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Neurorestorative Properties of Cerebral Dopamine Neurotrophic Factor in Alpha Synuclein Pre Formed Fibril Model

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The diagnosis Parkinson's disease is most commonly given at the age of 50 but the patient might have had the disease already for years.
The disease is commonly graded by the severity of the symptoms and how it limits normal life. The main brain regions of interest in Parkinson's are striatum and substantia nigra. The latter has axonal connections to striatum with its dopaminergic neurons.

Lewy bodies (LBs) are thought as a hallmark in Parkinson's disease. They are aggregations from proteins. The main protein in the aggregates is called alpha synuclein (asyn). It is an abundant protein in neuronal cells. If the conformation of this protein is changed to a misfolded from through metabolic change called phosphorylation, it will acquire amyloid features. This means it will start to aggregate and turn other alpha synuclein proteins into the misfolded form. It has been shown that by injecting these misfolded proteins as pre formed fibrils (pff) of alpha synuclein it is possible to mimic the pathology of Lewy bodies in alpha synuclein.

Neurotrophic factors (NTFs) are small proteins that promote neuron survival and control the number of neurons in the nervous system. NTFS are promising candidates for treatment of PD. Cerebral dopamine neurotrophic factor (CDNF) is a novel trophic factor and it has earlier been shown to be neuroprotective and neurorestorative in mouse, rat and monkey Parkinson animal models and is current in clinical phase I/II patient trials.

Aim of this thesis work was to study the neurorestorative properties of CDNF against the phosphorylation and spreading of misfolded alpha synuclein by injecting alfa synuclein fibrils to rat brain. After the pathology has formed, the animals were injected with CDNF in two different concentrations. After two weeks the brains were immunohistologically stained and LB were counted from striatal area of the brain. Results between groups werecompared and analyzed statistically.

Keywords	CDNF, Neurotrophic factor, Parkinson's disease, Alpha synuclein

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Parkinsonin tautia sairastaa miljoonat ihmiset. Tauti saadaan diagnosoitua potilaille yleensä yli 50 vuoden iässä, vaikkakin potilas olisikin saattanut sairastaa tautia jo useita vuosia. Oireet luokitellaan usein asteittain niiden vakavuuden ja elämän rajoittavuuden mukaan. Nykyiset lääkkeet Parkinsonin tautiin pystyvät lievittämään oireita, mutta eivät korjaa jo taudin aiheuttamaa hermosolujen kuolemaa.

Tämän opinnäytetyön tarkoituksena on selvittää CDNF (conserved dopamine neurotrophic factor) -hermokasvutekijän hermosoluja korjaavia ominaisuuksia aggregoitunutta alfa-synukleiinia ja sen leviämistä vastaan. Tavoitteena oli löytää mahdollinen hoitomuoto Parkinsonin taudissa ilmenevää alfa synukleiinin aggregaatiota vastaan.

Lewyn kappaleita on pidetty keskeisimpänä merkkinä Parkinsonin taudin tunnistuksessa. Ne koostuvat aggregoituneista proteiineista. Valtaosa proteiineista on alfa-synukleiinia, jota löytyy normaalisti runsaasti hermosoluista. Jos alfa-synukleiini saa virheellisen konformaation fosforylaation kautta, se voi saada amyloidisia ominaisuuksia. Tällöin proteiini muuttaa muita naiiveja alfa-synukleiineja samaan virheelliseen konformaatioon ja nämä proteiinin kiinnittyvät toisiinsa eli aggregoituvat. Eläinkokeissa on osoitettu, että injektoimalla näistä aggregaateista rakentuvia alfa-synukleiini-fibrillejä aivoon voidaan matkia Parkinsonin taudissa esiintyvää Lewyn kappaleiden leviämistä.

Työssä rotille tuotetiin Lewyn kappaleita injektoimalla alfa-synukleiinifibrillejä ja jälkikäteen injektoitiin CDNF-hermokasvutekijää eri pitoisuuksissa tai suolaliuosta verrokkina. Kahden viikon jälkeen aivot kerättiin, leikattiin koronallisesti ja Lewyn kappaleet värjättiin immunohistokemiallisesti. Leikkeistä laskettiin Lewyn kappaleet ja eri ryhmien tuloksia verrattiin keskenään käyttäen tilastollisia menetelmiä.

Verrokki- ja hermokasvutekijäryhmien välillä ei havaittu tilastollisesti merkitsevää eroa. Tuloksesta selviää, että kyseisillä pitoisuuksilla ja injektioalueilla ei pystytä saavuttamaan haluttua estoa aggregaatioiden muodostumiselle.

Tämä tutkimus antaa jatkoa varten tietoa uusien koeasetelmien muodostamista varten. Aivoinjektioissa on useita muuttujia ja parametrejä, joita optimoimalla erot yksittäistenkin kokeiden välillä voivat olla merkittävät. Tutkimuksen tuloksia hyödynnetään seuraavissa kokeissa koetettaessa löytää hoitomuotoja Parkinsonin tautiin.

Avainsanat	CDNF, hermokasvutekijä, Parkinsonin tauti, alfa-synukleiini

Contents

List of Abbreviations

PD	Parkinson’s disease
Asyn	Alpha synuclein
PFF	pre-formed fibrils
LW	Lewy body
CDNF	Cerebral Dopamine Neurotrophic Factor
MANF	mesencephalic astrocyte derived neurotrophic factor
NTF	Neurotrophic Factor
STR	Striatum
SN	Substantia nigra

1 Introduction

Parkinson's disease (PD) is a neurodegenerative disease that typically affects people over 60 years old. In 2015, there was an estimated 117 400 deaths worldwide due to PD. It has been estimated that the disease currently affects roughly 6.2 million patients as of 2015. The current number of patients has doubled since 1990 and it has been estimated that by 2040 the amount of PD patients will be doubled again [1]. It is not fully understood what causes PD, though genetics and environment both play roles in the disease development. Currently, there is no cure for Parkinson's and the current treatment can only alleviate the symptoms to a certain extent.

In PD, the dopaminergic (dopamine producing) neurons of the substantia nigra pars compacta start to degenerate. When the total amount of dopaminergic neurons falls under 80% [2], the patient starts to show the first motor symptoms of PD [1,3]. In addition, at the later stages of PD a protein abundant in neurons, alpha synuclein (asyn), phosphorylates and starts to aggregate into large protein clusters with other proteins in the brain. These large protein clusters are called Lewy bodies (LB). It is not clear are these LB a result of, or a reason behind, Parkinson's, but as the disease progresses in most patients, the amount of LB increases and they spread to a larger area of the brain [3,4].

