., 2024). This further underscores the intricate connections of the substantia nigra with the rest of the brain.

Specific involvement of DA receptors in the striatum of mice supported reinforcing or aversive behavior. The activation of the D_1 expressing medium spiny neurons in the striatum was associated with reinforcing behavior, as the mice continued to conduct behavior triggering this activation, whereas activation of medium spiny neurons expressing D_2 receptors was linked to avoidance of continual triggering of this activation (Kravitz et al., 2012). This suggests that activation of specific DA receptors can contribute to different behavioral responses.

Although, aversive stimuli was not associated with the same spike in DA as unexpected rewards elicit in the earlier studies (Schultz et al., 1997), DA still plays an important role in mediating aversive association learning (Likhtik & Johansen, 2019; Zafiri & Duvarci, 2022).

Although the picture is less clear, some DA-neurons, e.g., from the ventral tegmental area (and the substantia nigra (Menegas et al., 2018)), may fire upon aversive stimulus and others may not (Lammel et al., 2014). Especially DA's connection with the amygdala (which receives ventral tegmental input) has been implicated in aversive/emotional learning. In here, involvement of both D1 and D2-like receptors are deemed instrumental for aversive associative learning (Likhtik & Johansen, 2019; Zafiri & Duvarci, 2022).

However, some substantia nigra neurons have also been found to fire in response to aversive stimulus (Menegas et al., 2018). Utilizing mice, Menegas and colleagues (2018) demonstrated that lateral substantia nigra DA-neurons projecting to the tail of the striatum may encode the intensity and not simply the positive or negative value of a stimulus. A highlight being, that mice with lesions of DA-neurons in the tail of the striatum failed to avoid air puffs (aversive stimulus) paired with water (attractant), when these air puffs could be avoided as water without air puffs could be accessed elsewhere - implicating this DA response in reinforcing aversion (Menegas et al., 2018). Thus, reward and aversion responses may be supported by distinct dopaminergic populations, but nevertheless supported by dopaminergic neurons in the midbrain.

Parkinson's Disease patients have also contributed directly to understand the role of the dopaminergic neurons in the substantia nigra. A study investigated the ability of Parkinson's Disease patients undergoing electrode-implantation surgery for deep brain stimulation to learn in a probabilistic task paradigm. Here, patients had to choose the most likely outcome when matching pairs of items. With a goal of maximizing rewards, feedback was provided to the participants after their answer was chosen by the click of a button (left or right button) (Ramayya et al., 2014). The electrode-implant surgery allowed the researchers to induce a phasic stimulation of the substantia nigra neurons when one of the item pairs was presented. They found stimulation of the substantia nigra neurons during correct choice of the most likely outcome to impair learning of this association. The reverse setup (providing stimulation when the answer was incorrect) seemingly had no effect on learning (Ramayya et al., 2014). The authors suggests that the electrical stimulation impairs stimulus-reward association, because the resulting substantia nigra stimulation induces an action-reward association instead - in this case, patients will not learn to associate the correct item-pairing with the positive feedback, but instead their act of button press (Ramayya et al., 2014).

Results from a very recent study, where four Parkinson's Disease patients (also undergoing deep brain stimulation surgery allowing for electrode recordings of DA-change) were faced with different monetary offers, suggest that substantia nigra DA response may track the difference in value with respect to the previously given offer (Batten et al., 2024). A decrease in DA was observed if the offer was worse than the previous, whereas an increase was observed for a better offer. This, as the authors mention, resembles dopaminergic changes in reward-prediction error (Batten et al., 2024).

One important and possible confounding variable when investigating learning in Parkinson's Disease patients, could the impact on motivation by lack of DA. As Berke (2018) mentions, impairment in movement-initiation could be interpreted as lack of motivation (Berke, 2018). Therefore, if Parkinson's Disease patients are simply severely less motivated to do well on tasks, like those seen in the probabilistic designs where being correct is not a one-to-one association, this may be the underlying reason for the poorer results on these somewhat less straightforward tasks.

In conclusion, DA supports a diverse set of brain functions, including movement and learning – which evidence from the neuronal synucleinopathies, namely Parkinson's Disease patients, has supported. It is interesting, that specific receptors, such as the D_1 and D_2 are involved in aversive learning in the amygdala (Zafiri & Duvarci, 2022). Similarly, D_1 and D_2 receptors also play a role in the medium spiny neurons of the striatum of mammals, where they promote opposite behavioral responses (Kravitz et al., 2012). As these functions are linked to the brain areas targeted by the p25-associated synucleinopathies, like the substantia nigra, looking into more cognitive related dysfunction is of interest in this project. Interestingly, it seems, that DA's involvement in movement and learning are conserved across many species, including those much different from humans, such as the nematode *Caenorhabditis elegans* (Vidal-Gadea & Pierce-Shimomura, 2012).

8. The *C. elegans* Nematode is a Valuable Model Organism for Neuroscience

At first glance, the little nematode may seem to lack relevance for the study of human biology in relation to neuropsychology. However, despite the obvious differences between worm and man, *Caenorhabditis elegans* is widely used in many different scientific fields. These fields extend to, but are not limited to, immunology, cancer, drug efficacy, aging and neuroscience

(Kaletta & Hengartner, 2006). The nematode exhibit distinct forms of behavior, like learning and movement, that, as in humans, are reliant on a functional DA system (Vidal-Gadea & Pierce-Shimomura, 2012). Thus, it has many applications, where the focus of this thesis is of course its useability in studying the neuropsychologically related impairments in the neuronal synucleinopathies.

C. elegans was originally chosen by Sydney Brenner as a model organism for the many benefits of its physiology (Ankeny, 2001; Brenner, 2009). *C. elegans* is a small roundworm and member of the nematode family. The small size of *C. elegans* is helpful for storage of the animals, which not only limits cost, but also permits the use of many worms in experiments, making high throughput more obtainable (Ankeny, 2001). In addition, it feeds upon *Escherichia coli* using its pharynx, a feeding organ (figure 6), making it easy to maintain in a laboratory (Kaletta & Hengartner, 2006). The population largely consists of hermaphrodites that reaches approximately 1.3 mm in size in adulthood (Ankeny, 2001; Kaletta & Hengartner, 2006). In general, hermaphrodites are also the sex used for conducting experiments. On the other hand, the male worms are smaller and appear much less frequently in the population (only around 0.1 %) (Frézal & Félix, 2015). The hermaphrodites provide a way to easily obtain genetically identical offspring, as they reproduce through self-fertilization, where they impregnate themselves using their own sperm (Kaletta & Hengartner, 2006). A big advantage of having males is the ease of crossing different genetic backgrounds, and thereby making it relatively straight forward to generate new mutant strains (Brenner, 2009). *C. elegans'* short lifespan of two to three weeks also has its benefits when conducting experiments, as the time from egg to adult is approximately three and a half days (Markaki & Tavernarakis, 2020). Another physiological trait that makes *C. elegans* a useful model is the see-through cuticle (Markaki & Tavernarakis, 2020). Because of this feature, genetically engineered mutants that expresses fluorescent proteins allow the researchers to detect internal changes of the worm without having to go through lengthy preparations of the tissue.

Being the first animal to have its genome fully sequenced, genetic manipulation, either adopting an old-fashioned crossing approach or a genetic engineering approach, is a technique used in all fields of *C. elegans* science (Rapti, 2020). The *C. elegans* genome is estimated to have 60-80% homology (genes originating from the same ancestor) with humans. However, even genes with less similar sequences can be useful in research, as the most important feature

is not always the similarity in the sequence of base pairs, but the function(s) of the protein eventually produced (Kaletta & Hengartner, 2006).

8.1 The *C. elegans* Nervous System

Brenner originally chose *C. elegans* as a model with the field of biological neuroscience in mind (Ankeny, 2001). The hermaphrodite sports 302 neurons. Their male counterpart has a few more adding to their total tally of 387 neurons. In addition, 50 glia cells are also present in the animal (Rapti, 2020). The highest concentration of neuronal synapses is found in the worm's nerve ring. This structure is sometimes referred to as the *C. elegans* brain (Cook et al., 2023), or neuropil, which denotes a form of neuronal tissue where neurons are heavily interconnected (Moyle et al., 2021). As Bargmann (2012) puts it, the *C. elegans* nervous system in general even seems "overconnected", as it is possible, for the most part, to draw a connection from one neuron to any other in three synaptic connections. The low number of neurons comes in useful when investigating interneural communication for which the worm utilizes similar mechanisms as seen in mammals. As humans, *C. elegans* possess both chemical synapses and gap junctions (Varshney et al., 2011). The neurotransmitters themselves are to a large degree the same as those utilized by human neurons, as neuropeptides, acetylcholine, GABA, glutamate, serotonin (5HT), DA and others are also present in the worm (McDonald et al., 2006). Although, epinephrine and norepinephrine are not present, they possess similar invertebrate types, called octopamine and tyramine (Mills et al., 2012).

Using electron microscopy, one of the first major goals for using *C. elegans* was to map the connections of their entire nervous system – or in other words, the worm connectome (Ankeny, 2001). This has proved helpful in understanding how electrochemical signals provide the basis for information exchange possible underlying the animals' behavior – and thereby also in understanding how this works in humans. Most of the chemical synapses are formed *en passant* or "along the way", and thereby not with a direct endpoint as synapses are classically depicted (White et al., 1986). In their original article, White, Southgate, Thomson and Brenner (1986) divided neurons into different classes, which include sensory, inter and motor neurons. Although, as the authors themselves mentioned, some neurons play more than one function, the legitimacy of dividing neurons based on their function has, at least to some degree, been supported by later studies. As an example, the sensory neurons are generally ciliated at their dendritic tip, which reaches out towards the outer layer of the worm, termed the cuticle,

facilitating their ability to sense chemicals or touch (Bae & Barr, 2008). One example is the ASE neuron pair, that are especially involved in sensing various chemicals, as laser ablation of these neurons rendered the worms unable to relocate in response to a given chemical (Bargmann & Horvitz, 1991). Thus, some neurons seem to have more specialized functions than others.

The intracellular environment of the neurons is also of keen interest in *C. elegans* neuroscience. The cytoskeleton components, like the microtubules, are studied for their contribution to neuronal function and thereby behavior (Harterink et al., 2018). Overall, many of these structures are conserved between species, but variation do occur. One example is the microtubules. As in humans, they are made up of different version, or isotypes, of α- and β-tubulin, but the *C. elegans* isotypes differs, humans e.g., have more subtypes of β-tubulin than the worm (10 vs. 6) (Lu & Zheng, 2022). However, as in humans, microtubules provide vital stability and cargo transport in neurons. To facilitate cargo transport, axons are generally organized with microtubule plus end out (thought to support kinesin-mediated anterograde transport), whereas dendrites are mostly found with minus-end out microtubules (for dynein retrograde transport) – however, dynamic microtubule structures (without clear polarity) are also present (Baas et al., 2016; Harterink et al., 2018). As in humans, *C. elegans* microtubules are also subject to post-translation modifications, like acetylation, and are therefore used to study the effects of these in addition to the organization of the microtubules themselves (O'Hagan et al., 2022).

Taken together, many of the nematode's physiological features provide an interesting model organism in the attempts to answer neuroscience and neuropsychological-related questions. Despite the obvious differences seen in the worm compared to humans, enough similarities are apparent to allow for this type of research to contribute significantly to the understanding of the nervous system in higher order animals. These similarities are highlighted further in the following sections addressing the worm's dopaminergic system.

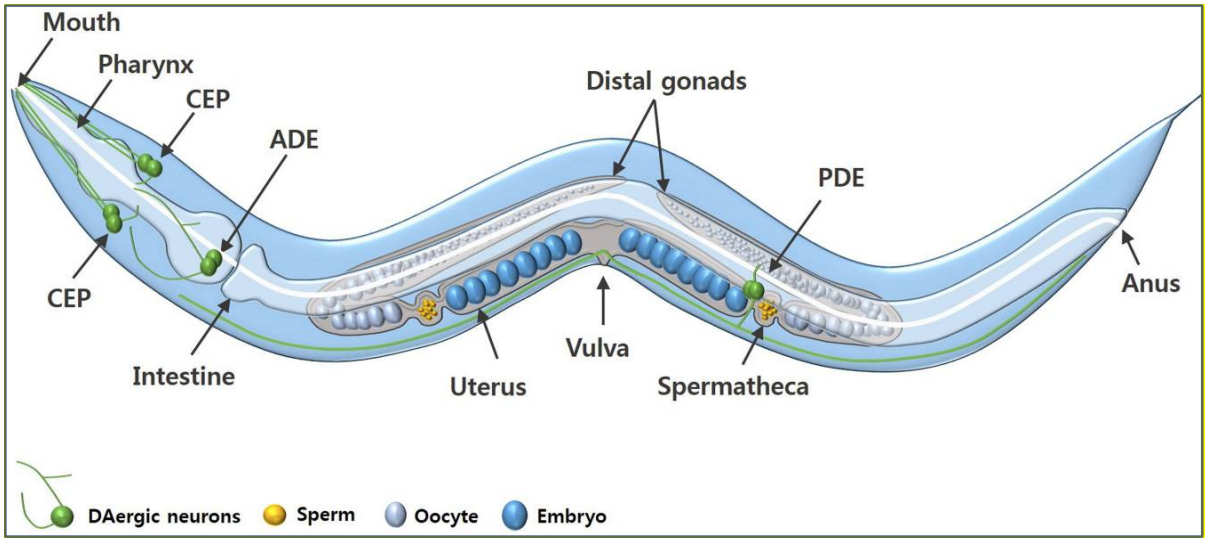
8.2 The Eight *C. elegans* Dopamine Neurons Function Similarly to Human DA-neurons

Of the 302 neurons in the hermaphrodite, eight are dopaminergic neurons (Suo et al., 2004). These not only function in a similar way to human dopaminergic neurons, as the nematode also utilizes similar G-protein coupled DA receptors to humans, but they are also responsible for

behavior like associative leaning (Raj & Thekkuveettil, 2022). All eight express tyrosine hydroxylase (the rate-limiting enzyme of DA-synthesis) – a common marker of DA-neurons also used in mammals (Vidal-Gadea & Pierce-Shimomura, 2012). Like humans, they also possess a homolog of the dopamine transporter 1, DAT-1, protein (Vidal-Gadea & Pierce-Shimomura, 2012). The worm's four most anterior dopaminergic neurons are termed the Cephalic (CEP) neurons, while the two Anterior Deirids (ADE) neurons reside not far behind. Finally, the two Posterior Deirids (PDE) neurons are found in the tail-end of the animal (McDonald et al., 2006; see Figure 6).

