PROGRAMA DE DOCTORADO EN NEUROBIOLOGÍA

DOCTORAL PROGRAM IN NEUROBIOLOGY

ROLE OF INSULIN-LIKE GROWTH FACTOR 2 IN DEGRADATION AND SECRETION OF ALPHA-SYNUCLEIN AND ITS IMPACT IN DOPAMINERGIC NEURON SURVIVAL IN PARKINSON'S DISEASE MODELS

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ACTA DE APROBACIÓN

DEDICATION

A mi abuelo, Fernando.

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ABBREVIATURES

rAAV: recombinant adeno-associated virus

Aβ: amyloid-beta peptides

AD: Alzheimer's disease

AKT: Activated protein kinase B

ALS: Amyotrophic lateral sclerosis

aSyn: alpha-Synuclein

ATCC: American Type Culture Collection

CMA: Chaperone mediated autophagy

CV: Crystal violet

DA: Dopamine

DAergic: Dopaminergic neurons

DARPP-32: dopamine and cyclic AMP- regulated phospho protein, 32 kDa

Dpi: Days post injection

FBS: Fetal bovine serum

GABA: Gamma-aminobutyric acid

GSK3β: Glycogen synthase kinase-3β

HBSS: Hank's Balanced Salt Solution

HD: Huntington disease

HEK: Human embryonic kidney cell line

IGF1: Insulin-like growth factor 1

IGF2: Insulin growth factor 2

IGF1R: IGF1 Receptor

IGF2R: IGF2 Receptor

IGFs: Insulin-like growth factors

IR: Insulin receptor

IHC: Immunohistochemical

L-DOPA: Dihydroxyphenylalanine

LAG3: Lymphocyte-activation gene 3

LB: Lewy bodies

LDH: Lactate dehydrogenase

mHTT: Mutant huntingtin

MTT: 3-(4,5-dimethylthiazole2-yl)-2,5-diphenyltetrazolium bromide

NAC: Non-amyloid-β component

paSyn: Phopho-aSyn

PD: Parkinson's disease

PFFs: aSyn pre-formed fibrils

PI: Propidium iodide

PI3K: Phosphoinositide 3-kinase

SMA: Muscular atrophy

SNpc: Substantia nigra pars compacta

TH: Tyrosine hydroxylase

ThS: Thioflavin S

UPDRS: Unified PD Rating Scale

WB: Westernblot

WT: Wild type

RESUMEN

La enfermedad de Parkinson (EP) es el segundo trastorno neurodegenerativo más común después de la enfermedad de Alzheimer (EA). Está caracterizado por la pérdida de neuronas dopaminérgicas (DAérgicas) en una región específica, conocida como Sustancia Nigra (SN). La degeneración progresiva de esta patología genera el desarrollo de síntomas motores y no motores que se observa en pacientes que padecen esta enfermedad. Esta enfermedad afecta un gran número de personas a nivel mundial y no existe una cura definitiva en la actualidad. Se estima que 10 millones de personas en todo el mundo padecen la EP reflejando la alta prevalencia, mientras que la incidencia aumenta con la edad.

En términos neuropatológicos, las neuronas DAérgicas son particularmente vulnerables a la acumulación intracelular de la proteína alfa-synucleina (aSyn). La presencia de agregados intracelulares de aSyn interfieren con la función normal de las neuronas causando daño celular. Es por esto que han sido considerados como principal factor neuropatológico debido a que inducen toxicidad neuronal generando deterioro de los procesos fisiológicos celulares y conduciendo a una neurodegeneración que culmina en la muerte neuronal. Sin embargo, se han descritos mecanismos celulares que participan en la degradación y secreción de esta proteína, promoviendo la supervivencia neuronal DAérgicas.

Los tratamientos actuales a menudo se centran en aliviar los síntomas motores causados por la patología, sin embargo, las investigaciones actuales buscan nuevos tratamientos que puedan detener la progresión de la enfermedad.

En este contexto, los factores neurotróficos desempeñan un papel esencial en mantenimiento del sistema nervioso, promoviendo la supervivencia neuronal, plasticidad sináptica, crecimiento y función. Es por esto que los factores neurotróficos han sido foco de interés

científico durante décadas, debido a su potencial para el tratamiento de enfermedades neurodegenerativas como Esclerosis lateral amiotrófica (ELA), la EA y la enfermedad de Huntington (EH).