In this study, the aim is to find out whether the protein cerebral dopamine neurotrophic factor (CDNF) affects the phosphorylation of asyn. By creating a fibrillar form of asyn, referred to as preformed fibrils of asyn (pff), it is possible to create PD-like symptoms to a rodent brain or to cultured neurons and use it as a disease model in research. The current assumption is that CDNF has neuroprotective properties and can prevent the spread of phosphorylated asyn in the brain.

Although it is yet unknown how the interaction of CDNF and asyn take place and will CDNF at some level block the spread of asyn if they are introduced to the brain at the same time. In the Institute of Biotechnology, there have been unpublished studies where the time points of pff and CDNF introduction have varied to see whether CDNF works differently whether the pffs are introduced before or after it.

2 Theory

2.1 Parkinson's Disease

PD is a neurodegenerative disease in which neurons and their neuronal pathways begin to degenerate. Most common symptoms of the disease are change of posture, tremor of limbs, difficulty to initiate movement and shaking hands. In addition, PD may include many other symptoms that affect directly or indirectly the quality of a patients' life. In table 1 is shown most commonly known symptoms. They can be divided to non-motor and motor symptoms. Motor symptoms affect the functions of both the autonomic- and somatic nervous system, movement in other words, whereas non-motor symptoms, such as depression, constipation and loss of olfaction, include problems with cognition, behavior, and sensory [2,6,7]. These symptoms can vary individually from patient to patient. It is usual, for example, to have stiffness as one of the first symptoms and despite that, some patients never have stiffness as a symptom [8,9,10]. It is also important to recognize that these symptoms are common for many other diseases and disorders, therefore the clinical diagnosis of PD is difficult to produce.

Table 1 Most common PD symptoms [7,8]

Lack of posture and/or balance
Dizziness
Low or Soft Voice
Constipation
Stiffness or trouble with moving
Trouble sleeping
Trouble smelling certain foods
Small and/or unclear Handwriting
Tremor
Loss of automatic movement (blinking of eye, smiling etc.)
Bradykinesia (slowed movement)
Mood disorders
Cognitive changes (trouble keeping focus)

2.1.1 Pathology

The current method of diagnosis is to define all the symptoms that a patient has and exclude other possible diseases. Like some other neurodegenerative diseases, PD cannot be simply diagnosed with any known neuroimaging tools, whereas they can be used to exclude other diseases when a diagnosis of PD is considered. Typically, during the first clear symptoms of PD, the loss of dopaminergic neurons is close to 80% [2] and currently there is no treatment to gain back these lost neurons and their connections.

The loss of neurons and their connections is occurring specifically in dopaminergic neurons and their axons in a brain region called substantia nigra (SN), region is marked on figure 1. These neuronal cells are playing a major role as the producers of dopamine, a

neurotransmitter that transmits signals regarding motor actions throughout the nigrostriatal pathway [11]. Neuron can communicate to one another by neurites. These can either send messages as axons or receive them as dendrites. The connection point for axons and dendrites is called synapse. Sending neurons part of synapse is called presynaptic terminal and receiving end is called postsynaptic terminal. The area between is called synaptic cleft. Once the message comes to presynaptic end, it releases vesicles containing a neurotransmitter molecules to synaptic cleft, such as dopamine. Postsynaptic end has neurotransmitter specific receptors that pass the signal once a correct molecule binds onto them. Figure 2 shows a simplified version of how dopamine works as a chemical neurotransmitter in the synaptic region.

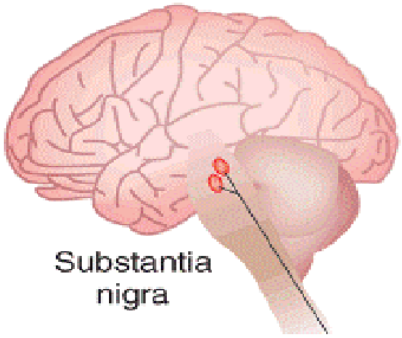

Figure 1. Location of substantia nigra in human brain. Edited from https://www.sciencenewsforstudents.org/article/explainer-nico-teen-brain Aug 11.2018

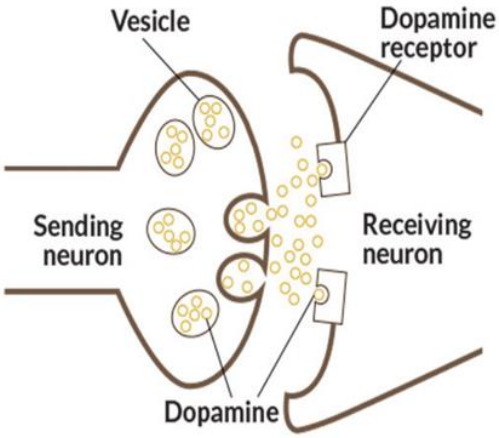

Figure 2. Simplified version of dopamine working as neurotransmitter. Once the sending neuron gets a signal to release dopamine vesicles, they spread into synaptic clench and from there dopamine attach to dopamine receptor. Upon activation, receptor passes on the signal[12] Neuroscience Gateway (May 2006) | :10.1038/aba1656 Aug 22.2018

2.2.2 Etiology

It is believed that both environmental and genetic factors play roles in the development of the disease. From environmental factors, head injuries, history of anxiety or depression, and pesticide exposure, seem to increase the risk or quicken the disease progression. In addition, it has been shown that drinking caffeinated beverages has a significant negative association in PD development. [12] Currently around 10 genes are known to associate with PD. There is no indication that a single gene or environmental factor is solely responsible for the disease, but rather a combination of factors. [13, 14]

2.2 Alpha Synuclein

Asyn has been found to be an abundant protein that is found in every brain region as well as in small concentrations throughout other tissues, such as muscle tissue. The gene that is responsible for asyn production is called SNCA. It is mainly localized in the presynaptic nerve terminals in neocortex, hippocampus, substantia nigra, thalamus and cerebellum. In figure 3 the localized high concentrations of asyn are shown in a monoclonal antibody staining in striatum, hippocampus, and substantia nigra.

Due to its nature, asyn binds to negatively charged phospholipids, such as cell membranes and to the neuronal mitochondria. These cellular asyn localizations have great variation depending on the region of the brain. All the properties of asyn are not known, but current studies suggest a role in regulation of dopamine release, working as a chaperone to assist folding and refolding of other proteins, movement of microtubules, and replenishing the levels of vesicles in the presynaptic terminals [13-18].