Figure 6

The C. elegans Dopaminergic Neurons



The *C. elegans* dopaminergic neurons are highlighted in green. The green circles represent the somas, whereas the green line represent the processes extending out from the somas. From *Caenorhabditis elegans: a model to investigate oxidative stress and metal dyshomeostasis in Parkinson's Disease* (p. 3), by PM Chege & G McColl, 2014, Front. Aging. Neurosci. CC BY 4.0. Reprinted with permission.

All four CEP neurons have dendrite-like processes that run towards the nose of the animal. In addition, their axons end in the nerve ring complex that is located just posteriorly to these neurons. Dendrites of the ADE-neuron pair project laterally to the side of the animal. Axons from both the ADE neurons are projected ventrally to form a ventral ganglion located posteriorly to the nerve ring (McDonald et al., 2006). The PDE neurons are somewhat similar to the ADE neurons, as their dendrite-like processes also project to the sides of the animal. Their axons project into the ventral nerve cord of the animal, which run along the animal

(McDonald et al., 2006). Commonly classified as mechanosensory neurons, the ciliated endings of the projections towards the outside of the animal, underlies the mechanosensationary ability of the dopaminergic neurons (Bae, 2008)

Interestingly, the expression patterns of tubulin isotypes are different between these three neuron sets, where the CEP and ADE neurons both express four isotypes of α-tubulin, the PDE neurons express six. On the other hand, both PDE and CEP express five β-tubulin isotypes, whereas the ADE neurons only express four (Lu & Zheng, 2022). Thus, the underlying microtubule architecture differs in these neurons.

As in humans, DA is both released through synapses as well as a volume transmitter in the nematode. DA also acts as a neuromodulator in the worm (Bargmann, 2012), and is found to act in neurons that are not in direct synaptic contact with either of the eight DA-neurons – demonstrating a similar role to DA in mammals (Chase et al., 2004).

The dopaminergic receptors are generally divided into four G-protein coupled receptor subtypes expressed in the *C. elegans* neurons. These include the DOP-1, DOP-2, DOP-3, and DOP-4. The DOP-1 receptor is thought to resemble the human D1-like receptors, while DOP-2 and DOP-3 resemble the D2-like receptors. The DOP-4 receptor is thought unique to invertebrates (Pandey & Harbinder, 2012; Wang et al., 2014). DOP-2 is thought to act as an autoreceptor, as it is expressed in the DA-neurons. In general, these receptors are expressed throughout the nervous system of the worm, allowing for the extrasynaptic actions of DA transmission (Vidal-Gadea & Pierce-Shimomura, 2012). Similar to G-protein coupled receptors in humans and other mammals, binding to these DA receptors also activates intracellular G-proteins (McDonald et al., 2006).

Underlined by the features described above, the dopaminergic systems of humans and the *C. elegans* nematode display conservation across species – both in way of transmission and the receptor subtypes present in the worm.

8.3 Dopamine is also Involved in Movement and Learning in *C. elegans*

The dopaminergic conservation continues from the molecular plane to the behavioral plane as DA, like in humans, is involved in movement and learning in *C. elegans* (Rahmani & Chew, 2021; Vidal-Gadea & Pierce-Shimomura, 2012).

Evidence for DA's role in movement in *C. elegans* comes from many different places. Motor deficits seen in Parkinson's Disease and Dementia with Lewy body patients could relate

some of the DA dependent movement-related phenotype in *C. elegans.* As an example, the basal slowing response in the worms refers to the decrease in the worms' speed when they sense a source of food (Sawin et al., 2000). Interestingly, DA underlies this motor function, as it has been demonstrated that ablation of dopaminergic neurons causes lack of basal slowing (Sawin et al., 2000). Interestingly, a more recent study using optogenetics found the dorsal CEP neurons to be more responsible for basal slowing response in comparison to the ventral CEP and PDE neurons – ADE neurons were not tested (Tanimoto et al., 2016). In addition, loss-of-function (i.e., a non-working mutation of a gene) mutants of tyrosine hydroxylase (*cat-2* in worms) that should be defective in DA synthesizes, also demonstrate impaired basal slowing response (Sawin et al., 2000).

Like humans, *C. elegans* also have different types of movement or gaits, where the switching between these relies on DA signaling, such as from swimming to crawling (Vidal-Gadea et al., 2011). This could again relates to movement disorder such as bradykinesia demonstrated by Parkinson's Disease patients (Bologna et al., 2020). Interestingly, the main culprit thought to underlie the neuronal death in the neuronal synucleinopathies, αSyn, has also been demonstrated to induce impairment in the initiation of swimming upon entrance into liquid when expressed in the eight dopaminergic neurons (Vozdek et al., 2022).

The similarities of DA functioning in humans and worms are not limited to movement, as DA also underlies associative learning in the nematode as it seems to do in humans (Schultz et al., 1997). As mentioned, *C. elegans* is capable of sensing various chemicals and adopting specific behaviors in response to them – a phenomenon termed chemotaxis (Bargmann & Horvitz, 1991). Often, chemicals may either elicit a positive response, where worms move towards the chemical, or negative, where they move away (McMillen & Chew, 2023; Rahmani & Chew, 2021). This ability is frequently quantified when assessing learning in *C. elegans*. For example, pairing an unconditioned stimulus, like a neutral chemical (one that does not strongly induce movement towards or away from it), with an unconditioned response, most often their source of food, can induce a positive chemotaxis response towards the chemical without the food, turning the chemical in question into a conditioned stimulus. In spite of its simple nervous system, associative learning is therefore within the *C. elegans* repertoire (Rahmani & Chew, 2021).

Raj and Thekkuveettil (2022) have demonstrated the need for a functional dopaminergic system to underlie this form of learning. In their study, worms would move towards butanol (a

weak attractant) after being conditioned with it in the presence of food. Genetic ablation of the DA-neurons implicated dopaminergic response in this form of olfactory learning, as these mutants demonstrated impaired learning (Raj & Thekkuveettil, 2022). Additionally, using GCaMP to indirectly measure DA release, they argue for an increase in DA release in conditioned animals compared to naïve worms. Furthermore, mutation of both *dop-1* and *dop-3* induced learning impairment, while neither mutation caused impairment on their own – implicating a relationship between these two antagonistic receptors (Raj & Thekkuveettil, 2022). DA is therefore pivotal for this type of associative learning.

As found in mammals, DA also seems to underlie and aversive forms of learning (Menegas et al., 2018). Besides facilitating an attraction towards a given chemical, like butanol, the worms' ability to form associations can also be hijacked to turn an otherwise inherently positive chemotaxis response into avoidance of the chemical (McMillen & Chew, 2023). One such paradigm is termed salt aversion (Rahmani & Chew, 2021). Normally, presence of 0.1 – 200 mM NaCl produces an attractive chemotaxis response towards the chemical, however, when *C. elegans* are conditioned with NaCl in the absence of food they start to avoid NaCl-sources (Saeki et al., 2001; Tomioka et al., 2006). Interestingly, both starvation and NaCl need to occur together to elicit the aversive response (Tomioka et al., 2006), whereas Saeki and colleagues (2001) suggests that salt aversion is a form of associative learning.

Calmodulin Kinase II (CaMKII) is associated with avoidance-related learning and memory formation through long term potentiation (where the synaptic strength of connected neurons increases following repeated stimulation (Magee & Grienberger, 2020)) in the hippocampus of rats (Whitlock et al., 2006). In the nematode however, Lim and colleagues (2018) found a loss of function mutation in the *cmk-1* (calmodulin kinase 1) gene responsible for learning impairment in salt aversion paradigm. This suggests that calmodulin kinase I and not II may play this role in the nematode (Lim et al., 2018).

Interestingly, the Acid Sensing Ion Channels (ASIC), a photon gated ion channel, expressed in substantia nigra dopaminergic neurons in humans, have been suggested to be involved in the neurodegeneration of Parkinson's Disease (Huang et al., 2015). In addition, although not in dopaminergic neurons, ASIC-channels are involved in the plastic response of long-term potentiation in the amygdala as measured by excitatory post-synaptic potentials in mice, which suggests that these channels could play a role in learning and memory formation (Du et al., 2014).

In nematodes, however, Voglis and Tavernarakis (2008) demonstrated the ASIC-1 channel (Acid Sensing Ion Channel 1) to, upon activation, facilitate DA signaling. In addition to the eight DA-neurons, *asic-1* is also expressed in tail-end PVQ interneurons. When *asic-1* is mutated, the worms are impaired in learning from 'aversive conditioning', as these mutants still show positive chemotaxis towards NaCl after conditioning. Administration of exogenous DA could rescue this learning deficit (Voglis & Tavernarakis, 2008). Based on this, the authors suggests that a role of the ASIC-1 channel is to suspend the activation and DA release from the dopaminergic neurons. This is further demonstrated by the detection of fluorescent intensities, that showed that the conditioning leads to enhanced DA release in the wild-type animals - an enhanced release that is impaired in the *asic-1* loss-of-function mutants (Voglis & Tavernarakis, 2008).

Hinting to the underlying G-proteins, that are also associated with human DA receptors, the $G\alpha_i$ (encoded by *gpa-14* in the worm) G-protein is also implicated in aversive learning. $G\alpha_i$ is expressed in concert with DOP-2 receptors in the worm's ADE dopaminergic neurons. Using both isoamyl alcohol and NaCl as a chemoattractant, they found worms with a putative *gpa-14* loss-of-function mutation to be defective in negative chemotaxis when conditioned with either of the chemicals in the absence of food (Mersha et al., 2013). Once again, the addition of exogenous DA during conditioning rescued this learning deficit. A double mutant of both the DOP-2 receptor and $G\alpha_i$ subunit did not demonstrate further impairment – which Mersha and colleagues (2013) suggest supports a synergistic role of these proteins as well as them acting upstream in the plastic chemotaxis response pathway. According to the authors, the DOP-2 receptor could act as an autoreceptor in the ADE neurons and thereby regulate dopaminergic release (Mersha et al., 2013; Vidal-Gadea et al., 2011). Although the G-protein encoded by *gpa-14* is not homologous to any mammalian G-proteins, it shows similarities to the human $G\alpha_i$ subunit that is activated by D2-like receptors (Pandey & Harbinder, 2012; Suo et al., 2004).

DA has also been implicated in a similar assay, termed gustatory plasticity, where the worms are exposed for a shorter amount of time to higher concentrations of NaCl than generally used in salt aversion learning (Hukema et al., 2008; Rahmani & Chew, 2021; Watteyne et al., 2020). Hukema and colleagues (2008) found worms carrying a loss-of-function mutation in either the *cat-2* gene required for DA-synthesis showed impairment in gustatory plasticity - thus, they did not avoid NaCl after being pre-exposed to it in the absence of food (Hukema et al., 2008). Finally, they also found DA receptor mutants, DOP-1, DOP-2 and DOP-3, to be

defective in gustatory plasticity, and thereby further demonstrate the involvement of DA in the behavioral plasticity of the animal (Hukema et al., 2008).

Thus, as in the substantia nigra in mammals, including humans, the DA system supports behavior, like movement and aversive associative learning in *C. elegans*.

8.4 The Molecular and Behavioral Features of *C. elegans* and its Dopaminergic System Makes it an Interesting Model of Neuronal Synucleinopathies

The neurological molecular and behavioral aspects highlighted in the sections above suggest that *C. elegans* is valuable model organism for the study of neuronal mechanism in the human brain, which is not surprising, given it was the initial reason for its choice. Seeing that it is capable of learning through association it can also provide insights into neuropsychology. In addition, it has been successfully applied as a model organism to research concerning several serious neurodegenerative diseases including Alzheimer's Disease and Parkinson's Disease (Markaki & Tavernarakis, 2020). In contrast to what one might think of an organism that only has eight dopaminergic neurons, the intricate interplay of DA receptors and G-proteins and the somewhat sophisticated behavioral features this system supports, makes it compelling for the study of synucleinopathies like Parkinson's Disease and Dementia with Lewy bodies (Maulik et al., 2017). This is also evident by the long history of Parkinson's research done in the nematode (Kaletta & Hengartner, 2006; Maulik et al., 2017). Many features of the DA system seen in humans, like neuronal degeneration, receptor dysfunction, relevant drug application and related behavioral deficits are available for study.

The *C. elegans* genome lacks a homolog of αSyn. Therefore, owing to the ease of genetic manipulation in the worm, one popular application is to overexpress the human αSyn in the *C. elegans* background causing several phenotypes (Markaki & Tavernarakis, 2020). As an example, overexpression of the human wild-type αSyn has been shown to cause degeneration of dopaminergic neurons (Lakso et al., 2003), providing a link to the proteins putative toxic role in Parkinson's Disease and Dementia with Lewy bodies.

In summation, *C. elegans* both possesses the necessary biology and demonstrate behavioral phenotypes, like movement and learning, relevant for neurodegenerative diseases like Parkinson's Disease. Furthermore, the nematode's physical characteristics, such as short lifespan and ease of genetic manipulation, make it a model applicable for the study of the underlying molecular change, that may give rise to the neuronal synucleinopathies. For these

reasons, *C. elegans* was chosen as the model organism for studying the neuronal synucleinopathy-related p25 protein.

9. The p25-model Used in This Thesis has Previously Been Shown to Cause Dopaminergic Impairment

The focus of this thesis, the p25-protein, has been the protein of choice in other master theses conducted under the supervision of Anders Olsen in the past. To investigate the effects of this protein, a plasmid containing the DNA-sequence of the human p25-protein fused to a sequence of the Green Fluorescent Protein (GFP) DNA was constructed. As a fluorescent protein, GFP can be used as a reporter protein, as it lights up when excited with a laser (Chalfie et al., 1994). In this case, it was therefore used to visualize the dopaminergic neurons. The *dat-1* gene is only expressed in the dopaminergic neurons, and it was therefore used as a promoter to ensure, that the p25::GFP protein product would be expressed in the worm's eight dopaminergic (Lakso et al., 2003). This plasmid was injected into the worm's gonad using a standard microinjection technique to obtain transgenic mutants (Berkowitz et al., 2008). Furthermore, the injected DNA-sequence was integrated into the worm's genome resulting in reliable inheritance of the genetic insert. This has resulted in the specific overexpression of the p25::GFP product in the worms' eight dopaminergic neurons – the base model of choice for this thesis.