Basado en esto, el factor de crecimiento de insulina tipo 2 (IGF2, por sus siglas en inglés) ha sido propuesto como un candidato prometedor debido a su potencial efecto neuroprotector demostrado en estudios preclínicos de enfermedades como ELA, EA y EH. A pesar de que IGF2 no ha sido estudiado extensamente como otros factores neurotróficos, hay evidencia creciente que sugiere que IGF2 tendría un papel importante en la fisiopatología y también como potencial tratamiento para las enfermedades neurodegenerativas. Sin embargo, la contribución de IGF2 en la patología de la EP aún no ha sido dilucidada.

El objetivo de esta investigación es evaluar el efecto de IGF2 como tratamiento en la patología inducida por aSyn en modelos de EP. Específicamente, demostramos que el tratamiento con IGF2 disminuye la toxicidad celular, promueven la secreción extracelular de aSyn y previene la muerte neuronal. Experimentos que fueron validados en una línea celular humana de neuroblastoma y en cultivos de neuronas corticales primarias expuestas a las fibrillas preformadas (PFF) de aSyn.

Estos hallazgos se respaldaron con resultados obtenidos en experimentos *in vivo*, utilizando un modelo murino. Brevemente, con el objetivo de generar un modelo idiopático de la EP, se inocularon aSyn PFFs en la región dorsal del estriado de ratones de tipo salvaje (wild type, por sus siglas en inglés). Posteriormente, para evaluar el efecto de IGF2 en la patología de aSyn, desarrollamos una estrategia terapéutica para administrar IGF2 en el cerebro del modelo murino de EP. Como herramienta genética utilizamos virus recombinantes que fueron inyectados en la SN para incrementar los niveles de expresión de IGF2.

Basándonos en los resultados obtenidos, demostramos que el tratamiento con IGF2 disminuye significativamente los agregados de aSyn en neuronas del cuerpo estriado en comparación con los ratones control. Además, se evidenció que la administración de IGF2 previene la pérdida de neuronas DAérgicas provocada por la neurotoxicidad de aSyn en la región de la SN.

Interesantemente, el uso de IGF2 además demostró que contribuye positivamente en la disfunción sináptica de esta patología, donde se ha descrito una disminución en el número de espinas dendríticas, lo que resulta en una disminución de la conectividad sináptica en el sistema nigraestriatal. Observamos que IGF2 tiene un efecto significativo en la prevención de la pérdida de espinas dendríticas en la SN en modelo de EP.

Finalmente, la terapia preclínica basada en IGF2 mostró resultados favorables, mejorando el déficit motor observado en el modelo de EP, lo que sugiere una contribución neuroprotectora de IGF2 en la progresión de esta enfermedad neurodegenerativa.

Esta investigación evidencia el impacto positivo del tratamiento con IGF2 en modelos celulares e *in vivo* de la EP. Los resultados sugieren que IGF2 proporciona un efecto neuroprotector en el desarrollo de la patología de la EP.

Como futuras proyecciones, continuar con el estudio de los efectos neuroprotectores de IGF2, favorecería a ampliar el conocimiento y avanzar en el desarrollo de terapias basadas en factores neurotróficos. De esta forma, posicionamos a IGF2 como un blanco terapéutico prometedor para el desarrollo de nuevas terapias para la EP.

SUMMARY

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease (AD). It is characterized by the loss of dopaminergic neurons (DAergic) in a specific region known as the Substantia Nigra (SN). Progressive degeneration of this condition leads to the development of motor and non-motor symptoms observed in patients with this disease. This condition affects a large number of people worldwide, and there is no cure. It is estimated that 10 million people worldwide suffer from PD, reflecting its high prevalence, while the incidence increases with age. Neuropathologically, dopaminergic neurons are particularly vulnerable to the intracellular accumulation of the alpha-synuclein (aSyn) protein. The presence of intracellular aggregates of aSyn interferes with the normal function of neurons, causing cellular damage. For that, been considered a major neuropathological factor because they induce neuronal toxicity, leading to impairment of physiological cellular processes and ultimately resulting in neuronal death. However, cellular mechanisms have been described that participate in the degradation and secretion of this protein, promoting the survival of DAergic neurons.

