Parkinson's Disease: An Experimental Approach to Model the Visuo-Spatial Learning and Memory Deficits and an Analysis of Evidence Implicating α-Synuclein in Lewy Body Formation

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PARKINSON’S DISEASE: AN EXPERIMENTAL APPROACH TO MODEL THE
VISUO-SPATIAL LEARNING AND MEMORY DEFICITS AND AN ANALYSIS OF
EVIDENCE IMPLICATING α-SYNUCLEIN IN LEWY BODY FORMATION

by

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of the Requirements for a Degree with Honors
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ABSTRACT

Parkinson’s disease (PD) is the second most common age-related neurodegenerative disorder. Given its prevalence, researching PD is important to world health. The well-known motor symptoms of PD are often accompanied by non-motor symptoms (NMS) including visuo-spatial learning and memory deficits. However, the mechanism whereby these NMS occur is unknown. PD is also characterized by the presence of protein aggregates termed Lewy Bodies (LBs). The mechanism whereby LBs form is unclear, although the fibrillization and aggregation of α-synuclein seem to be critical contributing factors.

This thesis reports development of an animal model of PD to study NMS using 6-Hydroxydopamine (6-OHDA) to produce partial bilateral lesions to the striata of mice. These lesions significantly reduced the tyrosine hydroxylase immunoreactivity in the striatum and hippocampus, mimicking the loss observed in PD patients. A deficit in visuo-spatial learning and memory in 6-OHDA treated mice was documented using the Barnes maze and Spatial Object Recognition test. Therefore, this model is expected to be useful for future study of the cause of these NMS.

An analysis of mechanisms reported to underlie LB formation revealed the potential importance of the protease calpain and the peptidyl-prolyl isomerase PIN1 in α-synuclein fibrillization. Data from the literature demonstrated that calpain cleaves α-synuclein, promoting its aggregation, and PIN1 potentially regulates calpain activity. Preliminary data reveal that calpain-2 cleaves PIN1 in vitro. The relationship between calpain and PIN1 should be examined further in regards to PD.
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INTRODUCTION

Parkinson’s disease (PD) was first described as a neurological disorder by James Parkinson in his 1817 work “An Essay on the Shaking Palsy” (32). PD currently affects approximately 6 million elderly adults worldwide with a mean age of onset of 55 years old. It is the second most common neurodegenerative disease after Alzheimer’s (7) and currently affects 1.5% of the global population over 65 (55). The vast majority (95%) of PD cases are idiopathic, while only 5% are genetically based (62).

The average lifespan is increasing with advances in modern healthcare. Therefore, higher prevalence and incidence of PD are expected to occur over time. Worldwide epidemiological studies give different estimates of PD, as it is difficult to compare rates of illness between countries. This discrepancy could be due to the variation in measurement method of prevalence and incidence or the difference in survival rates of various populations (58). Men have a higher chance of developing PD, with a relative ratio of 2.72:1 to women. Yet, the main factor known to promote sporadic PD is age (74). Wirdefeldt et al. performed a meta-analysis of the literature regarding potential environmental factors that could increase risk of PD. They find that there is mostly inadequate epidemiological data, with the exception of smoking tobacco or consumption of coffee reducing the risk of acquiring PD (86).

The prevalence and potential growth of PD is staggering. While PD has been studied for almost 200 years, much more is unknown. By unraveling the mechanisms underlying the pathology, treatments would be aimed at the cause and not just the symptoms.
Lewy Body (LB) Formation is an Integral Mediator of PD Pathology

PD is a neurodegenerative disease characterized by the progressive death of dopaminergic neurons originating in the substantia nigra pars compacta (SNpc) projecting to the dorsolateral putamen. This produces a striatal loss of dopamine, the hallmark of PD pathology (30). In addition to cell death, LBs are distinctive of the disease. These abnormal protein aggregates found in the cytoplasm of neurons are spherical of more than 15 μm in diameter, composed of a dense hyaline core. The core is granulovesicular in nature surrounded by a ring of radiating 8-10 nm fibrils. The central core appears differently than the rest of the inclusion when tissue is stained with haematoxylin and eosin. These morphological characteristics and specific staining allow for definitive identification of LBs as the primary diagnostic indicators of the disease (11, 20, 22).

Staining of PD patient’s brain tissue reveals the presence of LBs throughout the central and autonomic nervous system (22). LB formation and cell death are apparent in dopaminergic, noradrenergic, serotonergic, and cholinergic neurons in the SNpc, locus coeruleus, raphe, and nucleus basalis of Meynert/dorsal motor nucleus of vagas, respectively. LBs are also found in the cerebral cortex, specifically the cingulated and entorhinal cortices (20). Immunocytochemical staining and western blots of purified LBs reveal a predominance of the protein α-synuclein (6, 76). It is currently unknown as to what causes this protein to aggregate into LBs. By the time symptoms begin, LBs are
present in the SNpc and 60% of SNpc dopaminergic neurons have been lost with an 80% reduction in dopamine (11, 20).

James Parkinson depicted PD as “involuntary tremulous motion…lessened muscular power…a propensity to bend the trunk forwards, and to pass from a walking to a running pace,” describing the three cardinal motor symptoms of tremor, akinesia, and rigidity (64). Motor symptoms are directly related to striatal dopaminergic loss and the corresponding disruption of the motor circuit (11, 42, 46, 67).

Non-motor symptoms (NMS) also occur in up to 90% of PD patients and can often appear in the pre-motor stages of the disease (80). NMS include sleep disorders, olfactory dysfunction, neuropsychiatric issues of anxiety and depression, and cognitive impairment, to name a few (1, 15, 16, 49). Whereas the pathological mechanisms underlying PD motor symptoms are partially understood, the mechanisms of NMS are still largely unknown (29). LB formation in diverse brain regions could explain both motor and NMS. Indeed, the six-stage pathological system described by Braak et al. (based on the progression of LB development in diverse brain structures), would explain the appearance of NMS before motor symptoms, as the brainstem ( theorized mediator of olfaction, depression, cognition, pain, constipation, and autonomic vagal control) stains for LBs before the SNpc is affected (11, 47).
Development of a 6-OHDA Mouse Model to Examine Visuo-spatial Learning and Memory Deficits

Cognitive dysfunction, such as visuo-spatial deficits, can occur early in the disease and worsen as it progresses. PD patients perform poorly in tasks assessing the dysfunction of working memory that stores and processes visual and spatial information when compared to an age-matched healthy control and cognitively preserved group (46, 47). Understanding the underlying mechanisms of these symptoms could allow better treatment regiments to be developed for PD patients (53).

As experiments determining the cause of these NMS in humans pose many ethical and technical issues, animals are often used to model the disease. PD is one of the many human diseases that do not spontaneously appear in animals, but the characteristic features of PD can be imitated. Mice, rats, and monkeys are commonly used to study the disease by injection of a neurotoxin, such as 6-Hydroxydopamine (6-OHDA) (8, 12, 17, 20).

6-OHDA must be injected into a brain structure due to its inability to cross the blood brain barrier (30). Once injected, terminals of catecholaminergic neurons selectively take up the toxin. 6-OHDA accumulates in the cytosol and generates reactive oxygen species (ROS) and quinones that attack nucleophilic groups, causing the loss of dopaminergic neurons (20). The 6-OHDA injection induces stable lesions within one to three weeks, with no further depletion of dopamine, nor a chance at recovery (12). This makes 6-OHDA an excellent model for long-term behavioral analysis. The study of the NMS of
PD with 6-OHDA requires a partial bilateral injection of the toxin, causing a ~65% reduction of striatal dopamine, in order to avoid the confounding effect of motor imbalance on the cognitive performance and the possible compensation of one hemisphere (12, 54). This model has been used to examine cognitive impairment, depression, anxiety, pain, circadian deficits, and GI dysfunction (53). Based on the previous use of this toxin to study cognitive deficits, one aim of this work was to develop a functional 6-OHDA-mouse model of visuo-spatial learning and memory deficits for use in elucidating the underlying cause of these issues.

**α-Synuclein Aggregation Plays A Key Role in the Molecular Mechanisms**

**Underlying Idiopathic and Genetic PD**

A prominent hypothesis as to the underlying cause of PD pathology suggests that α-synuclein (Figure 1A) deposits into LBs causing SNpc dopaminergic death. α-Synuclein fibrillization via multiple mechanisms could contribute to aggregation.

α-Synuclein has extraordinary conformational plasticity. The protein remains unstructured, forms monomeric species, or amyloid fibers depending on its environment (Figure 2). The protein passes through intermediates forms, including spheres of 2 to 6 nm in diameter, chains of spheres (protofibrils), and circular protofibril rings in order to fibrillize. Its aggregation depends on nucleation, where a lag phase in polymerization is followed by exponential growth once aggregates have already formed (85).
The normal physiologic function for α-synuclein is only now being elucidated, and it has been suggested that it plays a role in the density of synaptic vesicles, plasticity, and modulating synaptic transmission (9). α-Synuclein is found in pre-synaptic terminal vesicles and portions of the nucleus interacting with lipid membranes. Davidson et al. showed through circular dichroism spectroscopy that binding with small (20-25 nm) vesicles composed of anionic phospholipids stabilized an α-helical secondary structure only in the N-terminal domain of α-synuclein. This promoted the formation of multimers, the precursors to fibril formation (5, 21).