Earlier work using this p25-model has made way for interesting discoveries. The expression of the p25::GFP fusion protein created was verified using western blot, that can be used to quantify protein (Vestergård, 2012). The consequence of p25-overexpression is morphological changes to dopaminergic neurons, and more concretely, the somas of the ADE neuron pair. They thin out and eventually end up being undetectable. Thus, these somas presumably degenerate with age (Vestergård, 2012; Christensen, 2013; Sørensen, 2014; Stenz, 2016; Fuglsang, 2017). It was however recently found that the degeneration was not as detrimental as originally thought (Rasmussen, 2023). Soma-degeneration of CEP and PDE neurons has not been detected – at least not to the same degree. Attempts has been made to introduce αSyn into the dopaminergic neurons of *C. elegans* to investigate the reported interaction of these proteins (Kovács et al., 2004). However, overexpression of αSyn under the DA specific *dat-1* promoter in the strain already expressing p25 led to a surprising rescue of ADE soma degeneration (Vestergård, 2012). This can be explained by 'promoter overload', where the promoter driving the expression does not work properly. This was supported by

western blots showing little or no presence of the p25::GFP product in this strain (Christensen, 2013). Instead, a strain overexpressing both p25 in the DA-neurons and αSyn in a pan-neuronal manner was created. This strain shows degeneration of ADE somas similar to what is observed in the p25-worms (Christensen, 2013).

The introduction of a deletion mutation in *tba-9* (encoding an α-tubulin subunit (Hurd et al., 2010)) to the p25-background caused a delay of soma degeneration, possible indicating the involvement of microtubules in this phenotype (Sørensen, 2014). Furthermore, investigation of a putative loss of function mutation in p25's interaction partner, *hdac-6* (involved in p25's interaction with microtubules), did not demonstrate further degeneration. Instead, it might cause later onset of ADE soma degeneration (Rasmussen, 2023).

To obtain mutations capable of suppressing this p25-induced degeneration, an EMS-screen (Ethyl Methanesulfonate – a mutagen) was conducted. This yielded mutants, that did not demonstrate ADE soma degeneration to the same degree as the p25-strain, suggesting that genetic pathways are indeed involved in causing this phenotype (Sørensen, 2014). Subsequent sequencing disclosed suppressor mutations to be in the *dlk-1* pathway, namely a *dlk*-1, a *pmk-3* and a *mak-2* mutation, which is involved in axonal repair after damage (Stenz, 2016; Fuglsang, 2017).

The behavioral consequences of p25-overexpression have been investigated by harsh touch and assessment of basal slowing response (Fuglsang, 2017; Rasmussen, 2023). The harsh touch assay revealed p25-worms to be impaired in response to being poked on the nose – quantified by the number of backwards bodybends it makes after being poked. The obtained suppressor mutants did not rescue the p25-worm's response to harsh touch (Fuglsang, 2017). The p25-worms were not impaired in basal slowing response (Rasmussen, 2023), suggesting the need for investigating other DA dependent behaviors.

9.1 The Aims of This Thesis

The purpose of this thesis is to investigate the ability of the p25 protein to induce impairment akin to those observed in the neuronal synucleinopathies, including neuropsychologically-related behavioral impairments. To this end, the above-mentioned model, where the human p25 protein is overexpressed in the worms' eight dopaminergic neurons will be used. As the morphological phenotype has been established, a possible behavioral affliction is of key focus in this thesis. In addition, investigating potential underlying molecular changes is also of

interest. In short, the overall aim is to evaluate the p25-overexpression strains as a model of synucleinopathies. To do this, the following hypotheses will be sought answered:

1. p25-overexpressing strains are impaired in aversive learning

Associative learning has been demonstrated impaired in Parkinson's Disease patients (Foerde & Shohamy, 2011). In addition, associative aversive learning is also linked to the dopaminergic system and the substantia nigra in mammals (Menegas et al., 2018), which is affected in both Parkinson's Disease and Dementia with Lewy bodies. Thus, the aim is to investigate whether overexpression of the human p25 protein causes impairment in associative learning, as this could reveal neuropsychologically relevant impairment in *C. elegans* brought about by the p25 protein.

As mentioned above, the p25-worms were not impaired in their basal slowing response. Interestingly, as Mersha and colleagues (2013) report, their strains were not impaired in basal slowing response either, but did show defects in aversive learning. Thus, to possible detect p25-related impairment in associative learning, the DA related salt aversion assay will be applied (Voglis & Tavernarakis, 2008). Furthermore, to investigate any exaggerated behavioral consequences of having the p25 protein and the disease-related αSyn present together, a strain expressing αSyn pan-neuronally (in all neurons) and p25 in the dopaminergic neurons will also be used in this assay.

2. The p25-protein co-localizes with microtubules

Microtubule malfunction has been implicated in the pathology of Parkinson's Disease and Dementia with Lewy bodies (Pellegrini et al., 2017; Power et al., 2017). As the p25-protein is capable of binding to microtubules, and in addition, potentially cause aberrant microtubule structures, the aim is to investigate whether these proteins co-localize inside the worm's dopaminergic neurons. This could potentially link to the previous results obtained using the *tba-9*-deletion mutation (Sørensen, 2014). To this end, antibody staining will be used to detect the presence and localization of p25 and microtubules of interest by binding to specific proteins (Duerr, 2013). This is done to provide clues as to how the p25-overexpression causes the observed morphological phenotype, and/or whether ectopic p25 may engage in a pathological complex with microtubules.

3. The p25-protein co-localizes with αSyn in a strain expressing both the respective proteins

Other than being able to bind to microtubules, the intrinsically disordered p25 protein can also bind to αSyn (Oláh & Ovádi, 2019). This could potentially hint to *in vivo* interaction of these two proteins, that are hypothesized to form pathological complexes capable of inducing αSyn-aggregation – a major hallmark of neuronal synucleinopathies (Simuni et al., 2024). Like hypotheses 2, antibody staining will be used to see detect possible co-localization and potentially aggregates of the two synucleinopathy-related proteins, p25 and αSyn. The strain described above, that expresses αSyn in a pan-neuronal manner and p25 in a DA-neuronal manner will be used for the staining.

10. Methods and Materials

10.1 *C. elegans* Strains and Maintenance

In the laboratory, worms are normally kept in a perish dish containing Nematode Growth Medium (NGM), which contains nutrients not only for the worm, but also the bacteria, they feed upon. To make the NGM medium, 2.5g/L soy peptone, 17g/L agar, 3g/L NaCl and 1L of ddH_2O was mixed before autoclaving for sterilization. After autoclaving, 5mg/L cholesterol in addition the following different salts were added such as the content reached 1mM $MgSO_4$, 1mM $CaCl_2$ and 25mM KH_2PO_4 - pH 6.0.

The NGM-plates were seeded with the OP50 *Escherichia coli* strain, that had been cultured in Lysogeny Broth (LB) media overnight to allow the *E. coli* to grow before being put on the NGM-plates. Worms were kept at 20°C.

Table 1

The Different Strains Used

Strain	Genotype	Mutation	Referred to as
Bristol N2	Wildtype		Wildtype
OLS152	vtIs7 [*dat-1*p::GFP] (in the AO N2 wild-type background)	BY250 backcrossed to the AO-lab N2 wildtype (expressing GFP in dopaminergic neurons)	GFP-control
MT15620	*cat-2* (n4547) II.	Deletion – putative loss of function of tyrosine hydroxylase	*cat-2*
BY250	vtIs7 [*dat-1*p::GFP]	GFP expressed in dopaminergic neurons	BY250
OLS325	aarIs1 [*dat-1*p::p25::GFP; *unc-119(+)*]	p25::GFP fusion protein overexpressed in dopaminergic neurons	p25
OLS342	aarIs2 [*dat-1*p::p25::GFP; *unc-119(+)*]; tmIs908	p25::GFP fusion protein expressed in dopaminergic neurons.	p25; αSyn

	[*unc-51*p::synWT + *unc-51*p::EGFP]	Wildtype αSyn expressed in body wall muscles and all neurons. Enhanced GFP expressed in body wall muscles and all neurons*.	
FX14478	tmIs908 [*unc-51*p::synWT + *unc-51*p::EGFP]	Wildtype αSyn expressed in body wall muscles and all neurons. Enhanced GFP expressed in body wall muscles and all neurons*.	αSyn-control

*The promoter, *unc-51*, was originally used to create a model expressing αSyn in all neurons (Kuwahara et al., 2008), but has been found to also be expressed in pharyngeal and body wall muscles (Ogura et al., 1994).

10.2 Crossing and Husbandry

I have earlier experienced unexpected phenotypes using the OLS93 strain i.e., a control strain to the p25 strain that only expresses GFP and no p25 in its dopaminergic neurons. This strain demonstrated severe movement impairment in a previously performed basal slowing assay, performing worse than the p25 and the wildtype strain (Rasmussen, 2023) To accommodate this problem, a new control strain was created by crossing. The BY250 strain, originally created by Nass and colleagues (2002), was ordered from the Caenorhabditis Genetics Center (CGC) and used for crossing with the AO-lab wildtype strain (N2). This ensures a more uniform genetic background between the new control (GFP-control) and the p25-strain, such that, ideally, the only difference should be the GFP and p25::GFP inserts.

To obtain males of the N2 strain utilized for crossing, N2 worms at the L4 stage were placed in an incubator, where they were kept at 31°C for six hours. This increases the risk of X chromosomal non-disjunction, which generate animals with one X chromosome – and hence males (Walsh et al., 2020). The BY250 was backcrossed with the AO-lab's N2 wildtype three times.

When offspring is produced through crossing, the offspring will be 50% hermaphrodite and 50% male (Walsh et al., 2020). Accordingly, after the first cross, males with GFP-expression were chosen to mate with N2 hermaphrodites. These were chosen, as these inherited the BY250 *dat-1*P::GFP insert allowing for visualization of the dopaminergic neurons. This was done two times. After the third cross, heterozygous hermaphrodites were allowed to self-fertilize. Thus, offspring of these hermaphrodites, were placed on individual plates (singled out) and allowed to self-fertilize. If all the offspring on a plate had fluorescent dopaminergic neurons, they were classified as homozygous and ready to use for experiments.

10.3 Strain genotyping

To ensure use of correct worms, the genetic background of the different strains used were verified by various methods. Pan-neuronal expression of αSyn was verified by fluorescent microscopy using an Olympus SZX16 microscope with CoolLED pE-300^{white} attached. The same was the case for the newly generated control (OLS152). The presence (or absence) of the human p25 gene or the *cat-2* deletion was verified by Polymerase Chain Reaction (PCR) using a S1000 Thermal Cycler (Bio-Rad). The difference between the αSyn-control and the p25; αSyn strain was determined by fluorescent microscopy, as the dopaminergic neurons of were more visible in the p25; αSyn strain (see below).

For PCR genotyping, a small number of worms from the strain of interest were placed in a PCR-tube containing a 1/100 concentration of proteinase K in lysis buffer (2.5 mM $MgCl_2$, 50 mM KCl, 0.45% NP-40 Tween 20, 10 mM Tris – pH 8.3 and 0.01% gelatin). The lysate-mix was placed in a -80° freezer for approximately 20-30 min. and then run on a lysis-program in the thermal cycler. These steps were done to break down the tissue and access the DNA. Afterwards, to upscale the DNA for detection, a primer-mix, containing 6 µl DreamTaq Green PCR Master Mix (2X) (Thermo Scientific) and 0.5 µl of each primer (table 2) were added to 2 mL Eppendorf tubes. Nuclease-free water was added until a total of 11.5 µl could be aliquoted to separate PCR-tubes. Finally, 0.5 µl of the lysis-mix from each strain was added to each of the separate PCR-tubes. The mix was run on the appropriate PCR-program. To detect the presence of a gene of interest, an electrophoresis gel (1% - 0.3 g agarose, 30 mL TAE buffer) containing the DNA samples were run, and subsequent imaging using the Bio-Rad ChemiDoc™ Imaging system was used to visualize the DNA.

Table 2

PCR Programs

Primers	PCR-program (minutes)
p25 forward primer: 5'-AGA TGC ACG GCA AGA ACT G-3′ **p25 reverse primer:** 5'-CCG GAC ACA TAG CCT GAC TC-3′	1. 95.0°C for 5:00 2. 95.0°C for 0:30 3. 59.0°C for 0:30 4. 72.0°C for 1:00 Step 2-4 was repeated 29 times. 5. 72.0°C for 5:00 6. 4.0°C for ∞
***cat-2* forward primer:** 5'-ACT TCC GTC CGT CTT GAG AA-3′ ***cat-2* wildtype reverse primer:** 5'-ACG AAC GAA AGC CTA ACG AA-3′ ***cat-2* mutant reverse primer:** 5'-GTT CTC GGC TAC TTT GGT GG-3′	1. 95.0°C for 3:00 2. 95.0°C for 0:30 3. 55.0°C for 0:30 4. 72.0°C for 0:50 Step 2-4 was repeated 30 times. 5. 72.0°C for 10:00 6. 4.0°C for ∞

A new set of primers for identifying the p25-gene was created during this thesis using primer3.ut.ee software.

10.4 Age Synchronization of *C. elegans*

To use worms of a similar age for experiments, worms were age synchronized. Gravid hermaphrodites were placed on plates and allowed to lay eggs for the time intervals indicated in the description of the different experiments. The eggs were then moved to new plates and allowed to grow to the desired age. An exception, however, was made for the wildtype and p25 strains used for the salt aversion experiments. Here the gravid hermaphrodites were removed from the plates, leaving the eggs in place instead.

10.5 Imaging of Worms

To image worms, using either Differential Interference Contrast (DIC) or confocal microscopy, worms were paralyzed in a drop of sodium azide (NaN_3) on a 2% agarose pad mounted to a microscope slide. A coverslip was gently placed on top of the worms and nail polish was used to seal the gap between the coverslip and microscope slide. Two Hamamatsu Orca-Flash 4.0 cameras with a 10 x UPLSAPO objective mounted to an Olympus IX83 inverted microscope run by the Olympus CellSens software were used to acquire images of the worms' neurons expressing fluorescent proteins. All imaging was acquired as z-stacks, where subsequent images are taken it increasing depths. This allows for making a 'scan' through the worm and make a 2D image where areas of interest further down are also visualized.

This protocol was used to visualize the dopaminergic neurons in the GFP-control and p25 worms, as well as the pan-neuronal expression of αSyn in the p25; αSyn and the αSyn-control strains. Synchronized (approx. two hours) six-day old worms were used. DIC images were taken with an exposure of 1 second, while the images showing GFP were taken with 500 millisecond exposure and a laser intensity of 10 %. A 60x magnification was used.