Current treatments often focus on relieving motor symptoms caused by the disease. However, current research is seeking new treatments that delay the progression of the disease. In this context, neurotrophic factors play an essential role in the maintenance of the nervous system, promoting neuronal survival, synaptic plasticity, growth, and adequate function.

For this reason, neurotrophic factors have been a focus of scientific interest for decades, given their potential for the treatment of neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS), AD, and Huntington's disease (HD).

Based on this, IGF2 has been proposed as a promising candidate due to its demonstrated neuroprotective effect in preclinical studies of diseases such as ALS, AD, and HD. Although IGF2 has not been studied as extensively as other neurotrophic factors, there is growing evidence suggesting that IGF2 may play an important role in the pathophysiology and as a potential treatment for neurodegenerative diseases. However, the contribution of IGF2 in PD pathology has not yet been elucidated.

The aim of this research is to assess the effect of IGF2 as a treatment in aSyn-induced pathology in PD models. Specifically, we demonstrate that IGF2 treatment reduces cellular toxicity, promotes the extracellular secretion of aSyn, and prevents neuronal death. These findings were validated in a human neuroblastoma cell line and in primary cortical neuron cultures exposed to a Syn preformed fibrils (aSyn PFFs) of aSyn.

These findings were supported by results obtained in *in vivo* experiments using a murine model. Briefly, to generate an idiopathic model of PD, aSyn PFFs were injected into the dorsal striatum region of wild-type (WT) mice. Subsequently, to assess the effect of IGF2 on aSyn pathology, we developed a therapeutic strategy to administer IGF2 in the mouse brain. Recombinant viruses were used as a genetic tool, and they were injected into the SN to increase IGF2 expression levels.

Based on the obtained results, we demonstrated that IGF2 treatment significantly reduces aSyn aggregates in striatal neurons compared to control mice. Furthermore, we observed that the administration of IGF2 prevents the loss of DAergic neurons induced by aSyn neurotoxicity in the SN region.

Interestingly, the use of IGF2 also showed a positive contribution to the synaptic dysfunction in this pathology. A decrease in the number of dendritic spines has been described, resulting

in a reduction in synaptic connectivity in the nigrostriatal system. We observed that IGF2 has a significant effect in preventing the loss of dendritic spines in the SN in the PD model. Finally, IGF2-based therapy showed favorable results in improving the motor deficits observed in the PD model, suggesting the contribution of IGF2 to the progression of this neurodegenerative disease.

This research demonstrates the positive impact of IGF2 treatment in cellular and in vivo PD models. The results suggest that IGF2 provides a neuroprotective effect in the development of PD pathology.

As future projections, continuing the study of the neuroprotective effects of IGF2 would contribute to expanding knowledge and advancing the development of therapies based on neurotrophic factors. This study position IGF2 as a promising therapeutic target for the development of new therapies for PD.

INTRODUCTION

1.1 Parkinson's Disease

"*Involuntary tremulous motion with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward and to pass from a walking to a running pace; the senses and intellect being uninjured*" was the first description used by James Parkinson in 1817 to describe this disease, which the called "shaking palsy" by the Latin term "*paralysis agitans*" after a detailed observation of six cases with the clinical features (Reviewed in Parkinson, 2002; Polymeropoulos et al., 1997). Seventy years later the neurologist Jean-Martin Charcot excluded resting tremor symptom and introduced the notion of muscular rigidity, then he suggested the name of the disorder as PD (Fahn, 2003; Gelfand, 1989).

PD is recently being considered not only as a motor disorder but also as a systemic neurodegenerative disease due to embraces a broad spectrum of motor and non-motor manifestations which are presenting and progressively worsening with time affecting the quality of life of the patients (Gökçal et al., 2017).

Since the characteristic symptoms of this disease are related to motor deficits, PD is classified as the most prevalent movement disorder worldwide and the second most prevalent neurodegenerative, chronic, and progressive disease after AD, begin the prevalence in men higher than in women (Aarsland & Kurz, 2010; Ding et al., 2022; Tanner & Goldman, 1996).

From this, the pathology is considered as a multifactorial disorder, epidemiology is composed by genes and environment factors contributing to risk of suffering PD.