α-Synuclein Interacting with Synphilin-1 Promotes Aggregation

α-Synuclein interacts with a variety of proteins (5, 85). Synphilin-1 (Figure 1B) was initially identified to interact with α-synuclein in a yeast two-hybrid screen, and the interaction was confirmed via NMR spectroscopy (87). Synphilin-1 gradually accumulates during development and immunohistochemical staining shows co-localization with α-synuclein in pre-synaptic terminal vesicles (52, 87). Synphilin-1 has also been detected in the core of LBs through western blot and immunohistochemical analysis of PD patient samples (84). Interaction between the two proteins possibly occurs through the ankyrin-like repeats and the coiled-coil of synphilin-1 (52).

An interaction at the ankyrin-like repeat has not been confirmed experimentally. However, when full-length synphilin-1 or its coiled-coil domain alone is over-expressed, the formation of α-synuclein aggregates is enhanced both in cell culture and mouse brain tissue. Xie et al. demonstrated through reciprocal fusion-protein fragment ‘pull downs’
that the coiled domain of synphilin-1 and the first 12 residues of α-synuclein’s N-terminus are necessary for the two proteins to interact. NMR spectroscopy suggests synphilin-1 exists in an antiparallel dimer attached by the two coiled-coil domains. α-Synuclein binds to synphilin-1 at the coiled-coil domain opposite the side of dimerization. Once α-synuclein is bound, it becomes more compact and structured, promoting aggregation (as seen in NMR solution structures of the α-synuclein/synphilin-1 complex). Indeed, synphilin-1 as a binding partner contributes to LB formation (87).

Gene Mutations in SNCA and SNCAIP Causing Familial PD via Enhanced Aggregation

Idiopathic and genetic PD may have a similar underlying pathology. Studying genetic PD is relevant to idiopathic cases with the assumption that the two forms share pathogenic mechanisms. α-Synuclein modifications enhancing its ability to form inclusions could play a vital role in both types of PD. This would allow the data from genetic models to be applied to all cases of the disease. Mutations in the gene encoding α-synuclein (SNCA) cause a familial form of the illness. Notably, each of the three missense mutations are located within the N-terminal lipid-membrane interacting amphipathic region of α-synuclein, highlighting its importance in aggregation (57, 85).

The gain-of-function A53T mutation provokes the formation of a beta-sheet stretch between amino acids 51 and 81 via loss of the induced α-helix between residues 51 and 66. This effectively expands the hydrophobic region, increasing the formation of oligomeric species, the precursor to eventual aggregation (5, 57). The A30P mutation causes a disruption of the first induced α-helix in the structure, decreasing α-synuclein’s
affinity for anionic phospholipids by ~50%. This could cause enhanced deposition of the A30P α-synuclein variant, but has not yet been experimentally confirmed (5, 57, 85). The most recently discovered mutation E46K also alters α-synuclein’s interaction with phospholipids. This variant enhances aggregate formation by exposing hydrophobic surfaces within the structure, allowing for increased intermolecular interactions (i.e. hydrogen bonding) (5).

Recently, a mutation in the synphilin-1 gene (SNCAIP) has also been identified to cause familial PD. It is unclear as to how mutation R621C causes changes in synphilin-1 structure or function. Marx et al. found that expression of R621C synphilin-1 mutant increased inclusion formation in SH-SY5Y neurons. This further suggests that synphilin-1 is an important mediator of LB formation (52).

Oxidative Stress and Calcium Dysregulation as Potential Determinants of Idiopathic PD

In an analysis of clinical data, Tanner et al. found a statistically significant correlation between the use of rotenone and the development of PD. The disease developed 2.5 times more frequently in those who were exposed to the pesticide (81). Rotenone acts as a mitochondrial complex I inhibitor, causing symptoms that mimic PD. Interestingly, this pesticide stimulates the formation of LBs in the brain (8). PD pathology has been attributed to mitochondrial complex I dysfunction. Parker and colleagues found a reduction of complex I activity in PD platelet mitochondria, while Schapira et al. found similar results in the SNpc of postmortem PD brain tissue through complex I activity
assays (25, 63, 72). This suggests that a mitochondrial deficit could play a role in idiopathic PD (20).

Tanner and colleagues found the same correlation with another pesticide known as paraquat (81). The chemical acts as an oxidative stressor, mediating the formation of ROS, such as superoxide radicals, hydrogen peroxide, hydroxyl radicals, and peroxynitrite. Mitochondrial respiration produces ROS that is normally cleared by the body. In a diseased state, ROS are increased. Markers of oxidative stress appear in the SNpc of PD patients, such as tyrosine nitration, increased carbonyl levels, and methionine oxidation of α-synuclein (27, 39, 44, 48).

Peroxynitrite production causes the formation of tyrosine-nitrated α-synuclein, found in LBs through the use of epitope-specific antibodies. In vitro co-incubation studies assessed by immunoelectron microscopy demonstrated that low concentrations of nitrated α-synuclein monomers increased unmodified α-synuclein’s propensity to aggregate. The nitration also caused a decrease in the rate of α-synuclein’s degradation by the 20S proteasome and calpain-1 seen via gel electrophoresis (39). The presence of carbonyl modifications is indicative of protein oxidation. Higher carbonyl content in proteins occurs during normal aging of the brain and corresponds to cognitive deficits in those areas. Floor et al. found higher carbonyl levels in soluble proteins from the SNpc of PD patients via dinitrophenylhydrazine assay and immunoblotting. This indicates an increase in ROS production accompanying PD (27, 44). It has been theorized that dopamine metabolism increases the formation of ROS. Leong and colleagues demonstrated that
incubating α-synuclein with dopamine caused the oxidation of the methionine residues in the α-synuclein detected via an increase in molecular weight corresponding to the expected change. The oxidation of these residues increased the protein’s propensity to aggregate, observed through higher molecular weight species apparent in gel electrophoresis (48). The data suggest an association between increased levels of oxidation and aggregate formation.

Calcium uptake and storage are highly important to the function of mitochondria. When oxidative stress occurs, mitochondrial membranes become disrupted, reducing the efficiency of calcium reuptake. This leads to higher intracellular calcium in the susceptible neurons. As SNpc dopaminergic neurons have low intrinsic calcium buffering, these cells are particularly vulnerable to altered cellular calcium concentrations (25, 26, 44).

Dysregulation of the Calpain/Calpastatin System Promotes α-Synuclein Aggregation

Higher levels of free intracellular calcium have been proposed to increase the activity of calpain, a non-lysosomal, calcium-dependent cysteine protease found in all mammalian cells (Figure 3A, B) (78, 89). Esteves and colleagues demonstrated through fluorometric calpain assays and calpain-specific breakdown products that there is an increase in calcium-dependent calpain activation associated with dysfunctional mitochondria in a cellular model of PD (25).
Cleaved forms of α-synuclein are often found in LBs. Calpain has been shown to cleave α-synuclein at several locations as shown by using epitope-specific antibodies in a western blot analysis. Cleavage may occur in the N-terminal region between amino acids 9 and 10 and between 57 and 58, and at the C-terminus between residues 122 and 123. Higher calpain activity due to dysregulated calcium from mitochondria may increase cleavage of α-synuclein and subsequent LB formation, as argued by Esteves (25). This is supported by the immunohistochemical detection of calpain in the halo surrounding LBs (23, 38, 56).

Bioinformatic analysis of clinical data by Allen and colleagues suggests that two single nucleotide polymorphisms (SNPs) in the gene of calpastatin (CAST), the endogenous inhibitor of calpain (Figure 3C), may predispose Caucasian individuals with European ancestry to idiopathic PD (2, 3, 20). A potential decrease in calpastatin function due to an SNP, and corresponding increase in calpain activity, could allow for increased cleavage and aggregation of α-synuclein.

*Can PIN1 Promote Aggregation of α-Synuclein Through Potential Regulation of Calpastatin and Synphilin-1?*

The mechanism whereby the calpain/calpastatin system is regulated is currently unknown. Recently, the peptidyl-prolyl *cis/trans* isomerase PIN1 (Figure 1C) has been suggested to regulate calpastatin’s ability to inhibit calpain through either binding or isomerization. PIN1 may promote aggregation by increasing the formation of cleaved α-synuclein species because of a reduction in calpastatin efficacy. This concept was
proposed using crude cell lysate pull downs, demonstrating interaction between PIN1 and calpastatin (51). However, this result has yet to be confirmed with purified proteins.