The free imaging software FIJI (Fiji Is Just ImageJ) was used to open and analyze fluorescent images. It was used to make max-projects of the z-stacks to generate 2D images to better visualize the neurons. Thus, in the images shown, it is possible to see neurons of various depths, as the images are generated from a 'scan' through the worms. Brightness and contrast were adjusted as necessary to optimally visualize areas of interest – e.g., to visualize co-localization of antibodies. It was also used to color the images to the intuitive color palette, e.g., as the antibodies used to detect GFP stained the GFP red.

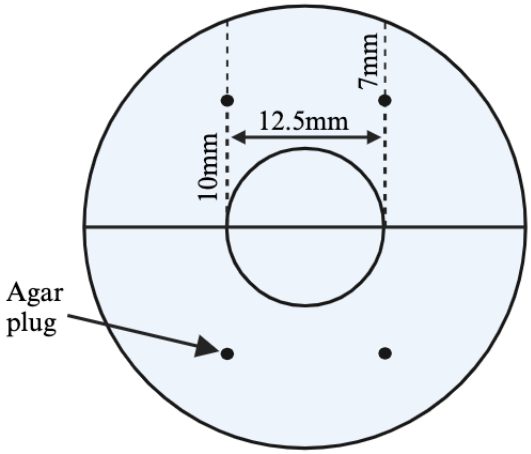
10.6 Salt Aversion Assay

The salt aversion assay used in this thesis was inspired and adapted from various papers (Adachi et al., 2010; Lim et al., 2018; Voglis & Tavernarakis, 2008). While four-day old worms (young adults) are commonly used in the literature, as ADE somas degenerate with age, six-day old worms were used for these experiments to have a bigger change of p25 having an impact. The obtain worms closer in age, worms were egg synchronized for 4.5 ± 0.5 hours. Worms were typically moved to new plates with fresh bacteria as necessary to avoid starvation. The experiments were performed while blind to the genetic background of the worms.

As in Lim et al. (2018) 35 mm plates were used. The assay plates used for chemotaxis were free of NaCl. 2% agar in ddH_2O was autoclaved and afterwards, 5 mM KH_2PO_4 – pH 6.0, 1mM $CaCl_2$ and 1 mM $MgSO_4$ were added. Approximately 4 mL media were added to each plate. The plate design (figure 7) was adapted from Adachi et al. (2010). As they used bigger plates for their assay, the dimensions were fitted to the smaller plates used in these experiments. However, to limit unspecific or random results, the start circle was allowed a bit larger.

Figure 7

Assay Plate Dimensions



The dimensions of the assay plates used for the salt aversion experiments are shown – inspired by Adachi et al., 2010. Created with BioRender.com.

To allow the worms to either move towards or away from NaCl, a gradient was made using plugs of agar containing 5 mM KH_2PO_4 – pH 6.0, 1mM $CaCl_2$, 1 mM $MgSO_4$ and 100 mM NaCl. These were placed at one end of the agar plate (Lim et al., 2018). Two control agar plugs, containing 5 mM KH_2PO_4 – pH 6.0, 1mM $CaCl_2$ and 1 mM $MgSO_4$, were placed opposite of the NaCl-containing agar plugs. All agar plugs were cut to a size of approx. 5 by 5 millimeters from agar-plates containing 4 mL of the respective media. The plugs were allowed to generate a gradient for three hours (Lim et al., 2018).

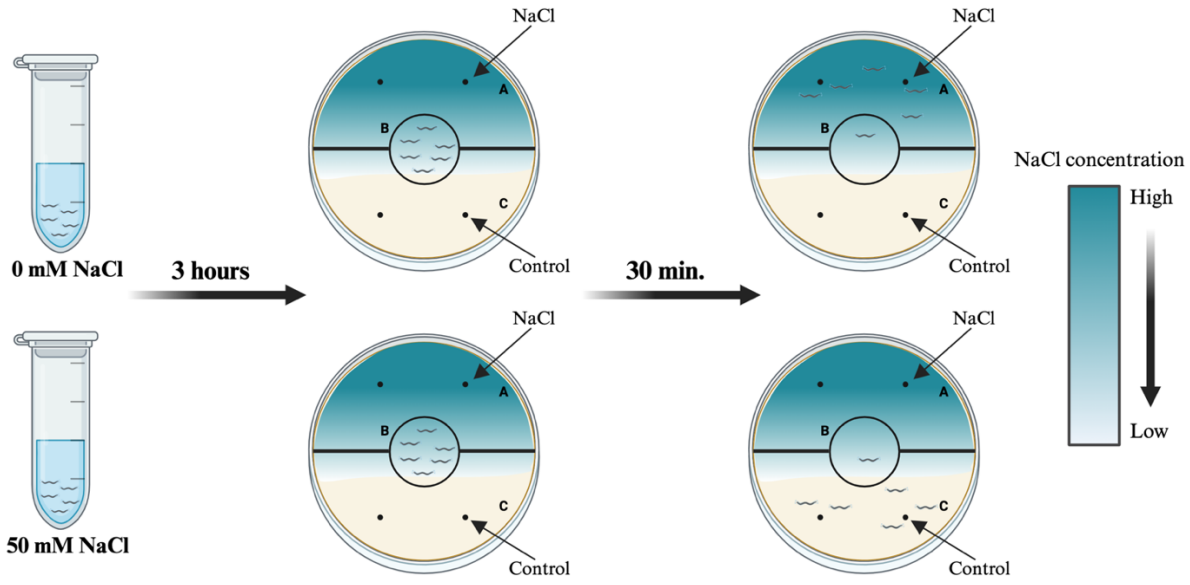
Before conditioning, the worms were picked from their plates and washed in their respective buffers three times. The mock-conditioning buffer, or 0 mM NaCl, contained 5 mM KH_2PO_4 – pH 6.0, 1mM $CaCl_2$ and 1 mM $MgSO_4$. While the NaCl-buffer contained 5 mM KH_2PO_4 – pH 6.0, 1mM $CaCl_2$, 1 mM $MgSO_4$ and 50 mM NaCl. For each round of the assay,

worms from each strain were either mock-conditioned or NaCl-conditioned for three hours, as a plateau of the chemotaxis index had been demonstrated to form after this time (Lim et al., 2018). However, to test a link to gustatory plasticity (Hukema et al., 2008), in one iteration of the experiment, the conditioning time was reduced to 15 minutes. The worms were conditioned at room temperature.

After conditioning, the worms were pipetted onto their respective plates. Excess buffer was removed using a Kimtech™ wipe. 1 µl of 0.5 M sodium azide (NaN_3) (used to paralyze and trap the worms) were spotted to each of the four spots where agar plugs had been placed (Adachi et al., 2010). If worms fell outside the circle, they were moved inside the circle with the Kimwipe™. If worms hit the sodium azide (NaN_3) during the transferring-process, these worms were censored from the count (this occurred in 2 out of 96 assays). The worms were allowed to move around the plate for 30 minutes at room temperature before scoring.

Figure 8

Salt Aversion Assay



The course of action for the salt aversion experiment. Worms were first picked from their plates and washed in their buffer (not depicted). Next, they were conditioned in their buffer for 3 hours, and placed on an assay plate. After 30 minutes, the worms would have moved around their plate, whereafter they were counted. The bluer, the higher concentration of NaCl. The control agar-plugs would also make a gradient (not shown). Created with BioRender.com.

A chemotaxis index was used to quantify the attraction or aversion of worms for NaCl. The assay plates were divided into area A, where NaCl agar plugs was placed, the starting circle was marked B and the area with control plugs was marked as C. The number of worms in each area was manually counted, and a chemotaxis index was calculated:

$$\frac{A - C}{Total\ number\ of\ worms\ (A + C)}$$

Thus, the chemotaxis index ranges from -1 to 1 (Adachi et al., 2010; Lim et al., 2018; Voglis & Tavernarakis, 2008). Worms at the center starting point (and the midline) were also counted, but not used for the calculation. When counting worms at the starting point, an artificial ceiling was made at 35+ worms, as it was difficult to reliably distinguish one worm from the other at that quantity. Although not in equal numbers, the salt aversion assays were performed in triplicates, where the used worms came from different offspring.

10.7 Antibody Staining

Antibody staining allows for visualization of the localization of proteins of interest (Duerr, 2013), also of targets, that do not normally fluoresce, like microtubules in this case. Four- and six-day old p25 and GFP-control worms were used to detect colocalization between tubulin and p25. To detect colocalization of p25 and αSyn, six-day old p25; αSyn and αSyn-control worms were used. Worms were synchronized by egg laying for approximately three and a half hours.

To gain access to the tissue needed for staining, a customized freeze-cracking approach developed in out lab was applied (Harders et al., 2018). This method utilizes cold temperatures to remove the outer cuticle of the worm, which mostly blocks the antibodies from reaching the desired target (Duerr, 2013). The worms were first placed in the S-basal liquid buffer (1 mM NaCl, 50 mM $KH_2\ PO_4$ – pH 6 and ddH_2O (Jensen et al., 2023)) on Superfrost™Plus Adhesion Microscope Slides (epredia) or Polysine™ (VWR) microscope slides. To make worms better adhere to the microscope slides, the worms were cut to release their germline (lower middle end of the worm) was cut with a needle. The worms were washed once in S-basal to remove bacteria, and a coverslip was placed on top. Excess buffer was removed. The slides were snap frozen on a metal block placed in a -80°C freezer for around 20-30 minutes and subsequently stored at -80°C.

To allow the antibodies to access their targets, the outer layer of the worm, called the cuticle, was ripped off. The slides were again placed on the metal block. A scalpel was used to flick off the coverslip, ripping off the cuticle in the process. The worms were then fixated in ice-cold methanol (-20°C). The slides were washed in PBS (Phosphate Buffered Saline – tablets, Sigma-Aldrich). Next, a Liquid Blocker PAP-pen (Daido Sangyo) was used to draw a hydrophobic square around the worms on the slide to help keep the liquid in place. Then, 2% skim milk (Marvel Original Dried Skimmed Milk) in PBS was used to block and limit unspecific binding. After at least 2 hours, the slides were washed in PBS.

The primary antibodies were added to 2% skim milk in PBS in the following concentrations: The anti-GFP rabbit polyclonal antibody (Abcam: ab290) was used to visualize GFP (1/2000). A mouse monoclonal anti-α-tubulin antibody (Sigma: T6199-200UL) was applied to detect tubulin (1/1000). The anti-α-tubulin antibody could be used to detect microtubules, as they band close together with the other β-tubulin subunits. The mouse monoclonal LB509 anti-alpha-synuclein antibody (Abcam: ab27766) was used to stain for αSyn (1/1000). The GFP antibody was used to detect the p25-protein. This is possible, as GFP and p25 together creates a fusion protein in p25-overexpressing strains; thus, wherever GFP is, p25 should also be. In addition, all strains produce GFP (table 1), however, GFP is typically lost during washing steps of the staining protocol (Scandella et al., 2020). This made it possible to use a GFP-antibody to detect the p25::GFP fusion, as the 'original' GFP signal should be lost. The access their targets, the primary antibodies were allowed to incubate at room temperature overnight.

The next day, the slides were washed in PBS, whereafter the secondary antibodies were added to 2% skim milk in the following concentrations: To detect the rabbit-based antibody (GFP in this case), a Cy5® goat anti-rabbit antibody (Abcam: ab6564) was used (1/1000), and to detect the mouse-based antibodies (αSyn and α-tubulin in this case), an Alexa fluor® 488 goat anti-mouse antibody (Invitrogen: A11001) was used (1/2000). The slides were incubated at room temperature for at least two hours. Afterwards, the slides were washed once in 1x Tris-Buffered Saline, 0.1% Tween® 20 Detergent (20 mM Tris, 150 mM NaCl, 0.1% Tween® 20 detergent). The slides were washed two times in PBS before being fixated with 2% paraformaldehyde for 15 minutes. The slides were finally washed once in PBS before a few droplets of Fluoromount-G™ (Invitrogen) were added to the slides. A coverslip was placed on top and excess Fluoromount was removed with a paper towel.

To image the antibody-stained worms, the confocal microscope and software described above was used. The magnification was 60x for both strains. The exposure for the GFP channel was set at 500 ms, while the laser intensity was set to 10 %. For the Cy5 channel, the exposure was 1 s, and the laser intensity was 25 %. Worms that were poorly stained (e.g., lacking much α-tubulin staining) were not used for the qualitative analysis.

10.8 Statistics

The statistical analyses applied to the data in this thesis was conducted using the open software program RStudio. Depending on the context of the salt aversion analysis, different types of analyses were used. First, normality of the residuals was visually inspected using QQ-plots etc. and tested for with the Shapiro-Wilk test. Approximately normal data was analyzed using a factorial ANOVA with a subsequent Tukey's Honest Significant Difference post hoc test or a Two Samples t-test – as commonly used to analyze salt aversion data (Lim et al., 2018; Voglis & Tavernarakis, 2008). For the non-normal data, a Kruskal-Wallis test was first conducted. Then, a post hoc Dunn's Test corrected using the Bonferroni-Holm alpha-adjustment was applied (Holm, 1979).

11. Results

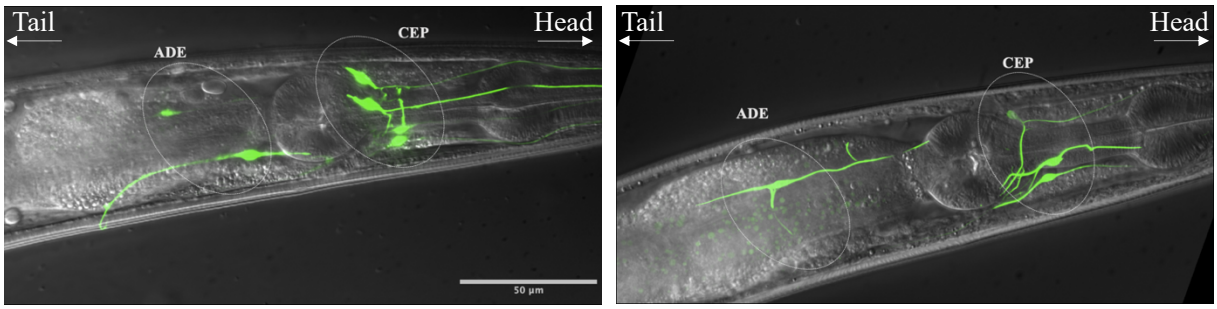
11.1 Degeneration of ADE Somas by Overexpression of p25 is Confirmed

Images of the GFP-control and p25 strains at day six were taken to simply verify that p25 can cause degeneration of ADE somas.