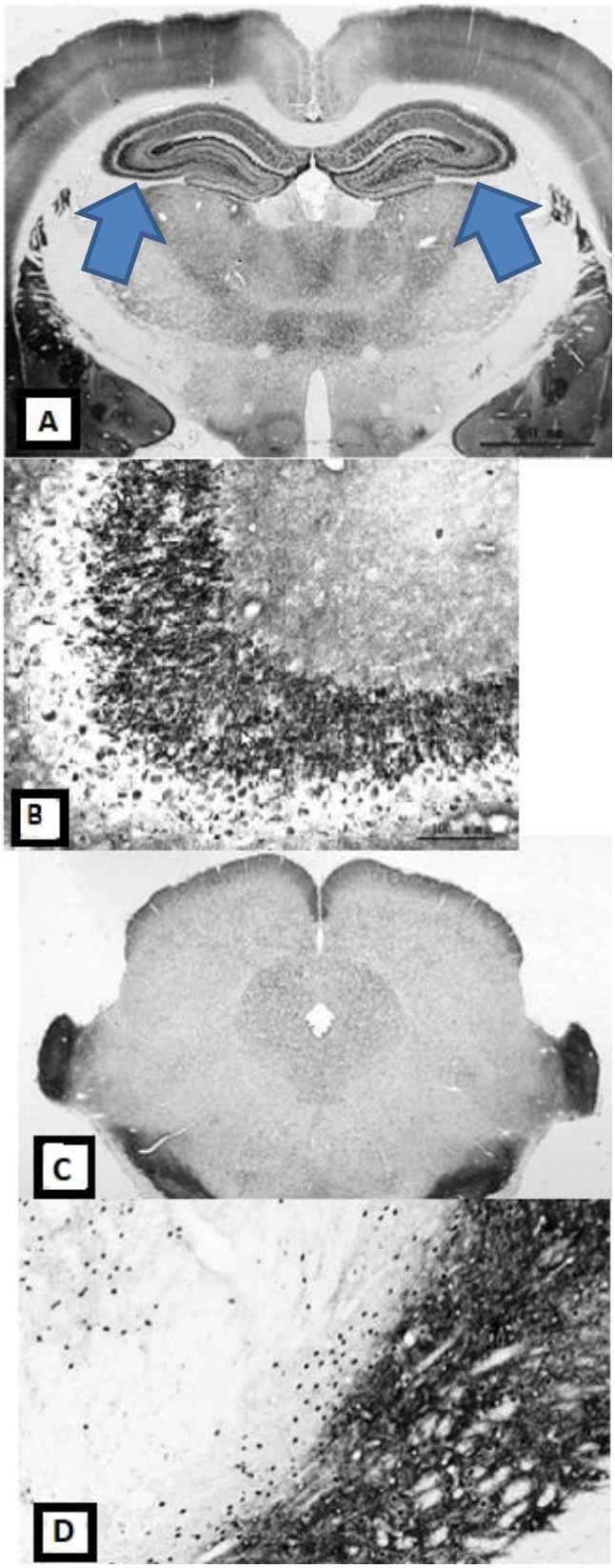

Figure 3. Localization of asyn in coronal rat brain section. A) hippocampus. B) closeup from hippocampal area. C) substantia nigra D) closeup from substantia. Pictures are modified from: Lesage S, Brice A (April 2009). "Parkinson's disease: from monogenic forms to genetic susceptibility factors". Human Molecular Genetics. 18 (R1): R48–59.

2.2.1 Alpha Synuclein Structure

In its soluble monomer form asyn does not have a singularly defined structure and so it can change depending on the surface it binds to [19]. A misfolded form of asyn is created by phosphorylation. It happens at the amino acid called Ser129 that transfers a covalently bound phosphate group from high-energy molecule ATP into asyn. Addition of a phosphate group into monomeric asyn can change its conformation and eventually affect its properties, such as solubility and binding of target receptors. In the case of asyn, Ser129 phosphorylation is behind nearly all aggregations and LB to the extent where it is used as a clear antibody marker to see asyn fibrils *in vivo* and in *in vitro* studies [19,20].

In other words, the protein misfolds into an aggregated form that does not complete the functions of the normal form of the protein. The aggregated form of asyn also has amyloid properties. It can convert other asyn proteins into the misfolded conformation and these aggregates can lock to one another in oligomeric pairs that will stack onto other pairs (figure 4, right). As a result, they form long column-like structures called fibrils that can be seen on left on figure 4. [19,20]

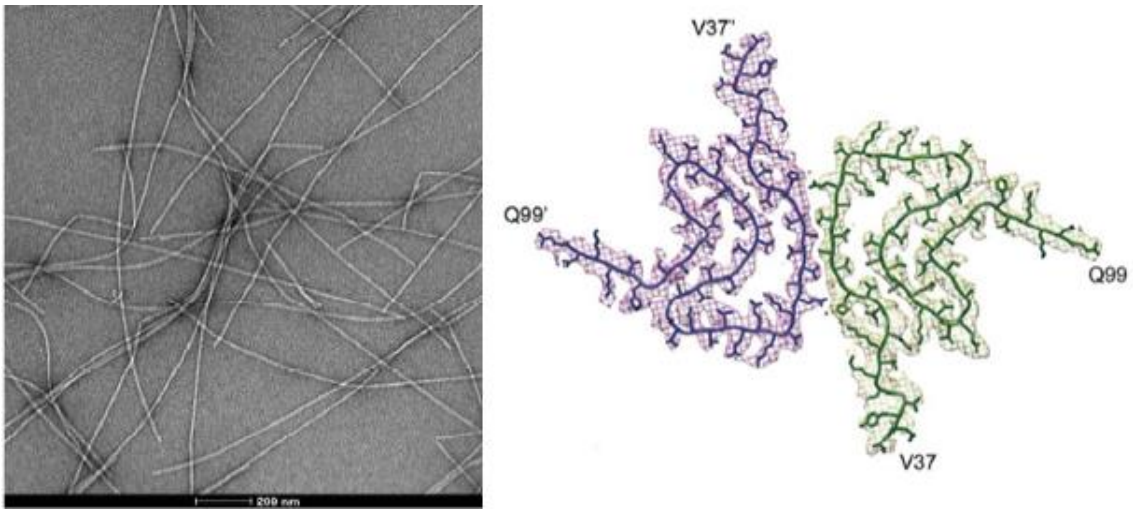

Figure4 **Left**: Transmission electron microscopy image of asyn fibrils. [20] **Right**: Atomic structure of two phosphorylated asyn oligomers. Pictures are from Filsy Samuel *et al* (2016) Effects of Serine 129 Phosphorylation on a-Synuclein Aggregation, Membrane Association, and Internalization

2.3 Braak Hypothesis

It has also been studied, and eventually Braak *et al.* created a hypothesis in 2003, that a pathogen, such as bacteria or virus, would be the cause of sporadic PD [22]. In this hypothesis, Braak's staging, the asyn aggregates can migrate from enteric nervous system that governs the function of the gastrointestinal track and the nasal cavity into the central nervous system [22,23]. Braak's staging is divided into six stages that each define the severity of asyn pathology spreading, starting from the peripheral nervous system as seen in figure 5.