Immunohistochemical staining documented that PIN1 was present in 50-60% of LBs. In examining PIN1’s role in α-synuclein aggregation, it was found that PIN1 does not act directly on α-synuclein, but on synphilin-1. Co-immunoprecipitation showed that PIN1 interacts with synphilin-1 directly at phosphorylated sites at S211 and S215 promoting interaction between the α-synuclein and synphilin-1. This was demonstrated by an increase in aggregation when α-synuclein is co-transfected with PIN1 into N27 dopaminergic cells (69). PIN1 could play a significant role in the development of LBs through multiple mechanisms.

PIN1’s Potential Regulation of the Calpain/Calpastatin System in PD is Pertinent to Examine Further

Calpain and PIN1 have both been implicated in α-synuclein aggregation via different mechanisms. PIN1 interaction with calpastatin would increase calpain’s cleavage of α-synuclein by reducing the efficiency of calpastatin, leading to increased LB development. The calpain system and PIN1 appear to be prominent in the molecular mechanisms underlying α-synuclein fibrillization and LB formation. Based on a literature review and preliminary data, this topic should be examined further in regards to its role PD pathology.
MATERIALS AND METHODS

Animals

Male and female C57BL/6J mice, 10 weeks old, weighing 25-30 g, were purchased from Taconic (Tornbjerg, Denmark). The animals were housed in groups of no more than five mice per cage and maintained in a 12 hour light-dark cycle at a stable temperature of 22°C, with food and water ad libitum. All experiments were carried out during the light phase and in accordance with the guidelines of the Research Ethics Committee of Karolinska Institutet and the Swedish Animal Welfare Agency, as well as the guidelines for the use of animals in biomedical research provided by the European Communities Council Directive 86/609/EEC.

6-OHDA Lesioning

Mice were anesthetized with a mixture of Hypnorm (BETAPHARMA), Midazolam (PANPHARMA 5 mg/ml) and water (1:1:2) injected intra peritoneal (i.p.) in a volume of 10 ml/kg, then mounted in a stereotactic frame (David Kopf Instruments, Tujunga, CA) with a mouse adaptor. The skull of the mouse was exposed and a researcher drilled one burr hole per side. Each mouse received a bilateral injection into the dorsal striatum as previously described (71) of 19 mM 6-Hydroxydopamine Hydrochloride (6-OHDA-HCl) (1 uL freshly dissolved in a vehicle 0.9% saline and 0.02% ascorbic acid) (Sigma-Aldrich, Sweden AB). The injections were made according to the following coordinates (mm) (65): anterior-posterior (AP): +0.6, medio-lateral (L): +/-2.2 and dorsal-ventral (V): -3.2. The injections were made by a metallic injector connected by a plastic tube to a 5 µl
Hamilton syringe mounted on a pump. After each injection, the injector was left in place for 3 minutes to allow complete diffusion of the drug. The control animals (sham) were subjected to the same procedure with the vehicle lacking 6-OHDA (1 µl). Before every new surgery all the equipment (injector, tube, and syringe) was cleaned with ethanol and water. After the injections, the skull was covered and the skin was disinfected and stitched with metallic clips. The mice were hydrated with a solution of 15% glucose injected i.p. in a volume of 10 ml/kg and placed under red light until they gained consciousness. After the surgery the animals were observed daily and fed with milk as considered necessary. Carprofen (250 µl dissolved in 0.9% saline at a concentration of 5 mg/kg) (Sigma-Aldrich, Sweden AB), an analgesic drug, was injected subcutaneously for three days following surgery and every 24 hours after as needed. The mice were given a recovery period of three weeks before behavioral evaluations.

**Statistical Analysis**

Data were analyzed using one- or two-way ANOVA, and post hoc comparisons between groups were made using Fisher’s post hoc analysis and t-tests with equal variances for groups of 2, when relevant. A p value of less than 0.05 was considered significant. * p < 0.05, ** p < 0.01 and *** p < 0.0001.
Behavioral Evaluations

*Barnes Maze*

The table consisted of a white circular open arena (125 cm in diameter) elevated to 89 cm by four metal legs. There were 36 circular holes around the edge of the table (4.5 cm in diameter) equally spaced (5.5 cm inter-hole distance) and placed 1.3 cm from the edge of the table (Fig 5A). The dark grey opaque plastic escape box (20.6 x 6.3 x 10.4 x 4.1 cm) was mounted in brackets under one of the holes. White opaque curtains surrounded the room to block extraneous cues. Extra-maze cues were a triangle (69.4 cm on each side) with 6 alternating white and black stripes (5.2 cm in width), a black circle (64.2 cm in diameter), and a square (65 cm on each side) with 5 white boxes (18.7 cm on each side) on a black surface, forming a checkered pattern (Fig 5B). The cues were placed on the walls of the room centered at 1.4 m high. Three round Honeywell Oscillating Table fans were placed 16.1 cm away and level to the table equidistant from each other and allowed to rotate on medium speed. The position of the fans was unchanged for the entirety of the experiment. Bright lights located on the ceiling illuminated the apparatus. The mouse was placed in a dark grey opaque plastic tube (12.3 cm tall, 11 cm in diameter), which was removed manually at the start of each session. A video camera above the field was connected to a video recorder and a monitor, recording the movement of the mouse. One mouse at a time was tested. To reduce intra-maze odor cues, each mouse was assigned a different hole for both habituation and training but maintained the same hole during the entire session.

The entire apparatus was cleaned with 70% ethanol between each mouse.
Mice received one habituation trial in which they were placed beside their respective escape hole (with the box underneath the hole) in a transparent glass cylinder for 4 minutes, and gently guided into the box for 1 minute. This procedure allowed the mice to experience the aversive bright lights and practice descending into the escape box. During the habituation trial, extra-maze cues were placed behind the curtain to prevent learning the escape hole’s location.

The acquisition phase consisted of 7-9 training days with two trials per day. For each trial, a mouse was placed into the start tube located in the center of the maze, the start tube was raised, and the trial ended when the mouse entered the escape box. After each trial, the mice remained in the escape box for 30 seconds before being returned to the holding cage located in a dark room adjacent to the test room for the inter-trial interval (10–15 minutes). For each session, mice were given 5 minutes to locate the escape hole, after which they were guided to the escape hole and coaxed to enter. Mice that still failed to enter the escape hole were placed directly into the escape box. If a mouse climbed back onto the maze after entering the escape box, the mouse was guided back until it re-entered the escape box. A mouse that fell into an incorrect hole was returned to its holding cage, and the trial was re-run after all the other mice had completed the respective training trial.

Learning was measured by latency, the time required for the mouse to locate, not enter, the escape hole (37, 61). An experimenter blind to the groups manually scored the latency.
**Spatial Object Recognition (SOR) Test**

The apparatus was a square field (55 cm on each side) with walls (50.2 cm high) made of dark gray plastic material. A visually uniform environment surrounded the open field. A dim light located on the ceiling illuminated the apparatus. A video camera above the field was connected to a video recorder and a monitor, recording the movement of the mouse. Five objects were simultaneously present in the field: (1) a red block (3.1 cm on all sides, 1.9 cm high); (2) a blue top (3.2 cm diameter, 1.3 cm high); (3) a yellow battery (4.2 cm long, 1 cm diameter); (4) an orange figurine (2 cm diameter, 3.5 cm high); and (5) a snowman figurine (2 cm diameter, 4.5 cm high). The initial arrangement was square with object 5 in the center (Fig 6A, S1-S3). The configuration was changed for the test by moving two objects: object 5 was placed below the previous location of object 1, which was itself displaced to the periphery of the apparatus so that the initial square arrangement was altered to form a polygon-shaped arrangement (Fig 6A, S4).

Mice were individually subjected to three successive 6-minute sessions, separated by a 3-minute delay (S1-S3), during which the subjects were returned to their home cages. On the next day (24 hours later), mice were subjected to only one 6 minute session (S4), with the objects displaced relative to the original arrangement as described.

Object exploration was evaluated by the time the animal spent in contact with an object. A contact was defined as the snout of the mouse touching an object. The duration of contact was expressed as mean per object. Response to spatial change was assessed by comparing the mean time in contact with the objects belonging to each category (displaced (DO) and non-displaced (NDO)) in S4 minus the mean time spent in contact with the same object category in S3 (DO S4-S3; NDO S4-S3) (68).
Molecular Assays for Mouse Studies

Western Blotting

Ten of the mice used in the Barnes maze were sacrificed via guillotine. The striatum and hippocampus were bilaterally dissected. The striata and hippocampi were sonicated in 750 µl and 500 µl of 1% SDS in water, respectively, and boiled for ten minutes.

Aliquots (5 µl) from sonicated striata or hippocampi were used for protein quantification with the bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL). Equal amounts of protein (5 µg) from each sample were loaded onto 10% Tris Glycine polyacrylamide gels (37.5:1 pore size). Proteins were separated by SDS-PAGE and transferred overnight to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Little Chalfont, UK) (1). The protein of interest was detected with mouse anti-TH antibody (1:3000, Millipore, Billerica, MA), and the PVDF membranes were incubated in horseradish peroxidase (HRP)-conjugated secondary goat anti-mouse antibody (1:30000, Millipore, Billerica, MA). The protein signal was visualized by enhanced chemiluminescence (ECL) (Pierce, Rockford, IL) and quantified using Quantity One software (Bio-Rad).