Figure 9

Overexpression of p25 Causes Loss of ADE Soma

a) GFP-control b) p25



Representative images of the GFP-control strain (a) and the p25 strain (b). Both worms are six days old. ADE and CEP neurons are encircled. The GFP-control worm still has two ADE somas, while only one ADE soma of the p25 worm is left.

The images verifies that p25 can cause degeneration of the ADE somas (figure 9). In this case the p25 worm only has one ADE soma left at day six, demonstrating the partial degeneration previously observed on day six.

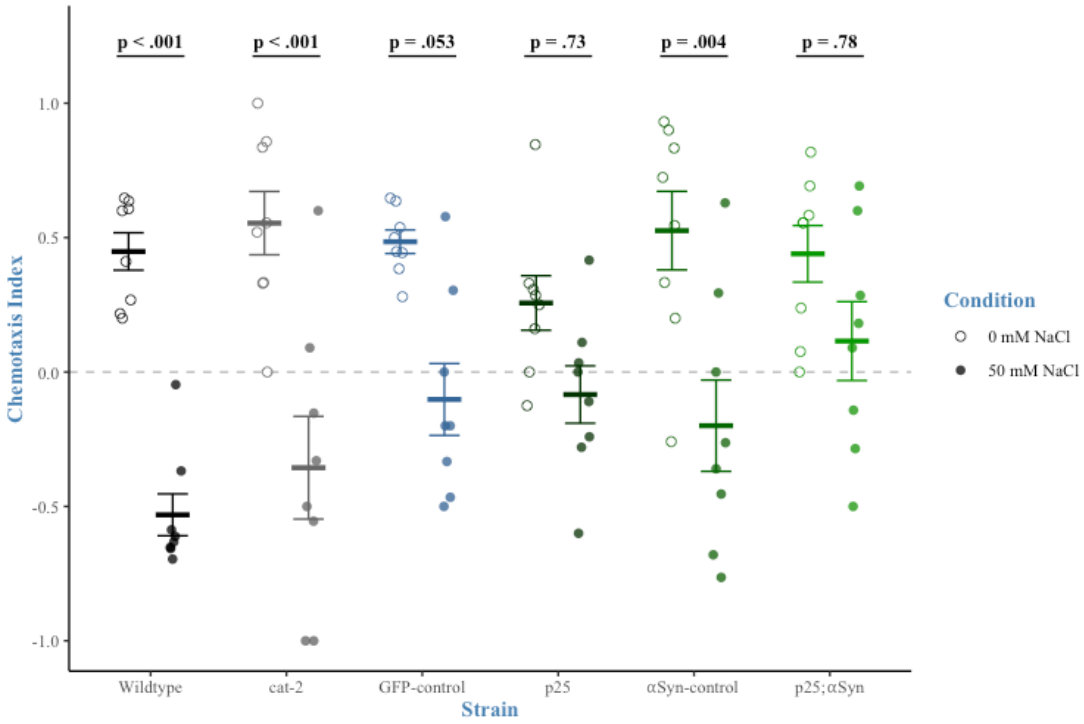
11.2 Overexpression of Human p25 May Cause Impairment in Salt Aversion Chemotaxis

Parkinson's Disease has been associated with various cognitive deficits, including learning and decision making (Perugini et al., 2018). In *C. elegans* salt aversion learning is associated with functional dopaminergic neurons (Mersha et al., 2013; Voglis & Tavernarakis, 2008). As p25-overexpression causes degeneration of the dopaminergic ADE somas (figure 9), the salt aversion learning assay was adopted to answer hypothesis 1 – that associative learning is impaired in worms overexpressing the human p25 protein. The calculated chemotaxis index was used to indicate what side of the assay plates the worms decided to move towards. A positive chemotaxis index indicates movement towards NaCl, whereas a negative chemotaxis

index indicates aversive movement. Thus, this index is taken to indicate whether worms learn to associate starvation with NaCl, and as a response, learns to avoid NaCl (Tomioka et al., 2006).

Figure 10

Overexpression of p25 May Cause Impairment in Salt Aversive Response



Results from the salt aversion experiments is shown. A lack of difference between being conditioned with or without 50 mM NaCl is observed in p25, p25; αSyn and unexpectedly, the GFP-control strains. $N = 8$ repeats per condition of each strain indicated by the dots – chemotaxis index was calculated based on 2.122 worms in total (see suppl. Table 1 for specific N per repeat). Error bars indicate SEM. The thick middle line indicates the mean.

To test differences in chemotaxis index between groups and conditions, and the interaction between these independent variables, a factorial ANOVA design was applied. No overall contributions is detected from the genetic background (strain) alone, $F(5, 84) = 1.522$, $p = .19$. As expected, the type of conditioning (with or without 50 mM NaCl) has a significant main effect on the chemotaxis index, $F(1, 84) = 80.563$, $p < .001$. In addition, the interaction between strain and condition also has a significant effect on the chemotaxis index, $F(5, 84) = 2.504$, p

= .036. Thus, as the chemotaxis index should be highly reliant on the type of conditioning, the overall F-statistics are as expected.

To investigate differences in means between groups, a Tukey Honest Significance Differences post hoc test was executed. First, in agreement with other papers (Saeki et al., 2001; Voglis & Tavernarakis, 2008), a significant difference is found between wildtype worms either conditioned with or without 50 mM NaCl ($p < .001$). This indicates that *C. elegans* worms can alter their decision, or learn, based on environmental differences. The *cat-2* mutants should by deficient in the production of DA, and therefore act as a positive control impaired in associative learning. Unexpectedly, the putatively DA deficient *cat-2* mutants also demonstrated significant differences between type of conditioning ($p < .001$). Although isoamyl alcohol was used as an attractant instead of NaCl, this contrasts with results from Voglis and Tavernarakis (2008), where they found the *cat-2* (e1112) strain to be impaired in aversive associative learning.

The difference between conditions of the newly generated GFP-control strain, which ideally should be similar to the wildtype strain, demonstrate a tendency towards being significant ($p = .053$). There is, however, a significant difference in chemotaxis index between the wildtype strain exposed to 50 mM NaCl and the GFP-control strain exposed to 0 mM ($p < .001$), whereas the opposite comparison (wildtype exposed to 0 mM and GFP-control exposed to 50 mM NaCl) is not significant ($p = .09$). This indicates that the reason for lack of significant difference between the two conditions of the GFP-control is the behavior of this strain after being conditioned with 50 mM NaCl. On the other hand, the condition-dependent difference in the p25 strain is, as hypothesized, not significant ($p = .73$). This suggests that p25-overexpression causes impairment in associating NaCl with starvation, as this strain do not strongly avoid NaCl after being conditioned with it in the absence of food. However, the lack of significant difference in the GFP-control strain complicates this conclusion, as a significant difference between the GFP-control's and p25's strains response to 50 mM NaCl should be significant to indicate more directly, that the p25 are impaired in salt aversion learning. This was not the case ($p = 1$ (rounded)).

When looking at the contribution of pan-neuronal expression of αSyn, the αSyn-control strain presented significant differences dependent on the type of conditioning ($p = .004$). Interestingly, the strain expressing both αSyn in all neurons and p25 in the dopaminergic neurons, p25; αSyn, did not reveal any difference between type of conditioning ($p = .78$). The

p25; αSyn strain is also the only one to demonstrate a positive chemotaxis index after being exposed to 50 mM NaCl (M = .11). This could imply that dopaminergic overexpression of p25 causes impairment in salt aversive learning, when expressed in an αSyn background. The only significant difference across strains of the same condition type is also between the wildtype and p25; αSyn strains exposed to 50 mM NaCl (p = .02). However, any difference observed between the αSyn-control and the p25; αSyn strains conditioned with 50 mM is not significant (p = .81).

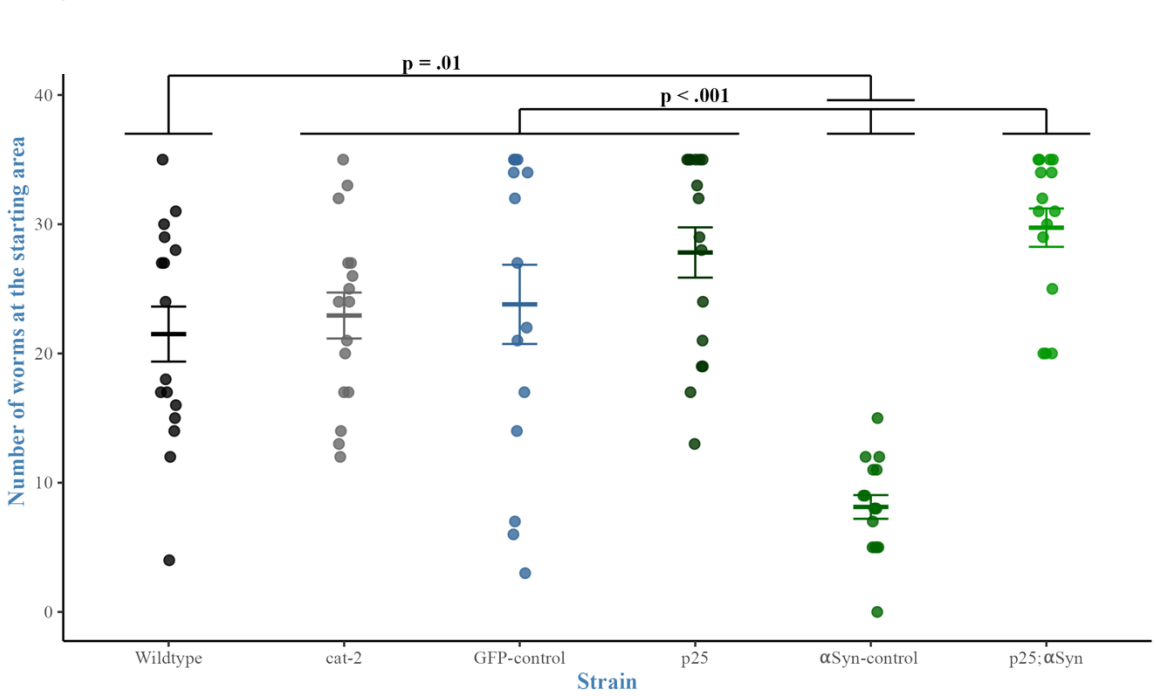
In general, the lack of differences across the two p25 strains and their respective controls may indicate, that the non-significant differences observed in the p25 strains are not only caused by the p25-strains' lack of learning to avoid NaCl, but also a drop in attraction towards NaCl when conditioned with 0 mM NaCl. However, both the p25 strain (M = .25) and the p25; αSyn (M = .43) demonstrate a positive mean chemotaxis index when exposed to 0 mM NaCl, which may indicate that both strains are able to sense and are attracted towards NaCl in the mock condition.

11.3 Lack of Movement Away From the Starting Area

During the experiments, it seemed to be the case that fewer worms moved away from the center of some strains compared to others (suppl. Table 1). Difficulties in moving away from the starting area could be related to the worms' movement, which is heavily linked to DA and movement impairments seen in Dementia with Lewy bodies and Parkinson's Disease. To investigate this, the potential group differences in the number of worms still present at the starting areas (or midline) after 30 minutes were analyzed. However, because of reporting errors, one entry of the number of worms at the starting area of the p25; αSyn strain (at 50 mM condition), and one entry of the GFP-control strain (at 50 mM condition) were omitted for this analysis.

Figure 11

Movement Away From the Starting Area



The number of worms still present at the starting area at completion of the assay. The αSyn-control has fewer worms at the starting area after 30 minutes of assay time (both 0 mM and 50 mM). A significant difference of $p = .01$ is present between the wildtype and αSyn-control strain, whereas a significant difference of $p < .001$ is found between the αSyn-control and the rest of the strains. $N = 16$ per strain, except p25; αSyn and GFP-control where $N = 15$, indicated by dots. Error bars indicate SEM. Mean is indicated by the thick middle line.

A Kruskal-Wallis test revealed an overall significant difference between the number of worms at the starting area, $H(5) = 40.373$, $p < .001$. A subsequent Dunn's Test for multiple comparisons was applied (alpha level adjusted using the Bonferroni-Holm procedure). The only significant differences found were between the αSyn-control and all other groups (figure 11). This could indicate that despite the pan-neuronal expression of αSyn, that this strain is better at moving after the being conditioned with or without NaCl. However, this result should be interpreted with some caution, as there is an artificial ceiling at 35. In addition, the number of worms pipetted in the starting area was not always the same; thus, in some cases there might simply be more worms at the starting area, because more worms were originally placed on the plate.

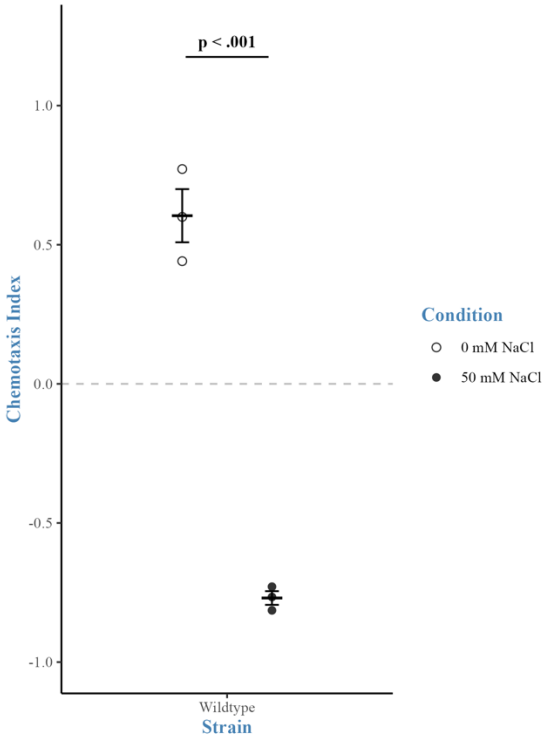
This could e.g., be the case for the wildtype worms, as these demonstrate clear salt aversion as described above.

11.4 Shorter Time is Enough to Induce Salt Aversion in Wildtype Worms

The gustatory plasticity assay closely resembles the salt aversion assay. It is however typically done with a higher concentration of NaCl (100 mM NaCl) and a shorter conditioning time (Hukema et al., 2008). Because I used a higher concentration of NaCl to induce aversion than the frequently reported 20 mM NaCl, I wanted to use the 50 mM concentration used here, to test if it could induce salt aversion after only 15 minutes rather than three hours - as seen in gustatory plasticity. Because the wildtype strain demonstrates clear salt aversion, I only used this strain for this experiment.