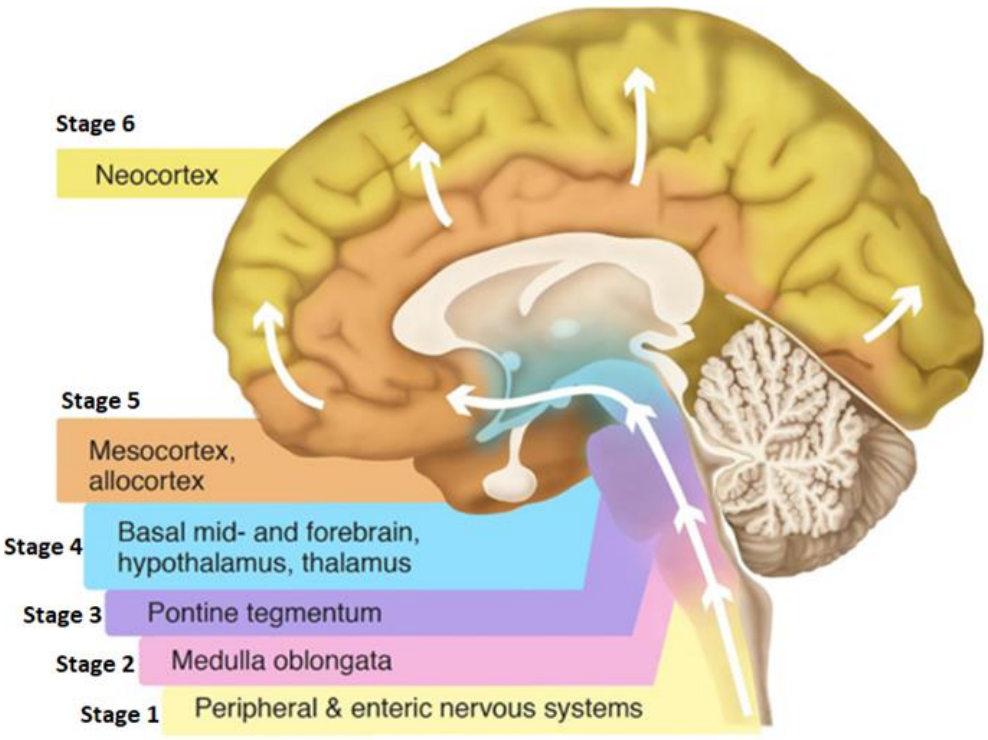

Figure 5: Stages of Braak model. According to it, the pathology is spreading in temporal pattern that ascends caudo-rostrally from the lower brainstem through the midbrain into the cerebral cortex. Picture modified from Noyce AJ, Bestwick JP, Silveira-Moriyama L, Hawkes CH, Giovannoni G, Lees AJ, Schrag A (December 2012). "Meta-analysis of early nonmotor features and risk factors for Parkinson disease"

2.4 Creating Alpha Synuclein Fibrils

The formation and spreading of asyn fibrils and LB can be achieved by injecting the fibrils into the brain. The fibrils will recruit the native asyn protein found in the brain tissue and cause aggregation, like that found in PD patient brains. It is also possible to produce these fibrils.

The common way to produce fibrils is to use viral vector-based models. By infecting a desired cell type with a virus vector that has coding to produce or overexpress asyn cells can be modified to produce soluble monomer asyn. Once an adequate amount of asyn has been produced, isolated and purified, they can be formed into fibrils by vortexing/shaking them for a week. By this time, the proteins have misfolded into an aggregated conformation and clumped together into fibrils as seen in figure 6 [24].