Immunohistochemistry

Mice were anaesthetized with pentobarbital (100 mg/kg, i.p., Sanofi-Aventis, France) and perfused transcardially with 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.5. After overnight post-fixation in the same solution, brains were cut with a vibratome (Leica, Nussloch, Germany) in 40 µm slices from hippocampus (anterior-
posterior (AP) between -1.94 and -2.06 mm, from bregma (intersection of the coronal and sagittal sutures on the vertex of the skull)) and striatum (AP -0.26 mm, from bregma).

**TH in the Striatum**

Slices were incubated in permeabilizing solution (0.3% Triton X-100, 1% bovine serum albumin (BSA), 5% normal goat serum in Phosphate-buffered saline (PBS)) for 60 minutes. After washing in PBS, sections were incubated overnight at 4°C with anti-TH mouse monoclonal antibody (1:300; Millipore, Stockholm, Sweden), rinsed again and incubated for 1 hour with goat Cy3-coupled anti-mouse secondary antibody (1:400; Jackson Laboratory, Bar Harbor, ME). Sections were then mounted in 2.5% 1,4-diazabicyclo [2.2.2] octane (DABCO) (Sigma-Aldrich, Sweden). After processing, images of the striatum were taken at 5x magnification using sequential laser scanning confocal microscopy (Zeiss LSM 510, Jena, Germany).

**Arc or pERK in the Hippocampus**

Slices were incubated in permeabilizing solution (0.3% Triton X-100, 1% BSA in Tris-buffered saline (TBS)) for 45 to 60 minutes. After washing in TBS, sections were incubated overnight at 4°C with anti-Arc mouse monoclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-pERK rabbit monoclonal antibody (1:200; Cell Signaling Technology, Beverly, MA), rinsed again and incubated for 1 hour with goat Cy2-coupled anti-mouse secondary antibody or goat Cy3-coupled anti-rabbit secondary antibody (1:400; Jackson Laboratory, Bar Harbor, ME). Sections were then mounted in 2.5% DABCO (Sigma-Aldrich, Sweden). After processing, images of the hippocampus
were taken at 10x magnification using sequential laser scanning confocal microscopy (Zeiss LSM 510, Jena, Germany).

**Molecular Assays for Calpastatin-PIN1 Studies**

**Purification of calpain, calpastatin, and PIN1**

BL21(DE3) *Escherichia coli* were previously transformed with two plasmids encoding recombinant six-Histidine tagged wild type (WT) rat calpain-2 (capn2-H6) in a pET24a vector and the requisite small subunit capns-1 in a pACpET vector. Other BL21(DE3) *Escherichia coli* were previously transformed with a pET24a plasmid encoding recombinant six-Histidine tagged double phosphomimic (PM2) calpastatin domain 1 (D1) or with a pMCSG7 plasmid encoding recombinant six-Histidine tagged human PIN1 (Addgene plasmid 40773 from Dustin Maly). All cells were induced to express recombinant protein with 0.5-0.6 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 14-18 hours at room temperature. Cells were harvested by centrifugation at 4000 xg for 15 minutes at 4°C. The pellets were washed with 0.15 M NaCl and recentrifuged under the same conditions prior to storing at -80°C.

For protein purification, calpain and calpastatin expressing cells were re-suspended in lysis buffer (50 mM 3-(N-morpholino) propanesulfonic acid (MOPS) pH 7.5 and 10 mM β-mercaptoethanol (β-ME)). For extraction of cells expressing calpain, the buffer also had 10 mM ethylene glycol tetraacetic acid (EGTA), 10 mM ethylene diamine tetraacetic acid (EDTA), and 0.2 mM phenylmethanesulfonylfluoride (PMSF)/benzamidine. The cells were lysed by sonication on ice (Branson Sonicator 450). Cells expressing calpastatin were subjected to three freeze (-80°C) thaw cycles in lysis buffer and the
supernatant from the final thaw was diluted with 50 mM MOPS pH 7.5, 5 mM EGTA, 5 mM EDTA, and 5 mM β-ME. PIN1 cells were re-suspended in 25 mM MOPS pH 8.0, 10 mM imidazole, 0.10 M NaCl, 0.4 mM EGTA, 0.2 mM EDTA, and 0.1 mM β-ME. All suspended cells or cell lysates were centrifuged at 30000 xg for 20 minutes at 4°C, and the supernatant with the protein of interest present was isolated (see below). Total protein was measured by Bradford assay. Concentration of calpastatin was also measured by A280 using an extinction coefficient of 4675 M$^{-1}$cm$^{-1}$ (19).

**Ion exchange and dye affinity chromatography:** Supernatant with calpain or calpastatin was loaded onto a DEAE Sephacel ion-exchange chromatography column pre-equilibrated with buffer A (50 mM MOPS pH 7.5, 5 mM EGTA, 5 mM EDTA, and 5 mM β-ME). For calpain, a wash with 0.15 M NaCl in buffer A removed weakly bound proteins from the resin. Elution by 0.5 M NaCl in buffer A yielded the protein of interest. The calpastatin peak was pooled and dialyzed against 25 mM MOPS pH 8.0, 10 mM imidazole, and 0.10 M NaCl. The calpain peak was pooled and loaded onto a pre-equilibrated Reactive Red Agarose affinity column. The bound proteins were eluted using distilled deionized water into final concentrations of 25 mM MOPS pH 8.0, 10 mM imidazole, 0.10 M NaCl, 0.4 mM EGTA, 0.2 mM EDTA, and 0.1 mM β-ME.

**Nickel affinity chromatography:** The pooled calpain peak, calpastatin eluted from DEAE, or PIN1 supernatant was loaded onto a pre-equilibrated Ni-NTA agarose affinity column (Qiagen). Bound proteins were eluted by increasing the imidazole in the buffer to 100 mM (calpastatin) or 250 mM (calpain and PIN1). Pooled calpain was dialyzed against 50 mM MOPS pH 7.5, 2 mM EGTA, 2 mM EDTA, 0.5 mM dithiothreitol (DTT) and 0.025% sodium azide. Pooled calpastatin was dialyzed against 25 mM MOPS pH 7.5.
Pooled PIN1 was dialyzed against PIN1 storage buffer (20 mM MOPS pH 7.5, 0.5 mM DTT, and 0.1 M NaCl). Calpain and PIN1 aliquots were frozen with liquid nitrogen and stored at -80°C. Calpastatin aliquots were frozen and stored at -20°C.

**SDS Polyacrylamide Gel Electrophoresis**

Protein purity and digestions were analyzed by electrophoresis on an 8%, 10%, or 12% Tris Tricine polyacrylamide gels (32:1 pore size) with samples that had been boiled for two minutes in SDS-PAGE sample buffer (0.0625 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 6.8, 10% glycerol, 5% β-ME, 2.5% SDS). Protein was visualized using 0.1% Coomassie blue and 0.05% amido black in 40% methanol and 10% acetic acid. Gels were imaged using the Chemimager 4400 Low Light Imaging System (Alpha Innotech Corporation) and saved as digital tiff files. Images represent original stained gels. PageRuler was used as protein molecular weight (MW) markers (Thermo Scientific, Waltham, MA). MW was analyzed by graphing the logarithm of the MW markers versus the measured Rf values. The resulting graph was used to estimate MW of the bands.

**Fluorescein isothiocyanate (FITC)-casein Hydrolysis to Assay Calpain and Calpastatin**

Assays were composed of 73 μM FITC-casein, 50 mM MOPS pH 7.5, 0.15 M NaCl, 10 mM CaCl₂, and 1 mM DTT. Varying concentrations of WT or PM2 calpastatin D1 diluted in 25 mM MOPS pH 7.5 or PIN1 diluted in PIN1 storage buffer were added as indicated. Incubation for 20 minutes at 25°C began when WT recombinant calpain-2 diluted in 50 mM MOPS pH 7.5, 2 mM EGTA, 2 mM EDTA, 0.025% sodium azide, and
1 mM DTT (MEEND) was added to the samples in a final concentration of 104 nM. Incubation stopped when cold trichloroacetic acid (TCA) was added for a final concentration of 3.5% and assays were allowed to precipitate overnight at 4°C. Samples were centrifuged at 16000 xg for 10 minutes at room temperature. The supernatant was collected and diluted by 0.5 M Tris pH 8.5. Assays were analyzed using a Perkin-Elmer LS50 B luminescence spectrophotometer (excitation at 488 nm and emission at 518 nm). Slit widths varied between assays (2.5-4.0 nm) but were consistent between samples in an experiment. Controls with or without enzyme were in triplicate; experimental samples were performed in duplicate.