Figure 12

Wildtype Worms Avoid NaCl after 15 min.



The conditioning of wildtype worms with 50 mM NaCl for 15 minutes can induce salt aversion alike to gustatory plasticity experiments. $N = 3$ in each condition – chemotaxis index was based on a total of 308 (see suppl. Table 2 for the specific N). Error bars indicate SEM. Mean is indicated by the thick middle line. A significance threshold of .05 is used.

A two-sample t-test revealed significant difference in chemotaxis index when conditioned in the presence of 0 (M = .604) or 50 mM NaCl (M = -.769), $t(4) = 13.922$, $p < .001$. Thus, 15 minutes is enough to induce salt aversion of the wildtype strain exposed to 50 mM NaCl.

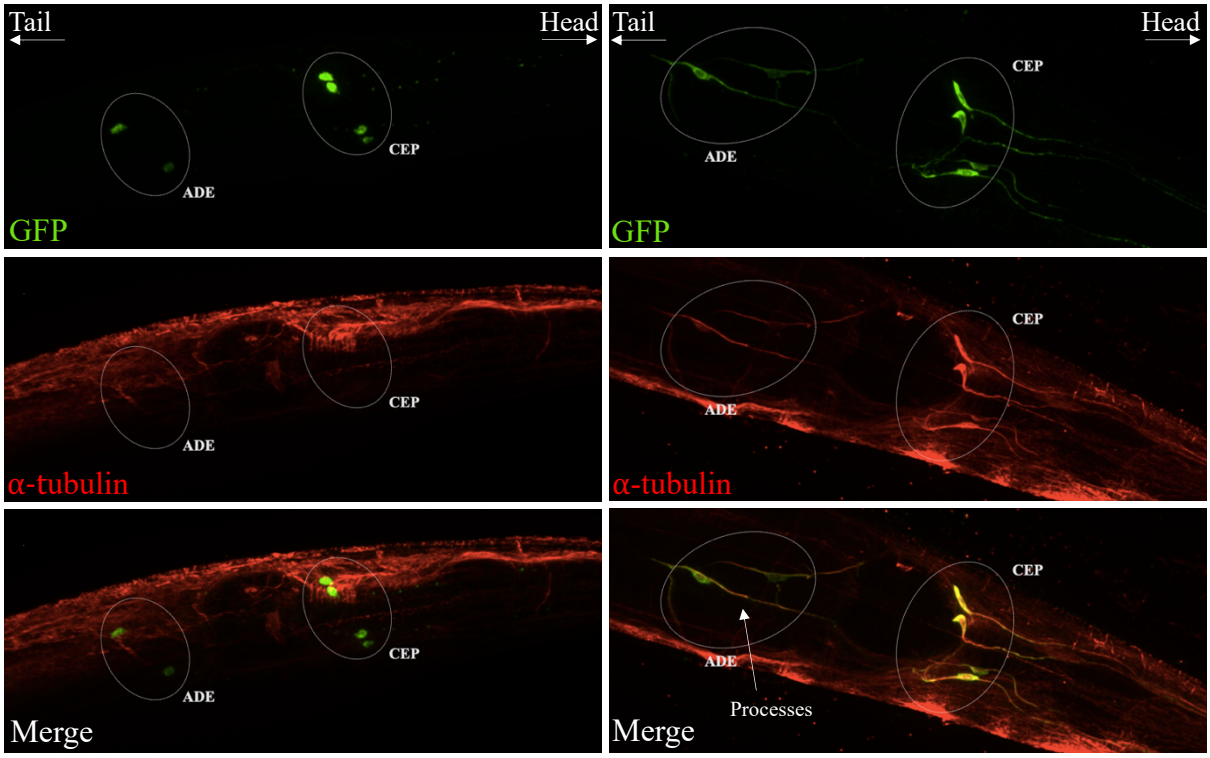
11.5 p25 Co-localizes with Microtubules

Although the results from the salt aversion assays are not entirely clear, both strains overexpressing p25 lack decisive differences in their response to the NaCl-conditioning. In addition, overexpression of the p25 protein has been shown to cause degeneration of ADE somas (figure 9). However, it is not known what underlying mechanisms through which p25 may interact and potentially cause pathology in the worms. As the p25 protein possess a binding site for microtubules (Tőkési et al., 2014), and can induce polymerization as well as aberrant structures of microtubules (Hlavanda et al., 2002), immunostaining for microtubules and p25 was done to investigate for co-localization of the two proteins (hypothesis 2).

Figure 13

Co-localization of p25::GFP and Microtubules is Detected at Day Four

a) GFP-control, day four ↓ b) p25, day four ↓



Representative antibody staining images of day four GFP-control worm (a) and day four p25 worm (b) stained with GFP (green) and α-tubulin (red) antibodies are shown. A merge of the respective staining is shown at the bottom. The anterior CEP-somas as well as the ADE somas are encircled. Arrows indicate the direction of the worm. Yellow indicates co-localization of the proteins stained for – as seen in the merge image of the p25 worm. The somas of the CEP neurons, and processes extending from both ADE and CEP somas show co-localization of the GFP and α-tubulin antibodies (that putatively stains for microtubules). The GFP-control worm shows little to no co-localization.

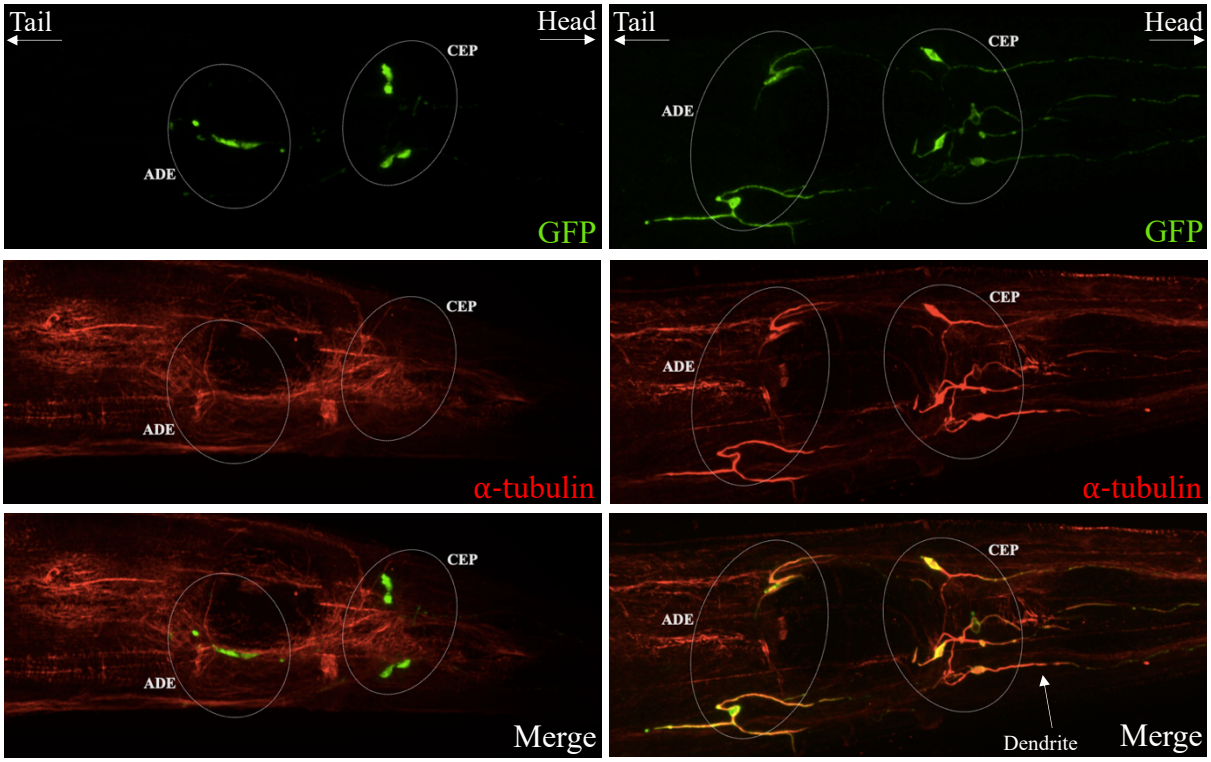
The qualitative observation of co-localization between microtubules and the p25-protein confirms hypothesis 2 – that the proteins can co-localize *in vivo* (figure 13). This simultaneously supports observations from various studies also demonstrating interaction of these (Szénási et al., 2017; Tőkési et al., 2014). However, one caveat is the observation, that the co-localization is not detected in all worms - at least not to a degree readily detectable by the applied method (see suppl. figure 1). However, the overlap in the GFP-control worms is not as clear and does not extend throughout the processes of the dopaminergic neurons, like

the ADE. As microtubules are important for and therefore present in almost all cells (Chakraborti et al., 2016), some co-localization could be expected.

Figure 14

Co-localization of p25::GFP and Microtubules is More Apparent at Day Six

a) GFP-control, day six ↓ b) p25, day six



The representative demonstrates immunostaining images with GFP (green) and α-tubulin (red) antibodies of a day six GFP-control worm (a) and a day six p25 worm (b). A merge of the respective staining is shown at the bottom. The directionality of the worm is indicated by arrows. Yellow shows co-localization. As seen in the merge image of the p25 strain, co-localization of α-tubulin and p25::GFP is pronounced in the somas as well as processes like dendrites.

As with the day four images, a little co-localization is also found in the control strains, but the co-localization of the p25::GFP fusion protein and microtubules in the p25-worms is observed more frequently and appear to extend more throughout the processes, such as the CEP dendrites (figure 14). In addition, the dopaminergic neurons can also be made out on the image only showing α-tubulin staining (b, red) in comparison with the equivalent GFP-control image, where they are not distinguishable. This is consistent with p25 causing microtubule polymerization in the dopaminergic neurons and thereby increasing their abundance. This is

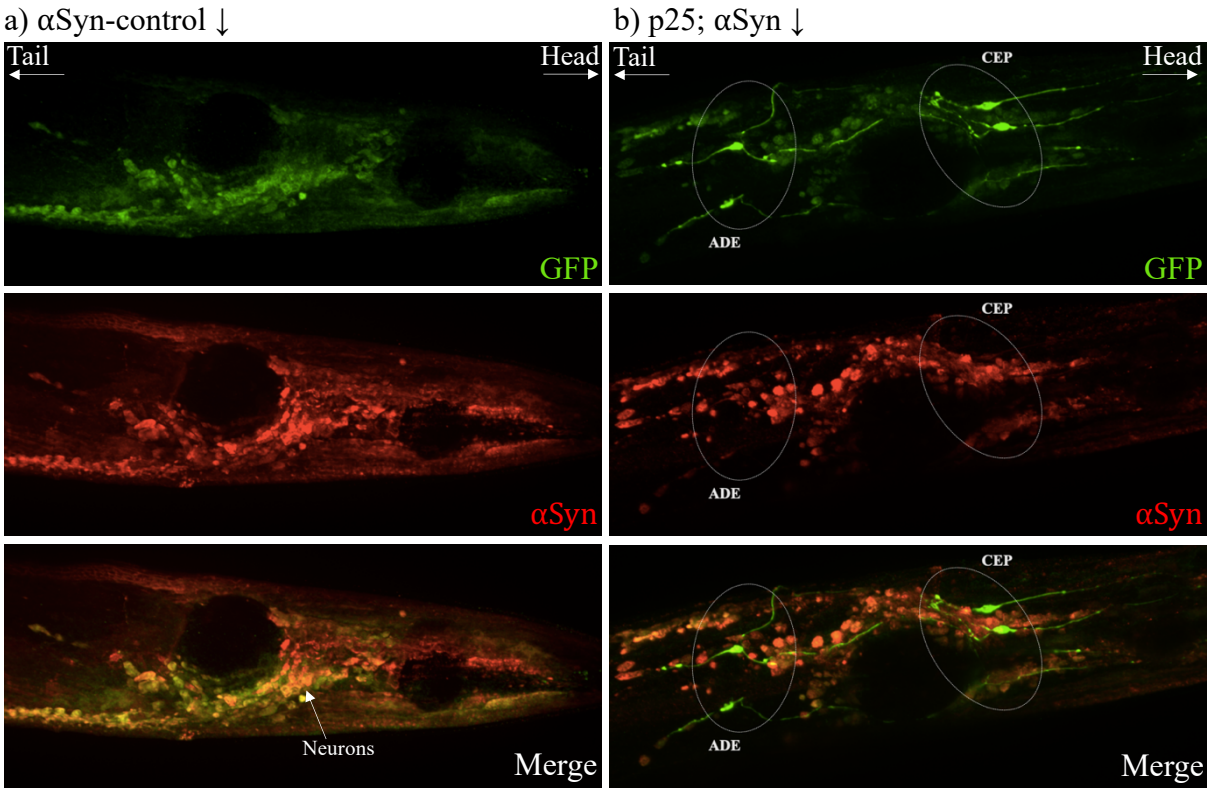
very interesting, as this finding potentially demonstrate why the ADE somas degenerate, which suggests a pathological role of p25 and microtubules when found in neuron. In addition, this also indicates that microtubule abnormalities underlie the somewhat impaired salt aversion demonstrated by the p25 strains.

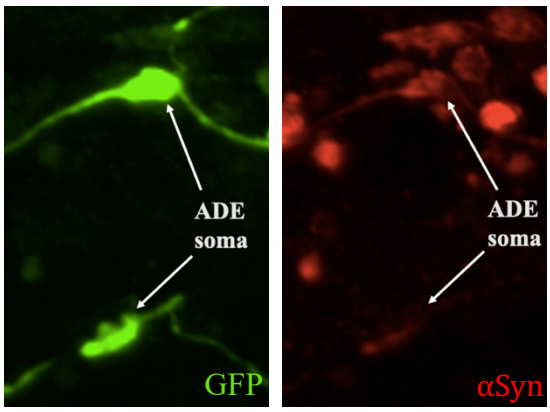
11.6 Co-localization of αSyn and GFP Antibodies Are Observed in Both the αSyn-control and p25; αSyn Strains

In addition to microtubule polymerization, one of the main interests for the p25-protein boils down to its potential role in causing αSyn aggregates and thereby being involved in the pathogenesis of the neuronal synucleinopathies (Oláh et al., 2020). The above salt aversion results further this interest, as the strain expressing both p25 and αSyn show a positive chemotaxis index even after being conditioned with NaCl in the absence of food. Hypothesis 3, that αSyn and p25 co-localizes in strains expressing both proteins in the worm's dopaminergic neurons, is therefore investigated by immunostaining.