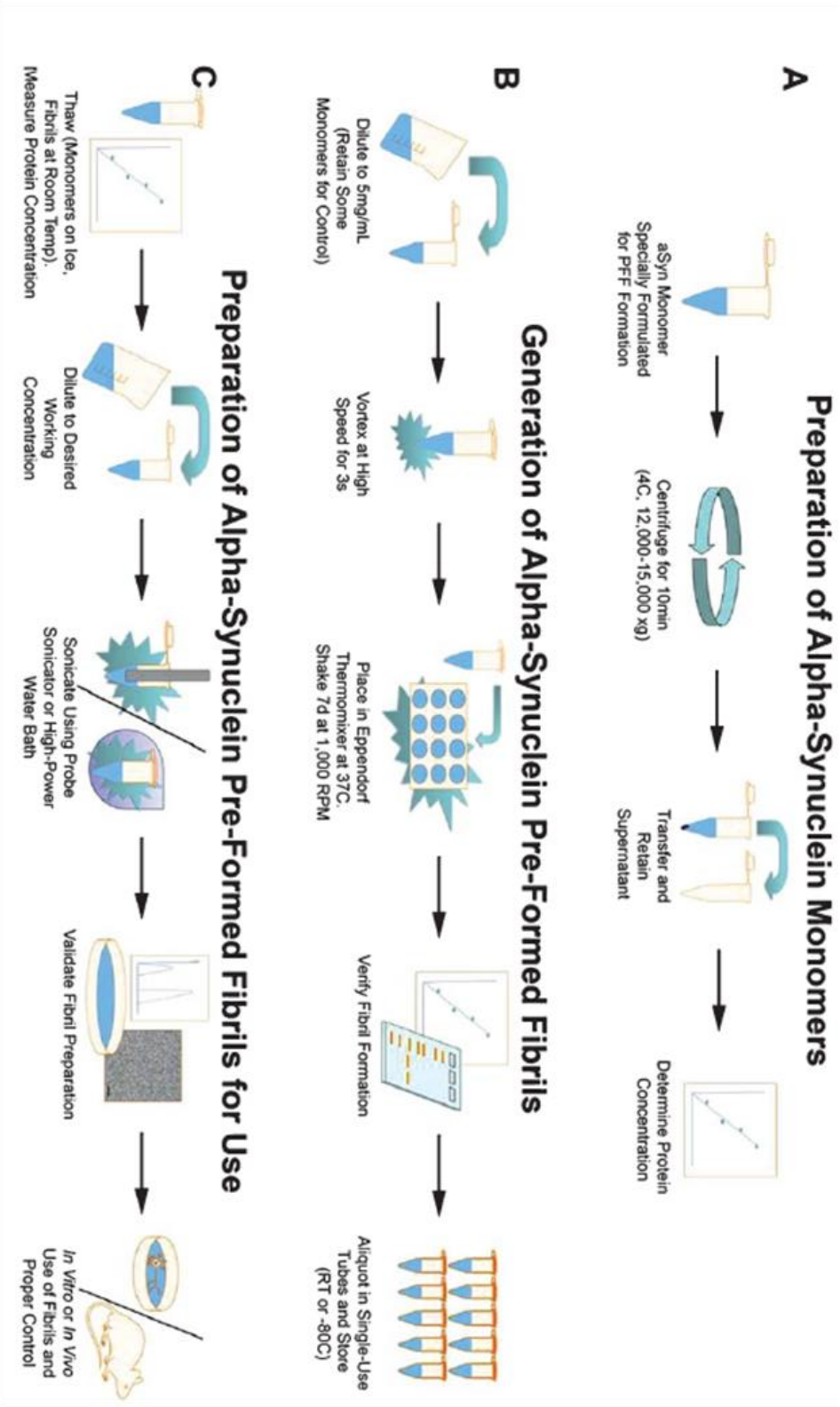

Figure 6: reparation, generation and using pre-formed asyn fibrils. Picture from Nicole K. et al (2008) Best Practices for Generating and Using Alpha-Synuclein Pre-Formed Fibrils to Model Parkinson's Disease in Rodents.

2.5 Asyn Models In Vitro Studies

In the case of media grown cells, asyn can be studied by generating induced pluripotent stem cells (iPSCs) that can be differentiated into desired cells, such as dopaminergic neuronal cells. Other option is to derive cells from animals (mouse neuroblastoma or dopaminergic neurons for example). In vitro studies provide an advantage compared to other models when the interest is at single cell or at cell-to-cell interactions. Conditions of the cell media can be easily altered and the cells themselves can be genetically manipulated for the study.

In the case of asyn phosphorylation, there are many tools for the in vitro research. As discussed earlier, the number of coding genes to produce asyn affects the amount of asyn produced and from there on the level of phosphorylated isomer of the protein. In vitro models the gene manipulation with transfections of the cells can be used to increase the number of asyn coding genes. In addition, it is possible to alter these asyn proteins so that their C-terminal end has an immunohistological tag. This way the identification and localization of monomer- and oligomer asyn are relatively easy to obtain. [25]

2.6 In Vivo Models

Animal models in general require a massive amount of work hours, money and time. In order to start in vivo studies the researcher needs be up to date with training, have a approval from supervising officials, have facilities that can provide good living conditions for the animals, and the list of requirements and responsibilities goes on. These requirements are listed in EU law and Finland has on top of that its own laws to make sure in vivo research provides the best possible care for the animals while still managing to get valuable data from the research at hand.

To understand how diseases function in complex system, such as in the mammalian brain, in vivo models are currently the only way to conduct studies. In the case of asyn phosphorylation, animal models provide insights, for example, how, where and in what time does the disease spread in the CNS. As established earlier, asyn expression can be increased, decreased or even stopped altogether (so called knock out animals). For PD, currently two models of 6-OHDA and MPTP are widely used in PD research, that both can specifically kill dopaminergic neurons in PD models. [26-28]

2.7 Neurotrophic Factors

In PD and other neurodegenerative diseases, the interest regarding neuronal protection has a huge focus now. With the current knowledge, neurotrophic factors (NTFs) seem to provide somewhat promising results against progressing degeneration. Currently there are four different neurotrophic factors in clinical trials in PD. [29]

NTFs are proteins that are secreted endogenous proteins. These proteins have multiple important functions regarding neuronal development, differentiation and maintenance of neurons. In this study, the focus is strictly in dopaminergic neurons that require their own NTFs whereas other regions and neurons need other NTFs for proper differentiation and normal cell function. More specifically, every NTF has a corresponding transmembrane receptor complex or complexes. In PD, it is known that specific NTFs can prolong the survival of dopaminergic neurons and give them some level of protection in toxin models in both in vitro and in vivo studies.

2.7.1 CDNF

In this study cerebral dopaminergic neurotrophic factor (CDNF, figure 7) was used. It belongs in to the same CDNF / MANF protein family with mesencephalic astrocyte derived neurotrophic factor(MANF).

Both CDNF and MANF are located and secreted in the endoplasmic reticulum(ER). The ER is a cell organelle responsible for synthesizing proteins, producing hormones and lipid metabolism. If ER receives a stress signal from accumulation of unfolded or misfolded proteins it will go into ER stress mode. This stress mode activates the unfolded protein response(URP) that tries to maintain cell homeostasis. If the state of stress is prolonged too long, it can result in cell death. [30]

The first comprehensive study about CDNF was published in 2007 by Lindholm *et al* in Nature. [32] For therapeutic standpoint, CDNF has unique dual mode of action by regulating UPR and ER stress intracellularly. Upon ER stress, it interacts with UPR regulating proteins, protects neurons from ER stress and can even rescue neurons from cell death induced by ER stress. [27.32-38]

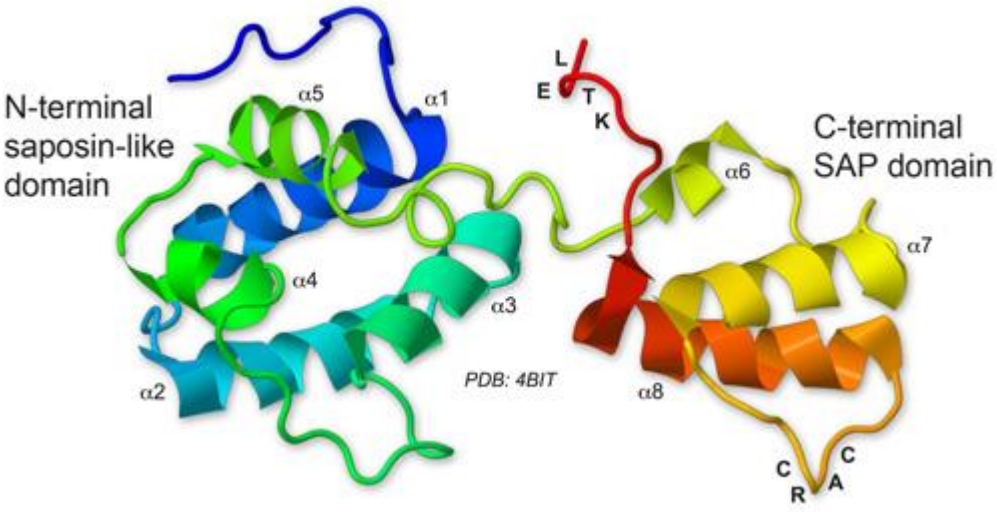

Figure 7 Molecular structure of CDNF. *CDNF has a unique sequence, 3D structure, mode of action and together with MANF forms a novel group of growth factors; Schematic illustration of the 3D structure of CDNF. Picture from: http://herantis.com/wp-content/uploads/2015/03/CDNF.jpg*