**Digestion of PIN1 by Calpain-2**

PIN1 was incubated with calpain in a molar ratio of 1:100 of calpain to PIN1 for 0, 5, 15, 30, or 45 minutes at 25°C in final concentrations of 50 mM MOPS pH 7.5, 10 mM CaCl₂, and 1 mM DTT. Controls were incubated at 0 and 45 minutes in the absence of calpain. Samples were quenched by the addition of SDS sample buffer with 5 mM EGTA and subjected to gel electrophoresis (see above) with a final concentration of 3 µg PIN1.

**Western Blotting**

SDS samples from PIN1 digestion by calpain-2 (0 and 45 minutes of both control and experimental) were loaded onto a 10% Tris Tricine polyacrylamide gel (32:1 pore size) and subjected to SDS-PAGE gel electrophoresis. Final concentrations of PIN1 loaded ranged from 0.5 to 2 µg as indicated. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) and blocked overnight in 3%
gelatin in 1% TBS. The protein of interest was detected with mouse anti-PentaHistidine HRP-conjugated antibody (1:1000, Qiagen). The protein signal was visualized by Clarity Western ECL kit (Bio-Rad).
RESULTS

Partial 6-OHDA lesion reduces dopaminergic innervation to the striatum and hippocampus.

Male and female mice were given a bilateral 6-OHDA lesion in the dorsal striatum. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthesis of dopamine, as it catalyzes the formation of L-DOPA. The loss of TH is representative of the death of the dopaminergic neurons. Therefore, PD is a TH-deficient syndrome as well a dopamine-deficient syndrome (35). To confirm the lesion’s efficacy, TH levels were assessed in the striatum and hippocampus of sham and lesioned mice. The western blot analysis shows a 70% decrease in the amount of TH in the striatum in lesioned mice compared to sham (Figure 4A). There is a corresponding 70% decrease of TH in the hippocampus (Figure 4B).

Immunohistochemistry was performed to visually verify the loss of TH in the striatum. The results show a visible, substantial decrease in the TH due to the lesion compared to sham, confirming the western blot results (Figure 4C). This is indicative of a successful partial lesion. Indeed, the model mimics the depletion of dopamine expected of PD NMS.

The Barnes Maze assesses visuo-spatial learning and memory in mice.

Once the lesion was confirmed, behavioral evaluations were used to assess if the mice possessed a visuo-spatial memory and learning deficit. The Barnes maze was determined to be potentially useful at evaluating the desired cognitive issues in the mice. To use the Barnes maze, it was necessary to develop a working protocol with the available apparatus
(Figure 5A). Over seven days, wild-type mice significantly increased their average speed in finding the goal hole by ~100 seconds (Figure 5C). Therefore, the protocol is effective at determining the function of visuo-spatial learning and memory in mice. This verification allows the continued use of the protocol for 6-OHDA lesioned mice.

**The Spatial Object Recognition (SOR) test assesses spatial learning and memory in mice.**

The SOR test was used to analyze another aspect of the mice visuo-spatial memory deficit. Wild-type mice were tested to determine the efficacy of the protocol for the SOR test. The ratio of exploration time for the displaced objects (DO) is high, at four seconds more exploration after the change. There is a slight reduction of one second in the non-displaced objects (NDO) exploration time, concomitant with the increase of DO exploration (Figure 6B). This demonstrates that the animal discriminated and remembered the spatial modification, confirming the working protocol, allowing for its use in further experimentation.

**Parkinsonian mice possess a visuo-spatial memory deficit.**

Once the protocols had been verified, the behavioral evaluations were available for use with lesioned mice. Visuo-spatial learning and memory in sham and lesioned mice were assessed with both the Barnes maze and the SOR test.

The mice learned the location of the hole over nine days of training on the Barnes maze, where both groups reduced overall time to find the hole by ~110 seconds. However, the
lesioned mice were eventually able to find the hole in 40 seconds compared to the sham mice finding the hole in only 10 seconds (Figure 7A). This demonstrates that the lesioned mice were unable to learn and remember the location of the goal hole relative to visual extra-maze cues as well as the sham mice.

The sham mice had a similar result to the wild-type mice in the SOR test with a 2 second increase in DO exploration and 0.2 second decrease in time examining NDO, demonstrating the expected spatial learning. The lesioned mice have a reduction in both DO and NDO exploration time of 1 to 1.5 seconds, with no significant difference between the two (Figure 7B). Therefore, the lesioned mice did not recognize and respond to the spatial change of the objects.

The model effectively mimics the visuo-spatial memory and learning deficits seen in PD patients. This allows the model’s use in examining the underlying mechanisms.

*Arc and pERK are not definitively associated with the memory deficits seen in the 6-OHDA lesioned mice.*

Visuo-spatial memory is believed to be located in the hippocampus. Activity-regulated cytoskeleton-associated protein (Arc), an immediate early gene, is found in many cognitive disorders. It is possible to measure and correlate the changes in expression of Arc to the effect of the disease on hippocampal function in animal models (75). Another functional marker to investigate the occurrence of plastic processes is phosphorylated extracellular signal-regulated kinase (pERK). ERK is an essential component of the ERK signal transduction mechanisms underlying behavioral memory formation (79).
To see if the lesion caused a reduction in synaptic plasticity, immunohistochemistry of the hippocampus was performed to assess the levels of Arc and pERK. The mice were sacrificed one hour post-stimulation with the Barnes maze.

Approximately one cell is visibly stained positive for Arc in both sham and lesioned samples (Figure 4D). This is not a definitive determination of the correlation between Arc and the effect of the lesion on visuo-spatial memory.

There are eight cells visibly stained for pERK in lesion and sham mice (Figure 4D). The low number of cells prevents analysis of the results. This gives a lack of clarity on the association between pERK and the memory changes due to the lesion.

While there are visuo-spatial deficits present in this model, further analysis needs to occur to determine the underlying pathology.

**Phosphomimic (PM2) and wild-type (WT) calpastatin domain 1 (D1) possess a similar ability to inhibit calpain-2 in vitro**

In order to examine PIN1’s potential regulation of calpastatin, it was necessary to create the proper conditions for PIN1 to act on the protein. PIN1 is a unique PPIase in that it is selective for phosphorylated serine (S) or threonine (T) residues followed by a proline (P) residue. Parallel FITC-casein assays were performed with calpastatin (D1) and its double phosphomimic (PM2), where S/T residues potentially targeted by PIN1 had been previously changed to E residues through site-directed mutagenesis (Figure 3D). Assays were performed to determine that PM2 retained the ability to inhibit calpain-2 activity. Calpain was inhibited equally by both WT and PM2 at concentrations of 20 nM, 40 nM, and 60 nM (15%, 25%, and 35% respectively). PM2 displayed more effective inhibition
at 80 nM (80% inhibition) and 100 nM (85% inhibition) when compared to WT (45% inhibition at 80 nM and 55% inhibition at 100 nM) (Figure 8). This indicates the ability to use PM2 calpastatin D1 in future assays examining calpastatin’s inhibition of calpain.

*Recombinant PIN1 is cleaved by calpain-2 at the N-terminus in vitro*

In order to determine if calpain-2 cleaves PIN1, the two enzymes were incubated under conditions that favored calpain-2 activity. The recombinant PIN1 is cleaved by calpain-2, seen by the loss of the control band at ~21 kDa (black arrow) and appearance of two new bands of a major product at ~18 kDa (blue arrow) and a minor product ~13 kDa (red arrow) (lanes 3-7) (Figure 9).

For an estimate of the location of cleavage on PIN1 by calpain-2, a western blot was performed using anti-PentaHistidine HRP conjugate antibody due to the presence of a six-Histidine tag on the N-terminal end of PIN1. The western blot demonstrates that PIN1 is cleaved at the N-terminus as seen by the loss of signal (lanes 7 and 8) and lack of a lower molecular weight band formation corresponding to the major or minor product (Figure 10).
DISCUSSION

A bilateral 6-OHDA lesion of the midbrain dopaminergic system produced a partial depletion of dopamine that mimics a relatively early stage of PD. The loss of dopamine is evidenced by a distinct reduction in TH immunoreactivity in the striatum and the hippocampus. The mice demonstrate decreased visuo-spatial learning and memory due to the lesion. These results did not find a definitive correlation between the lesion and Arc or pERK in the hippocampus. Overall, the 6-OHDA mice effectively model the visuo-spatial memory and learning deficit in PD.