Figure 15

Co-localization of the αSyn and GFP Antibodies is Observed in both αSyn-control and p25; αSyn Strains





Representative images are shown of the αSyn-control (a) and p25; αSyn (b) strains stained with GFP (green) and αSyn (red) antibodies. Both strains demonstrate co-localization in different neurons (the many different dots) as seen in the merge of the respective images. C) Zoomed in view of the p25; αSyn ADE somas (encircled in b). αSyn reactivity cannot be seen in the merge image due to a strong GFP signal, but ADE somas also faintly stain for αSyn (red). Age – six days. ADE and CEP somas are not encircled in the αSyn-control images, as they cannot be clearly distinguished.

The GFP and αSyn antibodies co-localize in both the αSyn-control and p25; αSyn strains (figure 15). The GFP antibody stains for both the p25::GFP fusion protein and the other GFP proteins (see section). The co-localization is observed in different neurons, and potentially even less in the dopaminergic neurons of the p25; αSyn strain – although some reactivity of both antibodies is shown in the ADE somas (figure 15, c). αSyn and EGFP is expressed under the same promoter in these strains (table 1), but not as a fusion protein, whereas the high degree of co-localization cannot explained by these proteins being physically fused together (Kuwahara et al., 2008 - see table 1). Due to this unexpected observation of the plentiful co-localization in both strains, the results are inconclusive and hypothesis 3 cannot be readily confirmed.

12. Discussion

12.1 The Salt Aversion Assay can be Used to Uncover Neuropsychological Impairment

To study the neuropsychological-related impairments in learning associated with dopaminergic dysfunction of the substantia nigra as well as demonstrated by some neuronal synucleinopathy patients, such as Parkinson's Disease patients, the salt aversion assay was conducted. This assay provides some interesting results to be discussed. On the face of it, overexpression of the human p25 protein appears to impair salt aversion learning (figure 10). However, a discussion of some potential confounding variables, that may impact the results, is warranted.

A strength of the salt aversion assay is that it allows to infer some form of motivation and decision making in an organism as simple as *C. elegans*. Decision making and motivation are both phenomena typically associated with DA in humans. Both have also been observed to be impaired in Parkinson's Disease (Berke, 2018; Perugini et al., 2018). A universal motivation for all organisms is the localization of sustenance. Thus, if a given *C. elegans* strain can form aversive associations, it should be motivated to move away from the NaCl since it is associated with the absence of food. The assessed behavior is therefore not random but requires the worms to decide on what side to move towards.

With these points in mind, the lack of significant differences in chemotaxis index between the NaCl and mock conditioning in the p25 and p25; αSyn strains, support the notion, that these strains could be impaired in associative learning caused by the dopaminergic overexpression of p25. The specific intracellular consequences of p25-overexpressing related to DA are unknown, however, as especially the ADE somas degenerate with age, one potential consequence is a lowered availability of DA. This could follow the results of Voglis and Tavernarakis (2008) who demonstrated, that worms unable to show an aversive response, demonstrated a significantly smaller increase in the release of DA compared to wildtype after conditioning. Thus, the lack of an aversive response in the p25-overexpression worms could be a consequence of lower levels of available DA. This could be related to the dopaminergic dysfunction in neuronal synucleinopathies (Simuni et al., 2024). In agreement, the role of the $G\alpha_i$ G-protein expressed in the ADE neurons in aversive learning (Mersha et al., 2013), points to the necessity of intact ADE neurons for behavioral plasticity. It is interesting, that involvement of DA in aversive learning is not limited to nematodes, but associated with intact DA-neurons in the substantia nigra of mice (Menegas et al., 2018). p25-overexpression thereby seem to afflict the conserved role of dopaminergic modulation of learning (Vidal-Gadea et al.,

2011), which is also proposed to be impaired in Parkinson's Disease patients (Foerde & Shohamy, 2011).

αSyn was introduced because of its strong relationship with the neuronal synucleinopathies (Simuni et al., 2024) . Whether the positive chemotaxis demonstrated by the p25; αSyn strain is evident of further impairment observed due to an interaction of the αSyn and p25 is difficult to say. This strain expresses αSyn in all neurons and potentially also in pharyngeal and body wall muscles (Ogura et al., 1994), which could be seen as problematic if it impacts the strains' behavior. However, the same expression patterns should be present in the αSyn-control, that do exhibit significant avoidance after NaCl-conditioning, suggesting that there is an additional effect of p25 and αSyn co-expression in the dopaminergic neurons. One thing needed to be addressed is the fact, that neither the p25; αSyn or the αSyn-control is backcrossed to the wildtype strain used in the AO-laboratory. This should be done to be able to definitively compare across strains. Ideally, the αSyn-control should be backcrossed to the newly generated GFP-control to also have expression of GFP driven under the *dat-1* promoter, like the p25; αSyn strain has.

In general, the salt aversion results should be interpreted with caution, as there are no significant differences between the p25 strains and either of their respective control strains, when conditioned in the presence of 50 mM NaCl. In addition, the GFP-control only demonstrates a tendency towards significance depending on type of conditioning potentially caused by large outliers, as a closer look at the data reveals two somewhat large outliers (figure 10 – see GFP-control 50 mM). Together, this suggests that the impairment in salt aversion learning demonstrated by either p25 strain is not strong enough to be able to definitive assert, that the p25 strains are impaired in associative learning.

A putative explanation as to why the learning impairment is not as clear as seen in other studies (Lim et al., 2018), could be that the p25 phenotype is not fully penetrant, as not all dopaminergic neurons degenerate. It could therefore be the case, that any possible remaining ADE, PDE and/or CEP neurons, that tend to survive, could be the reason that p25-overexpressing only causes a partial impairment in salt aversion learning. It is however interesting, that the p25 worms show different responses in the salt aversion assay despite the lack of total degeneration. This could indicate that intracellular mechanisms and structures, such as the microtubules, may be compromised before degeneration is observed.

The fact that the *cat-2* strain showed an aversive response was surprising, as this strain had earlier showed an impairment in the DA-dependent basal slowing assay on day six (Rasmussen, 2023). However, in several of the repeats using this *cat-2* strain, the chemotaxis response was based on the movement of very few worms (suppl. Table 1). This brought about the comparison of the number of worms that stayed at the center of the assay plate.

Ideally, the fraction of worms moved away from the starting area would have been used. However, since the artificial ceiling of 35 was used, this was deemed too unreliable. Thus, the comparison was instead done on the raw count data. Since no differences were found between the *cat-2* strain and most of the other strains except for the αSyn-control, some form of movement impairment away from start, does not seem responsible for the surprising *cat-2* results. However, since this comparison is very sensitive to the number of worms placed in the starting area originally, the salt aversion assay using the *cat-2* strain should ideally be repeated using more worms.

If the salt aversion assay is to be conducted in the future, a better control of the number of viable worms initially transferred to the assay plate, could allow for the investigation of whether the chemotaxis index is significantly different from zero. Instead of comparing means between strains, this method would directly measure if the worms of each group decided to move to one side or the other of the plate. This could more decisively assert whether the potential impairment seen in the p25-strains, is in fact an impairment. A one-sample t test can be used for this purpose. Additionally, rigorously monitoring the fraction of worms that moved away from the starting area, would subsequently make it possible to see if some strains demonstrate some form of movement impairment.

12.2 Association is Still Required for an Aversive Response Despite Shorter Conditioning Time

While a concentration of around 20 mM NaCl tends to be used for conditioning in the literature (Adachi et al., 2010; Lim et al., 2018), the higher concentration of 50 mM was used, as this had yielded the best results in the performed pilot studies. The result of the 15 min. assay, similar to gustatory plasticity paradigms (Dekkers et al., 2021; Hukema et al., 2008), revealed a clear effect of NaCl-conditioning in the wildtype worms. Because of the higher concentrations used in gustatory plasticity assays, it has been questioned if the assays actually induce an association or if, instead, the high NaCl instead makes the neurons abnormally

sensitive to NaCl (Rahmani & Chew, 2021). It could therefore be the case that the avoidance observed after the 15 min. assay is not a learned response. However, gustatory plasticity assays have been conducted with at least twice the concentration of NaCl than the 50 mM used here (Hukema et al., 2008). It could also be questioned whether 15 minutes is enough to induce an association between starvation and NaCl, or if some other nonspecific effect is at play. One potential nonspecific effect could be the increased osmolarity of the liquid, that increases as the concentration of NaCl increases. A high osmolarity has previously been shown to induce avoidance (Hukema et al., 2008). However, Saeke and colleagues (2001) have earlier demonstrated that even a 100 mM NaCl concentration do not induce non-specific effects caused by osmolarity. Thus, the 50 mM NaCl concentration used in this project should not be toxic to the worms in terms of osmolarity, and it is instead more likely, that the worms specifically respond to the presence of NaCl in the absence of food. Additionally, since the absence of food is a prerequisite for an aversive response even in gustatory plasticity (Hukema et al., 2008), some form of association needs to be formed between the two stimuli (NaCl and absence of food). Thus, the short version of the salt aversion assay used in this project may still be used to investigate impairment in learning.

12.3 p25 Co-localizes with Microtubules, but not with Alpha-Synuclein

The physiological role of p25 is to polymerize microtubules (Schofield & Bernhard, 2013). Thus, the very interesting result, that p25 seems to make use of its binding site associated with microtubules to co-localize with microtubules, seem to support the protein's moonlighting abilities as the p25 is expressed in the worm's dopaminergic neurons in contrast to oligodendrocytes (Oláh et al., 2020). The observed association of p25 and microtubules could therefore suggest that this complex is the main culprit of the observed p25-induced neurodegeneration and morphology of the ADE somas, as well as the potential functional impairment of salt aversion. It therefore seems to be the case, that p25-overexpression causes abnormal and pathological polymerization, that affects the health of the ADE somas.

The idea, that p25 and microtubules forms the main pathological complex is further supported by the fact, that the deletion of the α-tubulin subunit encoded by the *tba-9* gene has been shown to cause a later onset of ADE-degeneration in the p25-strain (Sørensen, 2014). In addition, as the various isotypes of tubulin subunits are expressed differently in the worm's dopaminergic neurons (Lu & Zheng, 2022), it could be the case, that the specific tubulin

subunits used to form microtubule structures in the ADE neurons makes them more susceptible to degeneration.

The p25 protein has been shown to increase the level of acetylated α-tubulin via its relationship with HDAC6 (Tőkési et al., 2010) which indicates stable microtubules. However, as stable microtubules tend to grow less than unstable ones, the results of the antibody staining for p25 and microtubules co-localization instead seem to indicate, that the p25 causes pathology by abnormal polymerization, as staining is observed throughout the dopaminergic neurons. In addition, the stabilization of microtubules has been associated with increased learning (Uchida et al., 2014), whereas the binding of p25 and microtubules is here proposed to be able to cause learning impairment in the p25 strains. This could suggest that it is not the known interaction of p25 with HDAC6, that is pathological in the case of p25-overexpression.

To confirm that the co-localization is not just an artifact of endogenously expressed GFP remaining active, which is typically quenched during the antibody staining protocol (Scandella et al., 2020), the experiment should be repeated while only adding the primary antibodies. As such, any remaining GFP signal would be caused by remaining active GFP, and not the staining process. However, since the GFP-control worms consistently show much less co-localization, if any, compared to the p25-strain, especially on day six, this suggests, that microtubules and the p25 protein do in fact co-localize.

The immunostaining for αSyn and p25::GFP was done to investigate for co-localization and to potentially observe aggregation of the proteins as previously observed in tissue samples from neuronal synucleinopathy patients (Kovács et al., 2004; Lindersson et al., 2005). However, the substantial co-localization of αSyn and the GFP proteins in neurons other than the dopaminergic ones in both p25; αSyn and αSyn-control worms complicates the interpretation of these images. In contrast, αSyn and p25 do not seem to co-localize to a substantial degree in worms' dopaminergic neurons (figure 15). This indicates that the somewhat more severe impairment in associative learning demonstrated by the p25; αSyn, is not caused by a direct interaction of these proteins. p25 have been found to co-localize more with microtubules when the full length p25 protein is endogenously expressed, as is the case for the p25; αSyn strain, which could explain the lack of co-localization of p25 and αSyn (Tőkési et al., 2014). Although a strain expressing αSyn under the *dat-1* promoter had been attempted without success (Christensen, 2013), it could be interesting to create a new strain that only expresses αSyn together with p25 in the dopaminergic neurons – e.g., by using the

CRISPR-Cas9 system which could prevent overexpression and thereby hypothetically also prevent 'promoter overload' as previously observed (Dickinson & Goldstein, 2016). This would not only make it easier to distinguish dopaminergic neurons following antibody staining, but also limit the potential confounding effects of αSyn expression in other neurons for behavioral investigations.

The immunostaining results demonstrate that microtubules are involved in the p25-related behavioral and physiological impairment seen in these models. This suggests that a focus on microtubule structures for the potential cause of neurodegeneration can provide valuable information for the study of causes for neuropsychological impairment.

12.4 *C. elegans* is a Model Organism Applicable for the Study of Neuronal Synucleinopathies and Related Neuropsychological Impairments

Neurodegenerative diseases, such as the neuronal synucleinopathies, are first and foremost the manifest consequences of an underlying protein pathology. Through the ease of genetical manipulation, the use of *C. elegans* provides a way to investigate directly *in vivo* these pathological cellular mechanisms, that are otherwise inaccessible to study in human. The purpose of this thesis has been to investigate the neuropsychological impairments brought about by dopaminergic overexpression of the human p25 protein as a model for neuronal synucleinopathies.

An important factor, when choosing an animal model for researching neurodegeneration as well as behavioral impairment through the use of genetic manipulation, is that the induced mutation displays similarities to the disease and symptoms, that is being studied (Kaletta & Hengartner, 2006). Despite the fact, that the nervous system found in *C. elegans* is much simpler than that of humans, the role of DA in both positive and aversive associative learning, e.g., mediated by the substantia nigra (Menegas et al., 2018) or DA receptors in the amygdala and striatum in mammals (Kravitz et al., 2012; Zafiri & Duvarci, 2022), is conserved between species (Raj & Thekkuveettil, 2022; Voglis & Tavernarakis, 2008). Although interpreted with caution, seeing that overexpression of the synucleinopathy-related p25-protein, especially when the p25; αSyn strain is considered, may cause impairment in aversive associative learning due to the protein's pathological effect on the worms' dopaminergic system, provides a neuropathological as well as a behavioral link to the impairments observed in Parkinson's Disease (Perugini et al., 2018).