CDNF has been proven to have both neuroprotective and neurorestorative properties in mouse, rat and non-human primate models of PD. [29,31,32] This combination of functions makes CDNF promising NTF to study in aim for developing possible therapies for PD. [32] Cerebral dopaminergic neurotrophic factor is patented by Herantis pharma and is currently in clinical trials for PD. CDNF was discovered by professor Mart Saarma at Institute of Biotechnology, University of Helsinki 2003.

3 Experiment Set Up

This experiment was performed on male rats that were divided to three different treatment groups: control with 10 animals, low dose with 12 animals and high dose with 12 animals. All groups received 4 µl or pff divided to two regions of substantia nigra. Control received phosphate-buffered saline (PBS) with no proteins in it, low dose group received 1.5µg/day for 30 days of CDNF and high dose group 3.0µg/day. After the dosing, tissues were collected, stained and scanned. LB's were counted from substantia nigra.

3.1 Animals

In this study 1 year old male Wistar rats were used. All surgeries and behavioural assays were carried out at the University of Helsinki Laboratory Animal Centre. All animal experiments were approved by the Finnish National board of animal experiments (ESAVI/5459/04.10.03/2011 and ESAVI/7812/04.10.07/2015) and were carried out according to the European Community guidelines for the use of experimental animals.

3.1.1 Surgery

Surgery was completed under inhalation anesthesia. Gas mixture consisted of isoflurane and oxygen. Lidocaine was given as local anesthetic before the surgery and carprofen as anti-inflammatory and buprenorphine as morphine analog was given to relieve pain right after the surgery. Rats were injected with pre formed fibrils in two locations in striatum. Injections were preformed using WPI needles with 2 micro liters per site, flow rate being 0.5 microliters per minute and total volume of injected protein was 4 microliters. Stereotaxic coordinates for these injections were A/P +1.6, M/L -2.8, D/V -6.2 and A/P 0.0, M/L -4.1, D/V -6.2. Both relative to bregma.

After 1 month of pre formed fibril injections ALzet minipumps connected to plastic tubing and brain cannula were installed in the STR: A/P +1.0, M/L -2.8, D/V -5.0. Alzet minipumps released continuous infusion of PBS and CDNF for 30 days.

There were three groups for this experiment: vehicle with PBS injected in the minipump, low dose of CDNF and high dose of CDNF.

3.2 Tissue Collection

After the behavioural studies were done to analyze the effect of CDNF to asyn related motor impairments, rats were sacrificed. Tissues were collected by following general guidelines. Rats were euthanized with CO^2, brains were removed carefully and stored in 4% paraformaldehyde solution overnight in cold room at +4°C. Paraformaldehyde solution was changed daily to make sure equal diffusion to the whole tissue. After multiple days the brains were moved to 20 % sucrose solution overnight to be ready for sectioning.

3.3 Sectioning

Brains were cut at a Leica CM3050 cryostat into 40 µm coronal sections. Striatum and SN were collected in this experiment and both regions were divided into equal sets of six in a 24-well plate containing PBS. After the sections were collected, they were stored in 1 ml cryovials (CryoPure,Startedt) containing approximately 900 µl of (20% glycerol and 2% DMSO in PBS) cryopreservant solution at -20°C freezer. These kinds of sections are called free floating sections(figure 8). The name comes from the fact that the samples are floating freely on a solution after they have been collected.