This mouse model of PD reproduces an initial stage of the disease, as suggested by the partial (~70%) reduction of TH immunoreactivity observed in the striatum. This protocol has been well established to reproduce the parallel degeneration of dopaminergic and noradrenergic neurons described in PD patients (11, 40, 42). This type of lesion allows the mice to develop NMS with a lack of overt motor symptoms (24, 35). The decrease in TH immunoreactivity produced by 6-OHDA affects not only the striatum, but also the hippocampus. Bonito-Oliva and colleagues found an 85% reduction of hippocampal TH in this model, which was abolished with the pre-treatment of desipramine, a noradrenergic reuptake inhibitor (10). This suggests a reduction of TH in the hippocampus is due to the lesion’s effect on not only dopaminergic, but on noradrenergic neurons that account for a high amount of TH positive cells in the hippocampus (7). The reduction observed in the present results only showed a ~70% decrease in hippocampal TH, possibly due to variability in the mice tissue composition and the lesion’s efficacy.
For the purposes of this study, the Barnes maze is preferred to measure visuo-spatial memory deficits. While the partial bilateral injection of the toxin did not cause overt motor symptoms, it may cause fine motor impairments that affect results in a wet maze, such as the Morris water maze. Therefore, a dry maze affords greater accuracy (59). Fox and colleagues measured visuo-spatial memory in a mouse model of traumatic brain injury by replacing the Morris water maze with the Barnes maze. The stress and physical demands on the mice due to the wet maze affected the results and made the behavioral deficit appear more prominent. Their results suggest that because of the trauma to the experimental mice, the Barnes maze was more effective at accurately measuring visuo-spatial memory (28). This promotes the use of the Barnes maze when the mice have impairments that can affect performance in a water maze, such as the 6-OHDA lesion.

It was necessary to determine a working protocol for the Barnes maze apparatus available. As apparatus design could influence results, a reproducible protocol was vital. O’Leary and colleagues found that a large table (122 cm) was more effective at accurately measuring visuo-spatial memory in rats due to the reliable use of extra-maze visual cues and strategy for spatial search. The apparatus used for this study had a diameter of 125 cm, giving credence to its efficacy (61). The protocol was effective at measuring the ability of the mice to learn the location of the goal hole using the extra-maze cues (60).

The SOR test is an effective measure of the mouse’s response to a spatial change. This is paralleled in the literature as a useful tool to measure response to spatial modifications in
mice. Roullet et al. reliably demonstrated that glutamate receptors play a role in processing spatial information using the SOR test to measure changes in mice treated with NMDA antagonists (68). If the mice have functional spatial memory, there is an increase in exploration of the DO in the test phase and corresponding loss of time exploring the NDO. The controls in the study by Roullet et al. mirror this work, further validating the present protocol (68). The SOR test could be used in further experiments due to the expected DO and NDO ratios.

The Barnes maze evaluation demonstrated that the lesioned mice did not reach the efficiency of the sham mice in finding the goal hole. The ~70% loss of dopaminergic neurons impaired the level of learning but not the ability to learn. Branchi et al. found that there were no deficits in 6-OHDA mice in the Morris water maze with a bilateral lesion of 45%. A ~75% loss is required for spatial deficits to be apparent, in line with the current results. Given that the Barnes maze is an associative task, the lesioned mice have a deficit in their ability to create a spatial construct based on visual cues. Mura et al. found similar deficits in visual spatial memory from subjecting rats with a bilateral 6-OHDA lesion to the Morris water maze, despite the drawbacks of a wet maze. Their findings are similar to the current results, where the experimental mice learned the task, but were unable to execute as well as the sham mice (59).

The SOR test demonstrated that the sham mice could react appropriately to the spatial change. They spent more time exploring the DO than the NDO. In contrast, the lesioned mice have the same exploration for both DO and NDO, showing their lack of response to
a spatial modification after 24 hours. The SOR is a non-associative task, and the data demonstrates a reduction in the animal’s ability to form spatial constructs without visual cues. Roullet and colleagues study revealed that an impairment of the mice after administration of the NMDA antagonist is similar to the results obtained with 6-OHDA lesioned mice (68). Leonibus and colleagues illustrated that the SOR test measures changes in PD mice that were treated with glutamate receptor blockades. Therefore, the SOR is a useful tool in PD research (49). In the present results, it seems that the sham mice had a reduced and more variable response to the spatial change than the wild-type mice used to validate the protocol. This could be due to effects of the surgery performed.

Spatial deficits are known to appear in this model (49, 59). The bilateral 6-OHDA injection allows other cognitive deficits to be studied. Bonito-Oliva et al. have demonstrated that a loss of synaptic plasticity in the hippocampus due to the same lesion in mice corresponds to decreased long-term recognition memory observed through the reduction of ERK signaling (10). It stands to reason that other such deficits would be apparent in the partial bilateral 6-OHDA model.

In the present results, only one cell in both sham and lesion samples stained for Arc is visible. This does not allow a clear determination as to whether or not the lesion caused a change in plasticity. The antibody’s efficacy at staining the samples was confirmed under identical conditions with tissue known to contain Arc (data not shown), confirming the antibodies function. Multiple cells are visible in the samples of the hippocampus stained for pERK, but the quantity is not sufficient for an accurate comparison between sham and
lesion samples. This result is not definitive, as there has been evidence of pERK and Arc involvement in the reduction of memory associated with PD (10, 73). It is possible that one-hour post stimulation is the incorrect timing of sacrifice to observe Arc or pERK, as thirty minutes post spatial learning stimulation is used reliably in protocols for observing the expression of both plasticity markers in rats (34). It is also conceivable that the stimulation was insufficient for Arc or pERK to be expressed. While it is known that the ERK signaling pathway is crucial to long-term recognition memory in the hippocampus, other pathways that were not examined may mediate visuo-spatial memory (10).

Partial dopamine depletion leads to an impairment of visuo-spatial learning and memory in a 6-OHDA mouse model of PD. This is a functional representation of NMS in humans, as it has been shown that patients suffer from similar impairments. PD patients did not perform as well as the age and education-matched controls in conditions requiring remembering locations in space. They also showed increased errors regarding working memory in forming a spatial construct (66). Braak staging suggests that LB formation stimulates the reduction in cognitive function. However, the 6-OHDA model does not create classic LBs, suggesting that neuronal degeneration plays a more prominent role in the deficits presently observed (47). This model is limited given that LB formation in various brain regions could contribute to the deficits in humans (43).

PD research is far off from finding a cause, let alone a cure. Further work should be done to determine the underlying mechanism behind this impairment. An assessment of the link between synaptic plasticity markers and the behavioral effects due to the lesion
should be done with the verified model. It is necessary to continue further experimentation with altered procedures to determine if Arc or pERK are affected by the lesion and give the requisite behavioral deficits in visuo-spatial memory. To assess if the ERK plasticity pathway is involved, it would be possible to treat mice with an ERK inhibitor or promoter to see if the deficit is worsened or improved, respectively. It is also necessary to examine different markers or pathways involved in synaptic plasticity. These could include the mTOR pathway, calcium/calmodulin-dependent protein kinase II (CaMKII), brain-derived neurotrophic factor (BDNF) or calcineurin (18, 79, 90). It could be possible to investigate the mTOR pathway’s involvement with an mTOR inhibitor. This could be followed by an attempt to ameliorate the effect with medication such as L-DOPA.

Data demonstrate that lower concentrations of PM2 and WT calpastatin D1 have a similar ability to inhibit calpain-2, diverging at higher amounts in vitro. Calpain-2 appears to cleave PIN1 at the N-terminus in vitro.

There was very little measurable difference in inhibition of calpain by calpastatin WT or PM2. Because phosphorylation in the cell is transient and the kinase that phosphorylates calpastatin is unknown, a phosphomimic increases the experimental feasibility. It has been previously shown that PIN1 can act on a phosphomimic (50). S/T residues were replaced with E residues, more favored by PIN1 than D residues. This was confirmed experimentally through similar enzymatic efficiency in coupled PIN1 activity assays when WT or phosphomimic substrates were utilized (50, 88). While the higher
concentrations of the two forms of calpastatin diverge in the ability to inhibit calpain, the similarity at lower concentrations allows for the use of PM2 in inhibition assays of calpain where calpastatin concentration is lower to see an effect of added variables.

It is unclear if PIN1 regulates calpastatin. This concept was first proposed using crude cell lysate pull downs. GST-PIN1 pulled calpastatin out of a cell lysate and the potential interaction was supported by calpastatin co-immunoprecipitating with an anti-PIN1 antibody (51). However, purified protein assays may prove more difficult. There could be multiple reasons why a direct experiment would not provide a clear result. If calpain is inhibited extensively by too much calpastatin, it is difficult to see any change in activity. In contrast, PIN1 requires large amounts of substrate because only 3-10% of the substrate is naturally in a cis conformation (50). It is reportedly unfavorable for proteins to remain in the cis conformation as it must be stabilized by surrounding folded structure, which is not possible with intrinsically unstructured proteins such as calpastatin. In order to observe an effect of PIN1, the substrate must be trapped in a trans conformation by selective proteolysis or a coupled assay. The calpastatin-calpain complex is tightly bound (19) and PIN1 may be unable to disrupt it. Further, the assays were performed under conditions favoring calpain, not PIN1, activity. It seems likely that calpastatin is acted on by PIN1, given its proclivity for unstructured protein substrates (50). Even in perfect in vitro conditions, PIN1 may not regulate calpastatin directly.