The similarities between species extends from neuropsychologically related behavioral functions to the mechanistic properties of the neurons. The association of the p25-protein with the microtubule network is a valuable finding since this offers an explanation as to what may cause the observed neuronal degeneration that leads to behavioral dysfunction. It also creates another link to the neuronal synucleinopathies, as these have been associated with abnormalities of the microtubule network (Mazzetti et al., 2024; Power et al., 2017). However, as interpreted from the antibody staining of microtubules where a polymerization process is proposed, it may not be likely, that the p25 expressions increases acetylated α-tubulin (through HDAC6), which has been observed in the substantia nigra of Parkinson's Disease patients (Mazzetti et al., 2024). This is however speculative, as no measure of acetylated α-tubulin was done. Thus, despite the inconclusive staining for αSyn and p25 co-localization, taken together, the results suggests that p25-overexpression is indeed able to cause both behavioral and dopaminergic degeneration through pathological interaction with microtubules.

The results of the experiments provide interesting ground for future research in p25-mediated pathology. Although the salt aversion experiments hints to a form of DA related behavioral impairment, evidence of a more decisive dysfunction related to the neuronal synucleinopathies would underscore the p25 protein's ability to cause disease-related impairment. Motor dysfunction is a main symptom of these diseases (Outeiro et al., 2019; Poewe et al., 2017). Seeing that DA also supports motor related functions in worms as they do in humans, a future approach could be to investigate the ability of the p25-worms to transit between swimming and crawling. Vidal-Gadea and colleagues (2011) found the transition between these two distinctive movement types to rely on the dopaminergic system, as genetic ablation of especially the ADE and PDE neurons of the worms, left mutants to lie still or move little compared to control strains after emergence from liquid. Furthermore, the authors suggests DA signaling from ADE and PDE neurons to target D1-like receptors in the anterior half of the worm to induce swim to crawl transition (Vidal-Gadea et al., 2011). As ADE neurons are the DA-neurons most affected by p25-overexpression, this experiment could potentially reveal motor dysfunction resembling, as mentioned, bradykinesia in Parkinson's Disease patients.

All in all, the work done in this thesis supports the useability of the *C. elegans* p25-overexpression model for the study of neuronal synucleinopathy-related impairments as well as DA-mediated neuropsychological behavior.

13. Conclusion

Dementia with Lewy bodies and Parkinson's Disease are both characterized by neuropsychological dysfunctions associated with dopaminergic impairment of the human midbrain. Many features of the human and mammalian DA system is conserved in the *C. elegans* nematode, and it was therefore used as a model organism to study disease mechanisms associated with the neuronal synucleinopathy-related p25-protein.

The salt aversion assay was used to investigate the neuropsychological impairments of the neuronal synucleinopathies, such as learning and decision making in Parkinson's Disease, related to dopaminergic dysfunction of the substantia nigra. The results indicate a possible impairment in the strains overexpression the human p25, as no differences are found between worms based on the type of conditioning in these strains. The pan-neuronal expression of the human αSyn in addition to the p25 protein may cause further impairment. However, the results should in general be interpreted with caution, as no difference between either of the p25 strains and their respective controls is apparent after being conditioned with NaCl. In addition, 15 minutes of conditioning with 50 mM NaCl is enough to induce an aversive response – at least in the wildtype strain.

The immunostaining experiments demonstrated clear co-localization of microtubules and the p25-protein. This exiting result suggests that disruptions to the microtubule network brought about by p25-overexpression is the main impairment, that underlies neuronal degeneration and behavioral dysfunction. Although the p25; αSyn could be more impaired in salt aversive learning, the results from the current immunostaining experiments do not point to a clear direct interaction of these proteins, suggesting that other explanations are needed for this potential additive effect.

As the p25-protein can induce dopaminergic degeneration and microtubule malformations in *C. elegans*, it offers an interesting model for the study of intraneuronal consequences of the neuronal synucleinopathies. Moreover, the fact that *C. elegans* are capable of showing change in behavior akin to associative learning seen in higher order mammals, including humans, demonstrates the p25 model's useability in not just neuroscientific research, but also neuropsychological research.

14. References

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15. Supplementary figures

Supplementary Table 1

Results of the Salt Aversion Experiments

Strain/condition	A	C	B	CI
1_Wildtype_0	20	5	14	0,6
1_Wildtype_50	5	28	16	-0,696
1_Wildtype_0	18	12	24	0,2
1_Wildtype_50	8	38	15	-0,652
2_Wildtype_0	26	15	28	0,268
2_Wildtype_50	18	39	17	-0,368
2_Wildtype_0	45	11	30	0,607
2_Wildtype_50	9	40	17	-0,632
3_Wildtype_0	27	6	31	0,636
3_Wildtype_50	7	29	27	-0,611
3_Wildtype_0	14	9	27	0,217
3_Wildtype_50	10	11	18	-0,047
3_Wildtype_0	24	10	35+	0,411
3_Wildtype_50	6	29	4	-0,657
3_Wildtype_0	28	6	29	0,647
3_Wildtype_50	13	59	12	-0,587
1_p25_0	8	4	28	0,33
1_p25_50	15	14	35+	0,034
1_p25_0	12	1	35+	0,846
1_p25_50	8	8	19	0
1_p25_0	18	10	35+	0,285
1_p25_50	10	8	35	0,11
2_p25_0	12	12	33	0
2_p25_50	9	16	17	-0,28
2_p25_0	17	9	35+	0,307
2_p25_50	12	15	29	-0,11
3_p25_0	7	9	35+	-0,125

3_p25_50	17	7	19	0,416
3_p25_0	18	13	32	0,161
3_p25_50	4	16	21	-0,6
3_p25_0	10	6	24	0,25
3_p25_50	11	18	13	-0,241
1_p25; αSyn_0	19	5	20	0,583
1_p25; αSyn_50	5	9	32	-0,285
1_p25; αSyn_0	6	6	31	0
1_p25; αSyn_50	12	16	30	-0,142
1_p25; αSyn_0	14	4	34	0,555
1_p25; αSyn_50	3	9	20	-0,5
1_p25; αSyn_0	10	1	29	0,818
1_p25; αSyn_50	13	9	20	0,181
2_p25; αSyn_0	13	8	35+	0,238
2_p25; αSyn_50	9	5	31	0,285
2_p25; αSyn_0	7	2	35+	0,555
2_p25; αSyn_50	8	2	35+	0,6
3_p25; αSyn_0	7	6	34	0,076
3_p25; αSyn_50	6	5	25	0,09
3_p25; αSyn_0	11	2	35+	0,692
3_p25; αSyn_50	6	4	?	0,692
1_αSyn-control_0	14	7	5	0,333
1_αSyn-control_50	7	12	0	-0,263
1_αSyn-control_0	28	1	8	0,931
1_αSyn-control_50	4	21	9	-0,68
1_αSyn-control_0	17	5	12	0,545
1_αSyn-control_50	3	8	5	-0,454
1_αSyn-control_0	9	6	8	0,2
1_αSyn-control_50	11	6	7	0,294
2_αSyn-control_0	22	2	8	0,833
2_αSyn-control_50	2	15	9	-0,764

2_αSyn-control_0	10	17	11	-0,259
2_αSyn-control_50	22	5	12	0,629
3_αSyn-control_0	25	4	11	0,724
3_αSyn-control_50	11	11	15	0
3_αSyn-control_0	19	1	5	0,9
3_αSyn-control_50	8	17	5	-0,36
1_GFP-control_0	18	8	3	0,384
1_GFP-control _50	10	15	21	-0,2
1_GFP-control _0	16	9	6	0,28
1_GFP-control _50	4	12	14	-0,5
1_GFP-control _0	21	8	7	0,448
1_GFP-control _50	5	10	27	-0,333
1_GFP-control _0	28	6	17	0,647
1_GFP-control _50	15	4	34	0,578
2_GFP-control _0	26	10	22	0,444
2_GFP-control _50	15	8	34	0,304
2_GFP-control _0	15	5	35+	0,5
2_GFP-control _50	4	11	32	-0,466
3_GFP-control _0	27	6	35+	0,636
3_GFP-control _50	12	18	?	-0,2
3_GFP-control _0	20	6	35+	0,538
3_GFP-control _50	6	6	35+	0
1_cat-2_0	12	1	21	0,836
1_cat-2_50	1	2	14	-0,33
1_cat-2_0	2	1	12	0,33
1_cat-2_50	3	9	17	-0,5
1_cat-2_0	10	5	17	0,333
1_cat-2_50	2	7	24	-0,555
2_cat-2_0	19	6	25	0,52
2_cat-2_50	11	15	24	-0,153
2_cat-2_0	3	3	27	0

2_cat-2_50	4	1	35+	0,6
2_cat-2_0	7	2	20	0,555
2_cat-2_50	0	8	13	-1
3_cat-2_0	3	0	26	1
3_cat-2_50	0	5	33	-1
3_cat-2_0	13	1	27	0,857
3_cat-2_50	6	5	32	0,09

The results of the salt aversion assay are shown. The numbers 1, 2 or 3 denote the round of experiment. 0 or 50 indicates concentration of NaCl. A indicates the number of worms at area A of the plate after 30 minutes, where the NaCl agar plug had been. C indicates the number of worms at the area A after 30 minutes, where the control agar plug had been, and B indicates the number of worms at the starting area after 30 minutes. Finally, CI denotes the Chemotaxis Index.

Supplementary Table 2

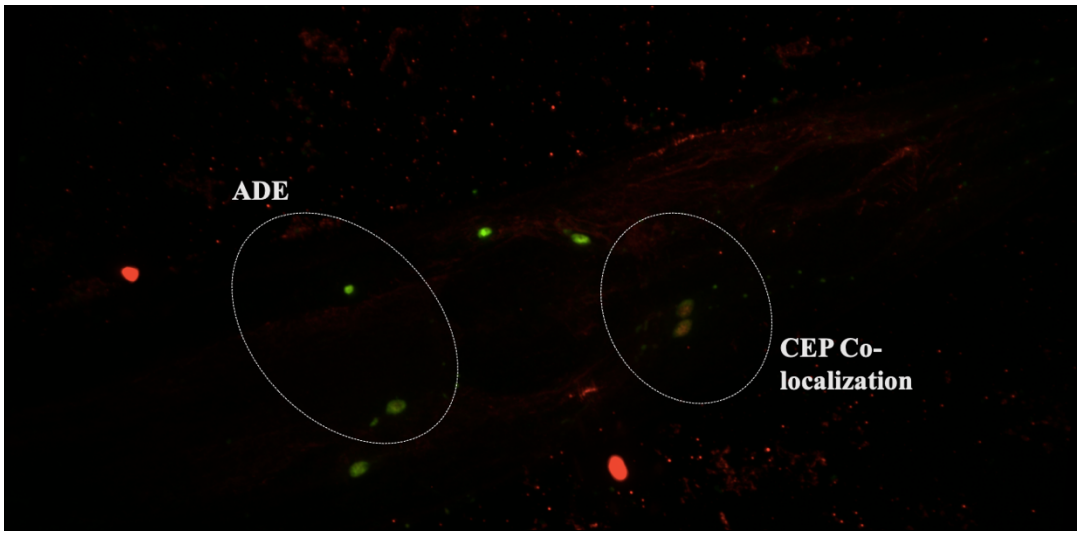
Results of the 15 Minutes Salt Aversion Experiments

Strain/condition	**A**	**C**	**B**	**CI**
15_ Wildtype _0	31	12	7	0,441
15_ Wildtype _50	5	32	3	-0,729
15_ Wildtype _0	56	14	1	0,6
15_ Wildtype _50	5	49	2	-0,814
15_ Wildtype _0	39	5	4	0,772
15_ Wildtype _50	7	53	2	-0,766

The results from the salt aversion experiments, where the conditioning time was reduced to 15 minutes are shown. Condition is indicated by 0 or 50 NaCl. As above, A, B and C denotes the number of worms at the area their respective areas after 30 minutes. A – where the NaCl plugs had been. C – where the control plugs had been. B – the starting area after 30 minutes. CI indicates the Chemotaxis Index.

Supplementary figure 1

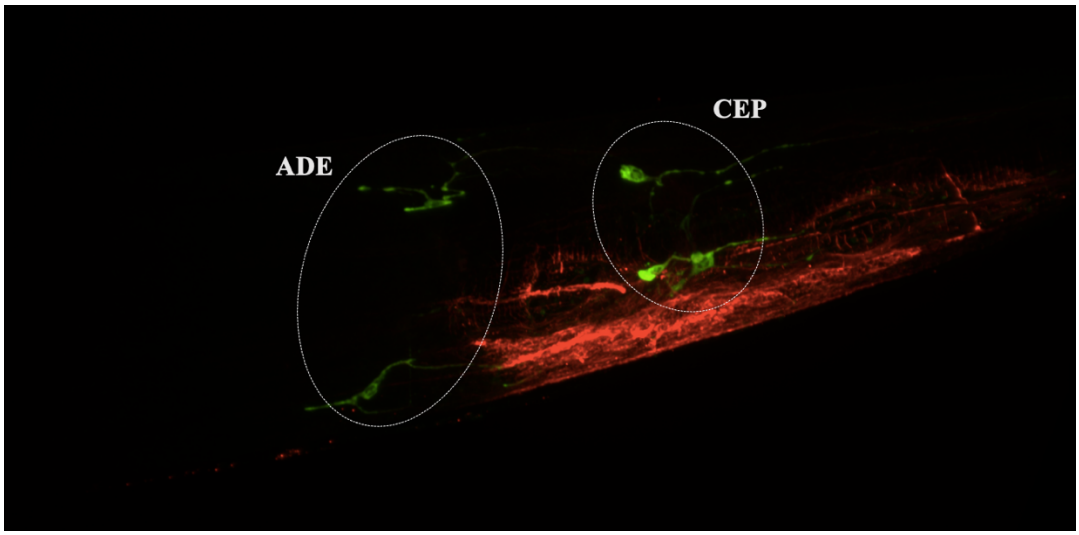
Co-localization of Microtubules and GFP Antibodies in GFP-control



The co-localization of the GFP (green) and α-tubulin antibodies (red) in CEP-somas of a day four GFP-control worm. The co-localization is similar to what is observed on day six (not shown).

Supplementary figure 2

No Co-localization of GFP and Microtubules in the p25 Strain



The lack of co-localization of the GFP (green) and α-tubulin antibodies (red) in a day four p25 worm. Images that show no co-localization on day six are similar (not shown).