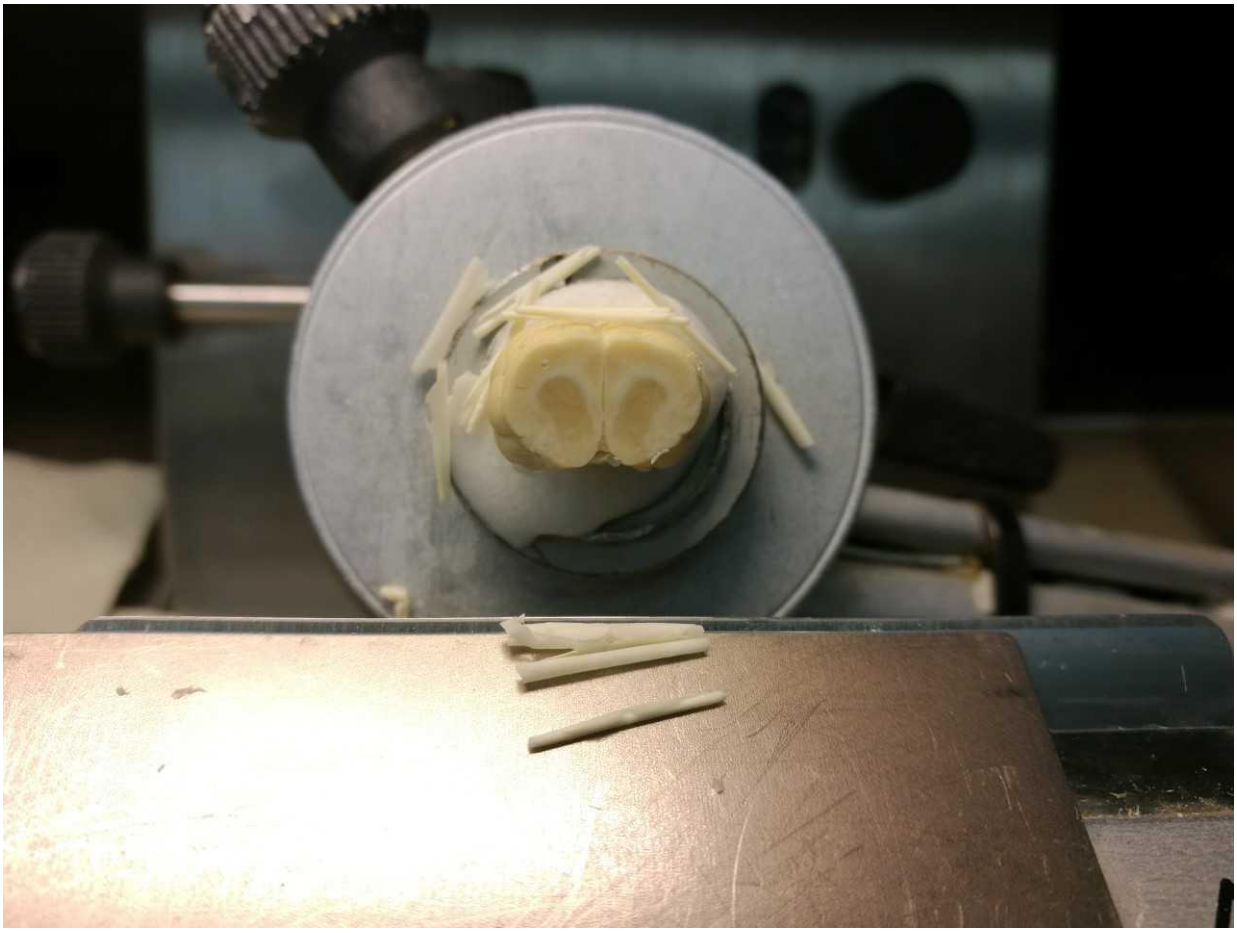

Figure 8 Brain sectioning process in cryostat.

3.4 Immunohistological Staining

Primary antibody used in this experiment was Anti-Alpha-synuclein (phospho S129) antibody [EP1536Y] (ab51253). This antibody was produced in rabbit, so the secondary antibody was anti-rabbit (VECTASTAIN Elite ABC HRP Kit (Peroxidase, Rabbit IgG)). Same Vectastain kit was used for Avidin/Biotinylated enzyme Complex(ABC). In figure 9 the use of antibodies is explained. Appendix 2 the protocol for staining is explained.

For the primary antibody to bind onto the corresponding antigen, it is necessary to incubate the primary antibody minimum of 8 hours. After the staining is completed the samples must be dried overnight and after the coverglass is mounted, the glue must dry overnight as well. Therefore, the steps are divided from day 1 to day 4.

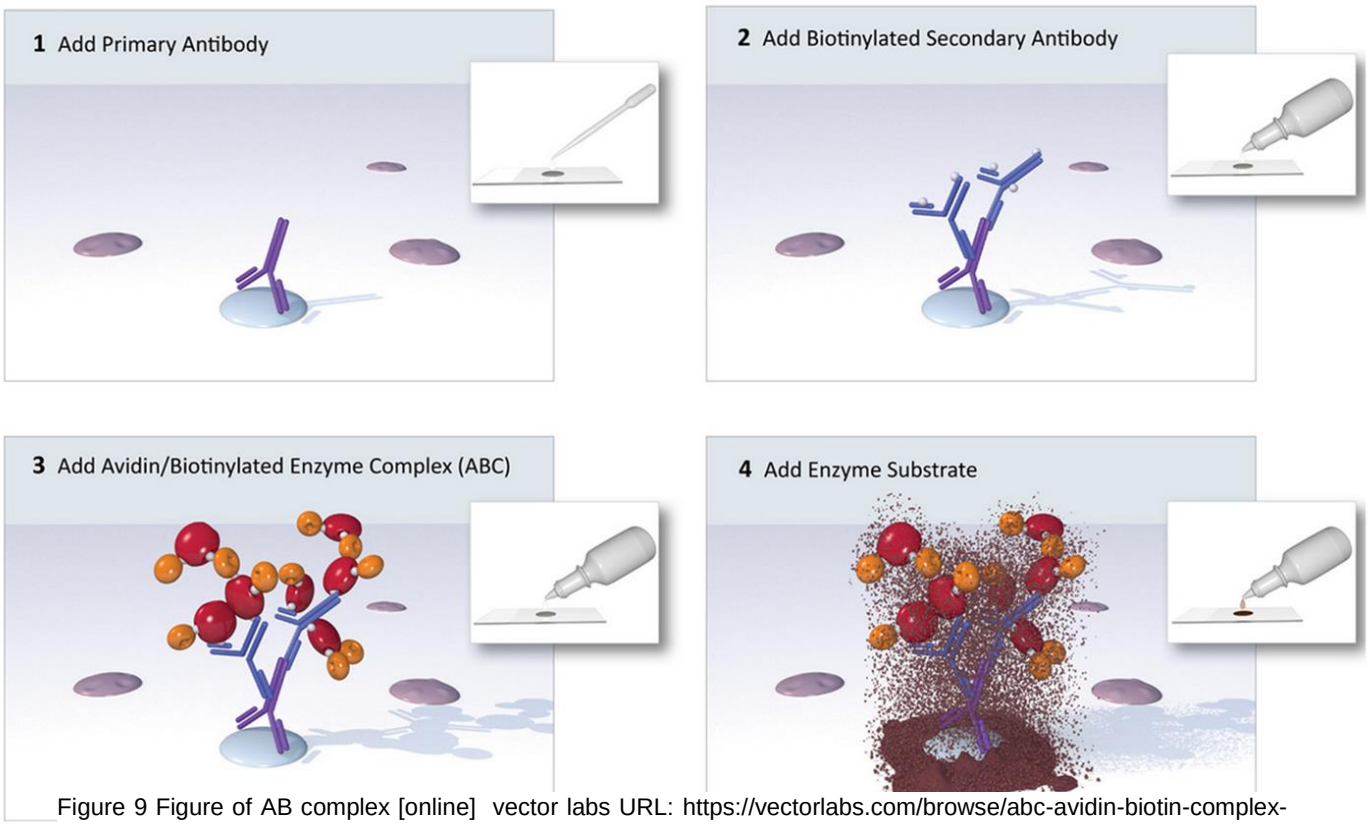

Figure 9 Figure of AB complex [online] vector labs URL: https://vectorlabs.com/browse/abc-avidin-biotin-complex-kits Nov 27.2018

3.5 Sample Analysis

Sample slides were scanned with 3DHISTECH scanner and analyzed by manual counting by a PhD student. Slides were scanned with single layer 20x magnification and CaseViewer software was used to analyze the sections. 5 to 19 sections were used from each brain from substantia nigra A/P -4.5 to 6.0 relative to bregma.

Sample analysis was done by comparing the number of LB's between animals that were given asyn pff + PBS, asyn pff + low dose CDNF and asyn pff + high dose of CDNF.