Another reason why PIN1 might not have any effect on calpastatin is potential cleavage by calpain. Data show that calpain cleaves recombinant PIN1 in vitro. PIN1 as a substrate
is relatively surprising, as calpain often prefers large proteins to cleave. There are two bands in the gel of potential significance. The band at ~18 kDa is the major product formed in the degradation of PIN1. The human PIN1 construct has a histidine tag followed by a tobacco etch virus protease cleavage site. It is possible that the ~18 kDa band represents PIN1 after calpain-2 has cleaved off both tags leaving the PIN1 protein intact, corresponding to the molecular weight change predicted by ExPasy. This cleavage would not be physiologically relevant. The minor product ~13 kDa band is intriguing. If calpain cleaves PIN1 between the WW and PPIase domain, there would be the production of a ~13 kDa protein fragment, similar to the size of the remaining catalytic domain (as measured by ExPasy). While the specific location of cleavage is still unknown, western blot data demonstrate that the N-terminus is cleaved, as neither the ~18 or ~13 kDa band appears on the membrane, further suggesting the removal of the affinity tags. Prediction of cleavage sites by the calpain.org database did not correspond to the observed truncation of PIN1. As calpain cleavage sites are highly variable, this result could be due to the difficulty in accurate predictions.

The method by which PIN1 binds and isomerizes its substrates is unclear (41). In some cases, the WW domain is necessary for the PPIase domain to function (as seen through western blot analysis of characterized substrates with only the PPIase portion present) (41). Therefore, cleavage between the two domains could effectively attenuate PIN1 activity, although this has not been confirmed experimentally. Liu argued that calpastatin’s ability to inhibit calpain is reduced in the presence of PIN1. This would likely cause a concomitant increase in calpain activity. If calpain were to then cleave
PIN1, potentially removing it’s catalytic activity, a novel feedback regulation mechanism appears wherein calpain regulates itself.

Grant and colleagues demonstrated that calpain is actively cleaving spectrin (measured by calpain-specific spectrin breakdown products) for at least 2 weeks post 6-OHDA lesion in the SNpc and striatum of a rat model (33). In a SH-SY5Y cell model of PD stimulated with MPP⁺ and rotenone, a rise of calcium concentration (measured via increased emission after addition of ratiometric dye Fura-2AM) activated calpain in the dopaminergic cells as measured by calpain-specific spectrin breakdown and immunohistochemical staining with an antibody against active μ-calpain. Inhibition of calpain activity can be neuroprotective in PD. When calpain was inhibited with SNJ-1945, damaging pathways such as oxidative stress (measured by caspase 10, Cox-2, and p10) and proteolysis of spectrin (examined by changes in calpain specific breakdown product) were attenuated (45). In a rat model of MPP⁺ and rotenone, mitochondrial dysfunction also promoted an increase in intracellular calcium (measured by an increase in emission after adding ratiometric dye Fura-2AM), inducing an increase in calpain-specific spectrin breakdown products. Calpeptin (a calpain inhibitor) significantly blocked apoptosis occurrence, conferring cytoprotection (examined through a reduction in DNA ladder formation) (70). A rat 6-OHDA model of PD given L-DOPA therapy had up to ~60% reduced severity and amplitude of L-DOPA induced dyskinesia when calpain was inhibited by MDL28170 or roscovitine (measured by the percentage of asymmetry in the stepping and cylinder test) (13). While spectrin breakdown is not a very accurate measure of calpain activity, these findings combined suggest that calpain plays a role in
the neurodegeneration due to PD, and may play a much larger role in PD development and response to therapy than previously believed.

Figure 11 is a compilation of potential molecular mechanisms underlying LB formation from the literature and preliminary data. SNPs can play a role in PD pathogenesis. This is evidenced by the SNP in the gene encoding NADH dehydrogenase 3 decreasing susceptibility of PD in those with Caucasian ancestry (57). In contrast, two separate bioinformatics studies analyzing available clinical data compellingly suggest that SNPs in CAST play a role in the pre-disposition to develop idiopathic PD, although unconfirmed experimentally (2, 3). Liu et al proposed that PIN1 reduces calpastatin’s ability to inhibit calpain either through binding or isomerization, but a crude lysate pull down is not definitive.

Mitochondrial dysfunction leading to oxidative stress causes α-synuclein to fibrillize through nitration and carbonyl formation (62). Oxidatively modified α-synuclein is more likely to aggregate than unaltered α-synuclein (20). Other diseases, such as Huntington’s disease (HD), may have a similar mechanism. However, unlike PD where complex I is affected, HD develops reduced activity in complex II, III, and IV. This also increases the oxidative stress, suggesting a role for ROS in neurodegenerative disease (14). Mitochondrial dysfunction causes calcium dysregulation (seen by an increase in emission from the staining with Indo-1/AM dye) in PD, increasing the activation of calpain (seen through increased cleavage of a fluorogenic substrate) to cleave α-synuclein (25). This also occurs pathogenically in HD, where calcium signaling is affected in the
mitochondria, leading to increased calpain cleavage of the N-terminal end of the huntingtin protein (83).

Calpain cleaves α-synuclein in a variety of locations, which could have significance for aggregation (23, 38). α-Synuclein C-terminus is a negative regulator of the protein’s aggregation. When the C-terminus is cleaved by calpain between residues 122 and 123, there is a propensity of the protein to aggregate faster. It also acts as a nucleation site for more inclusions to form (observed through sedimentation analysis) (9, 23). It has also been shown through high-pressure liquid chromatography that calpain cleaves α-synuclein after amino acid 57 within the amphipathic region of the protein. This portion of α-synuclein is crucial to fibrillization (seen via ion-mobility mass spectroscopy), and the fragment is therefore less likely to aggregate (82). When calpain cleaves α-synuclein between residues 9 and 10 aggregation may be attenuated, but this has not been confirmed experimentally. The loss in aggregation may also be due to a loss in α-synuclein/synphilin-1 binding, as the 12 N-terminal residues of α-synuclein are necessary for this interaction. The change in aggregation due to cleavage by calpain at either end of α-synuclein suggests both a neuroprotective and neurodegenerative role of calpain in PD (38, 85).

PIN1 contributes to aggregation of the α-synuclein into LBs (31). This occurs through the binding of synphilin-1 at phosphorylated residues (illustrated by reciprocal co-immunoprecipitation of wild-type and non-phosphorylatable (control) forms of synphilin-1) (69). PIN1 increases the propensity of synphilin-1 to bind and aggregate with α-
synuclein’s N-terminal end (4). PIN1 is up regulated in human PD midbrain as well as in cell culture and animal models of PD. It has also been found in LBs by immunohistochemical staining. PIN1 increases α-synuclein aggregation in the N27 dopaminergic cell line induced with MPP⁺ (seen through immunohistochemistry). When cells are pre-treated with a PIN1 inhibitor, the aggregates do not form. This suggests that PIN1 is necessary for inclusion formation (31, 69). Synphilin-1 may increase α-synuclein’s aggregation through stabilization, increasing half-life and insolubility. Synphilin-1 inhibits the degradation of wild-type α-synuclein by the 20S proteasome, examined through a reduction in cleavage products compared to control at identical time points in gel electrophoresis (4, 31). Marx and colleagues recently discovered a mutation of R621C in synphilin-1 that may have caused PD in two un-related patients. However, given that the mutation is located in a region that has an unknown function separate of it’s binding with α-synuclein, this mutation could cause PD by another, unknown mechanism (52).