4 Results

Once the data was collected the data was analyzed with ANOVA- and other statistical tests to see whether the results had statistical differences. The results are shown in figure 10 and statistical analyses are shown in figure 11 (appendix 1 and 2). ANOVA is used to compare the differences of means among more than 2 groups. This is done by comparing the amounts of variation between the groups with groups own variation. Mathematical equation (1) for ANOVA is shown below.

$$x\,ij = \mu\,i + \varepsilon\,ij \quad (1)$$

Where *x* is a individual data point, *i*'s and *j*'s donate the group and individual observation, ε stands for unexplained variation and μ is population mean for each group. Main focus in ANOVA is significant P-value. It shows if one groups mean differs from other groups in a wanted level. In this test the P-value used was 0.05, so the test shows if the group differs from others in 95 % probability. In this experiment the groups did not differ, as seen in figure 11.

PBS	CDNF Low	CDNF High
Y	Y	Y
16.928570	6.285714	2.833333
7.333333	20.578950	32.571430
34.000000	16.888890	21.090910
34.666670	24.800000	31.000000
35.111110	19.555560	36.666670
48.125000	26.666670	25.142860
27.000000	24.875000	34.363640
47.200000	31.666670	17.800000
7.555556	38.700000	34.818180
33.777780	25.777780	21.444440
	40.125000	15.928570
	36.250000	6.857143

Figure 10. Lewy body counts for every animal. Groups are divided in columns.

Data sets analyzed	A : PBS
ANOVA summary	
F	0.6597
P value	0.5241
P value summary	ns
Significant diff. among means (P < 0.05)?	No
R square	0.04082

Figure 11 Results from statistical tests

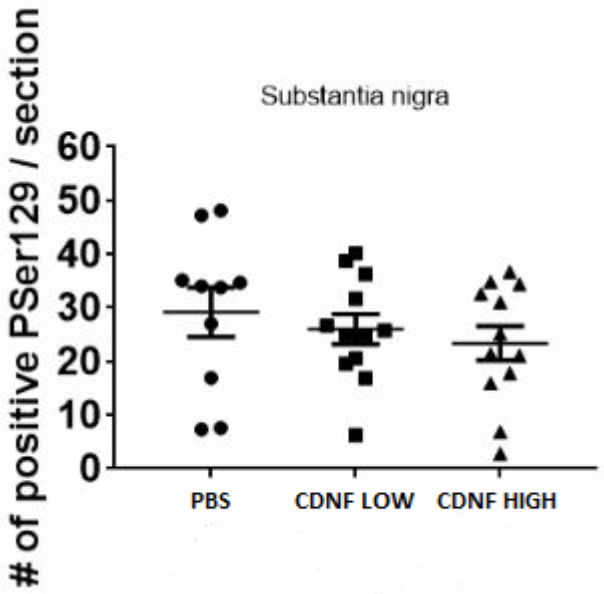

Figure 12. Results from LB counts. There was no statistical difference between PBS and CDNF-treated groups.

5 Discussion

The results do not show difference between the three groups. Based on this it would seem like CDNF does not have significant effect on pff formation and spreading. Although CDNF is currently in clinical trials for PD patients, further studies are needed to reveal, can CDNF reduce asyn aggregates and how it would affect asyn. Even if the idiopathic neurodegeneration could be controlled, the future damage from pff asyn spreading will pose a problem.

Current studies regarding PD and alpha synuclein are making it possible to understand more of neurodegenerative diseases and their pathology. Even though there is no simple answer, it seems that in future it would be possible to come up with a cure, therapy, or some sort of alleviation for PD patients.

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Figure 4 Pictures are modified from: Lesage S, Brice A (April 2009). "Parkinson's disease: from monogenic forms to genetic susceptibility factors". Human Molecular Genet-ics. 18 (R1): R48–59

Inclusion counts for groups

PBS	CDNF 1,5 µg/24 h	CDNF 3 µg/24 h
Y	Y	Y
16.928570	6.285714	2.833333
7.333333	20.578950	32.571430
34.000000	16.888890	21.090910
34.666670	24.800000	31.000000
35.111110	19.555560	36.666670
48.125000	26.666670	25.142860
27.000000	24.875000	34.363640
47.200000	31.666670	17.800000
7.555556	38.700000	34.818180
33.777780	25.777780	21.444440
	40.125000	15.928570
	36.250000	6.857143

Results from statistical analysis

Data sets analyzed	A : PBS
ANOVA summary	
F	0.6597
P value	0.5241
P value summary	ns
Significant diff. among means (P < 0.05)?	No
R square	0.04082

Staining protocol

Day 1

1. Wash samples 3 * 10 minutes in PBS solution in a shaker
2. Wash samples 30 minutes in 0.03 % H_2O_2 in PBS solution
    a. This is to remove the endogenous peroxidase enzyme
3. Wash samples 3 * 10 minutes in PBS solution in a shaker
4. Wash samples 1 hour in blocking solution
    a. Blocking solution is 4 % bovine serum albimun and 0.3 % of Tween 20 in PBS solution
5. Incubation in primary antibody solution overnight (o/n)

1/10 000 dillution of pSer129-antibody in blocking solution

Day 2

6. Wash samples 3 * 10 minutes in PBS solution in a shaker
7. Incubation in secondary antibody solution
    a. 1/200 secondary antibody diluted in blocking solution
8. Wash samples 3*10 minutes in PBS solution
    a. before the start of this wash make the ABC solution for step 9 by mixing one drop of both A- and B solutions in PBS and letting it incubate for 30 minutes (i.e. through step 8)
9. Incubate samples in ABC solution
10. Wash 3*10 minutes in PBS solution
11. DAB staining
    a. From DAB Peroxidase (HRP) Substrate Kit: 3 drops of buffer solution, 5 drops of DAB and 3 drops of H_2O_2 solution in 5 ml of MQ water. This solution is light sensitive, so the staining has to be done in dim light.
12. Wash 3*10 minutes in PBS solution
13. Place samples in glass slide and let dry o/n

Day 3

14. Dehydrate samples
    a. wash 30 seconds in 70 % EtOH
    b. wash 2* 1-2 minutes in 94 % EtOH
    c. wash 2* 2 minutes in absolute EtOH
    d. wash 2* 5 minute in xylene
15. Mount cover glass using coverquick 2000

a. apply few drops of coverquick 2000 on samples before the xylene evaporates
b. apply coverglass
c. press all air bubbles gently away from the samples

16. Let the slide dry o/n in ventilated space
17. Scrape excess glue
18. Store in appropriate casing