Further work is needed to confirm if PIN1 affects calpastatin. It would be necessary to promote the cis conformation of calpastatin through selective proteolysis of the trans conformer of calpastatin or in the presence of lithium trifluoroethanol, which promotes stabilization of the cis conformer. PIN1 activity also needs to be confirmed experimentally so that calpastatin could be pre-incubated with PIN1 under conditions in which it is active. This would allow a high amount of calpastatin present with PIN1, and dilution to a much lower concentration of calpastatin to inhibit calpain. At the same time, it would prevent calpain’s cleavage of PIN1 having an effect on the PIN1-calpastatin
interaction. A confirmation of the cleavage site would be necessary by Edmann degradation of the major cleavage product. If calpastatin still does not appear to have an effect, it would be prudent to examine if PIN1 and calpastatin bind directly. Indirect binding would require *in vivo* studies. The first step would be determination of PIN1 and calpastatin’s localization in the cell. Once localization has been confirmed, an induced increase in calcium may reveal clues as to the signaling pathways involved in the calpastatin-PIN1 interaction. To confirm PIN1 and calpastatin’s role in PD, knockdown or over-expression of either protein in a model may lead to novel insights.
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Figure 1: Protein map of α-synuclein, synphilin-1, and PIN1. (A) α-Synuclein: an unstructured, heat stable, charged protein of 140 amino acids. The N-terminal region of the protein contains an amphipathic region and a hydrophobic non-amyloid component (NAC) domain with six highly conserved repeats (KTKEGV), characteristic of all of the synuclein family members. It is followed by an acidic C terminus. Arrows indicate amino acid missense mutations causing familial PD. Adapted from Moore et al. 2005 (57). (B) Synphilin-1: a 919 amino acid protein with ankyrin-like repeats in its N-terminal portion, a coiled-coil domain, an ATP/GTP binding site, and a C-terminus containing a conserved region between 612-689 of unknown function. Insert shows an interspecies comparison of the amino acid region containing the missense mutation associated with familial PD. Adapted from Marx et al. (52) (C) PIN1: an 163 amino acid protein containing an N-terminal WW binding domain and a C-terminal catalytic peptidyl-prolyl isomerase (PPLase) domain. Adapted from Stanya et al. (77).
Figure 2: α-Synuclein Fibrillogenesis. Intrinsically unstructured monomers of α-synuclein are induced to oligomerize into protofibrils (spheres, chains, or rings) rich in β-pleated sheets. The structures fibrillize into amyloid-like filaments that deposit into Lewy bodies. Figure adapted from Moore et al. (57).
Figure 3: Calpain-2 structure with map and calpastatin map with sequence comparison. (A) Ribbon structure of calpain-2. Progression of the rainbow indicates N to C terminus. Sourced from PDB 1DF0. (B) Domain map of calpain-2: composed of a large subunit (~80 kDa), made up of domains 1 through 4, and a small subunit (~28 kDa), comprised of domains 5 and 6. Domains 4 and 6 contain penta-EF hand domains that bind to calcium. Calpain undergoes a conformational change after calcium binding, forming the active site cleft for substrate cleavage in domains 1 and 2. (C) Domain map of calpastatin: the 673 amino acid intrinsically unstructured protein contains a leader domain followed by four identical inhibitory domains. Each domain is able to inhibit one calpain and is composed of three subunits A, B, and C. Calpastatin subdomains A and C attach to the penta-EF hands, while the B domain blocks the active cleft of calpain in the presence of calcium (19, 36). (D) Calpastatin domain 1 amino acid sequence comparison between wild type (top line) and double phosphomimic (bottom line). Mutations of T135E and S243E bold and underlined. Subdomains bracketed in red.
Figure 4: Partial 6-OHDA lesion reduces dopaminergic innervation to the striatum and hippocampus and has no definitive connection to changes in Arc and pERK. (A) Quantification of TH immunoreactivity in the striata of sham-lesioned (sham) mice and 6-OHDA-lesioned (lesioned) mice. Data is calculated as percent of sham and shown as means ± SEM (n = 10 per group). Student t-test indicates a significant group effect (p < 0.0001). *** p < 0.0001 vs sham. (B) Quantification of TH immunoreactivity in the hippocampi of sham and lesioned mice. Data is calculated as percent of sham and shown as means ± SEM (n = 10 per group). Student t-test indicates a significant group effect (p < 0.0001). *** p < 0.0001 vs sham group. (C) Immunofluorescence images depicting TH-positive cells in the striata of sham and lesioned mice. (D) Immunofluorescence images depicting Arc and pERK-positive cells in the hippocampi of sham and lesioned mice.
Figure 5: **Visuo-spatial memory can be assessed using the Barnes maze.** (A) Image of the Barnes maze apparatus used. (B) Visual extra-maze cues used for spatial association with the location of the goal hole. (C) Latency of wild-type mice to find the goal hole in the Barnes maze over seven days. Data are calculated as time (in seconds) to find the hole, and are shown as means ± SEM (n = 14). Repeated measure (days of training) one-way ANOVA indicates a main effect of the time \( F(1,13) = 6.637, p < 0.0001 \).
Figure 6: Visuo-spatial memory can be assessed using the Spatial Object Recognition (SOR) test. (A) Arrangement of objects in training sessions 1-3 (S1-S3) and test session 4 (S4). (B) SOR test performed with wild-type mice 24 hrs after familiarization. Data indicate the time (in seconds) spent exploring the displaced object (DO) or non-displaced object (NDO) and are calculated as a ratio of the test session (S4) minus the last training session (S3) e.g. DO S4-S3; NDO S4-S3. Bars represent means ± SEM (n = 8). Student t-test indicates a significant group x object exploration (p < 0.012). ** p < 0.01 vs displaced object same group.
Figure 7: **Visuo-spatial memory is reduced in lesioned mice versus sham mice.** (A) Latency of sham-(sham) and 6-OHDA-lesioned (lesioned) mice to find the goal hole in the Barnes maze over nine days. Data are calculated as latency (in seconds) to find the hole, and are shown as means ± SEM (sham n = 8, lesioned n = 7). Two-way ANOVA with repeated measures (days of training) of time versus the group shows a significant interaction \[ F_{(8,8)} = 3.808, p < 0.05 \]. One-way ANOVA shows that both groups learned the position of the hole [sham: \( F_{(1,7)} = 9.088, p < 0.0001 \) and lesioned: \( F_{(1,6)} = 5.066, p < 0.001 \)]. (B) SOR test performed in sham and lesioned mice 24 hrs after familiarization. Data indicate the time spent (in seconds) exploring the displaced object (DO) or non-displaced object (NDO) and are calculated as a ratio of the test session (S4) minus the last training session (S3) e.g. DO S4-S3; NDO S4-S3. Bars represent means ± SEM (sham n = 8, lesioned n = 7). Two-way ANOVA indicates a significant effect of the lesioned (p < 0.0007) and between DO and NDO (p < 0.02). One-way ANOVA indicates a significant effect between DO and NDO within the sham group (p < 0.02), no significant interaction between NDO and DO in the lesioned group. There is no significant interaction between the sham and lesioned group (p < 0.07). * p < 0.05 vs displaced object same group, Fisher post-hoc test.
Figure 8: Phosphomimic (PM2) and wild-type (WT) calpastatin domain 1 (D1) possess a similar ability to inhibit calpain-2 in vitro. Inhibition of calpain-2 by PM2 calpastatin D1 (blue line) and WT calpastatin D1 (red line). Concentrations of calpastatin ranges from 0 to 100 nM, as shown. Data (n=1) calculated as percentage of calpain-2 activity after subtraction of the background from the average of duplicates at each concentration. Controls performed in triplicate.
Figure 9: Recombinant PIN1 is cleaved by calpain-2 in vitro. SDS-PAGE analysis of recombinant PIN1 digest by calpain-2 stained with Coomassie blue and amido black. Full-length recombinant PIN1 appears at 21 kDa (black arrow) incubated with calpain-2 for 0, 5, 15, 30, and 45 minutes (lanes 3, 4, 5, 6, and 7, respectively). PIN1 incubated without calpain-2 as a positive control for 0 and 45 minutes (lanes 1 and 9, respectively). Experiment performed with Nabeel Hashmi.
Figure 10: Recombinant PIN1 is cleaved by calpain-2 at the N-terminus in vitro. Western blot analysis of PIN1 digestion by calpain-2 using an anti-PentaHistidine horseradish peroxidase (HRP) conjugated antibody. Full-length recombinant PIN1 appears at 21 kDa. All samples loaded in duplicate at low (0.5 µg: odd lanes) and high (2 µg: even lanes) concentrations. Positive control (PIN1 incubated without calpain-2) at 0 minutes (lanes 1, 2) and 45 minutes (lanes 9, 10). Negative control (untagged casein) in lanes 5, 6. PIN1 digested by calpain-2 at 0 minutes (lanes 3, 4) and 45 minutes (lanes 7, 8).
Figure 11. Schematic of Potential Molecular Mechanism Underlying α-Synuclein Aggregation in PD. SNPs in the gene of calpastatin disrupt its ability to inhibit calpain. PIN1 isomerizes/binds to calpastatin, preventing its inhibition of calpain. Disrupted calcium signaling from the mitochondrial dysfunction activates calpain. Calpain cleaves α-synuclein at the C-terminus. Cleaved alpha-synuclein binds to synphilin-1 or forms fibrils. Oxidative stress from dysfunctional mitochondria produces reactive oxygen species (ROS), causing fibrillization of α-synuclein. Fibrils or complexes form aggregates. CAST = gene of calpastatin, α-SYN = α-synuclein, SPH-1 = synphilin-1.
AUTHOR’S BIOGRAPHY

Caitlyn Ahlberg (known as Cat) was born in Los Angeles, CA. She attended Venice High School until leaving to live in France for six months as a foreign exchange student at Lycée Scheurer Kestner. She returned to LA fluent in French, and studied at El Camino Real High School before graduating from Westchester High School. She then left for the University of Maine, Orono to study biochemistry with a minor in chemistry. She studied abroad again for a semester of her third year performing biomedical research at the Karolinska Institutet in Stockholm, Sweden, which sparked her interest in Parkinson’s disease. She has taught Organic Chemistry laboratory for three semesters and tutored in Calculus 2 and 3. Her interests are in cooking, swing dancing, and traveling.

Upon graduating, she plans on attending medical school to utilize her fantastic education from the University of Maine.