PD affects 1% of the people over 60 years old in industrialized countries and more than 4% of the population over 85 years (de Rijk et al., 2000; Reeve et al., 2014). The project number in most populous countries is expected to increase the number of PD cases, from 4.1-4.6 million in 2005 to 8.7-9.3 million by 2030 (Dorsey et al., 2007). To date, 10 million people worldwide suffering from this disease with no cure, while incidence increases with age. In Chile, there are around 40.000 people affected with this disease (Chaná C et al., 2013).

Regarding etiology of PD, is subdivided into two groups depending on the origin or cause of the disease. Above 15% of people with PD is represented by genetic PD or familial cases, which are mainly composed of mutations in more than 20 genes (such as, SNCA, PINK1, PARK7, PARK2, LARK2) (Blauwendraat et al., 2020; Funayama et al., 2023)**.** Sporadic or idiopathic cases are 85% proximately, lacking a hereditary genetic component. In this case, intracellular accumulation of proteins within neurons called Lewy bodies (LB) is the principal histopathological characteristic for sporadic cases. In addition, in idiopathic PD, aSyn was described as the major protein component of the LB, and one of the major proteins related to this pathology (Spillantini et al., 1997a). Also, LB have been observed in post-mortem brain tissue of PD patients, defining the pathological characteristics of vulnerable neurons, and causing neurodegeneration (Simon et al., 2021; Spillantini et al., 1997b). The main component of sporadic diseases are environmental factors, which in turn interact with genetic components that are present, thus these pathologies are described as multifactorial but of unknown origin (Ball et al., 2019a; De Miranda et al., 2022; Lill, 2016; Warner & Schapira, 2003).

Also, environmental factors such as the exposure to pesticides are thought to play a crucial role in disease onset and has been described as risk to develop PD pathology. Further, chronic exposure to metals and pesticides is also associated with an early onset of PD in

patients without genetic family history of PD being the severity of disease a dose-dependent to exposure through the time (Ascherio et al., 2006).

Epidemiological studies from 1990s accumulate evidence showing a genetic component in PD cases. Several autosomal dominant and recessive mutations have been described showing high penetrance in PD (Klein & Westenberger, 2012; Lesage et al., 2020).

Then, researchers have developed different models that recapitulate the pathological characteristics of PD aiming to develop novel treatments that can either delay disease progression or alleviate its symptoms (Lotharius & Brundin, 2002).

PD is the major cause of parkinsonism. PD is defined by a combination of six motor symptoms: resting tremor, bradykinesia (slow and decreased amplitudes of movement), loss of postural reflexes, rigidity, freezing or akinesia (difficulty to initiate movements), and flexed posture (Jankovic, 2008; Moustafa et al., 2016a)**.** In other hand, a broad spectrum of non-motor symptoms presented in PD patients involved several dysfunctions such as autonomic disorders (constipation and urogenital dysfunction), and impairment of olfaction as well. Also, mood disorders like depression (50% of cases) (Shulman et al., 2001), anhedonia, apathy, memory complaints, cognitive decline (30-40% of cases), and the most frequent, sleep disorders called hyposmia or rapid eye movement sleep behavior disorder (RBD) (Poewe, 2008; Tandberg et al., 1998)**,** as well as complex behavioral disorders. Several of these non-motor features often can precede the onset of motor symptoms dysfunction at the moment of it diagnosis (Siderowf & Stern, 2008; Tolosa et al., 2007)**.** For example, constipation, sleep disorders and daytime sleepiness are frequently perceived more than 10 years before motor symptoms. In this context, this phenomenon could provide clues to early symptoms for early diagnosis of PD (Schapira et al., 2017).

Unified PD Rating Scale (UPDRS), is a rating tool, based in a score, used to gauge the severity and progression of PD. Commonly, neurologists use UPDRS for evaluating the response to medications used to decrease the signs and symptoms of PD (Goetz et al., 2008; Kulisevsky et al., 2013; Martínez-Martín et al., 1994; Martinez-Martin et al., 2015). Also, the motor section of UPDRS score is linearly associated to neuronal density and neuronal loss observed in PD pathology (Greffard et al., 2006).

DAergic neurons are part of the nigrostriatal circuit which has been associated with the regulation of movement (Bourdy et al., 2014). The degeneration of the DAergic neurons in SN is considered to disrupt neurotransmission in the basal ganglia circuitry, begin the basis for several movement disorders and therefore underlying the main motor symptoms of PD. The flow information through the basal ganglia come from the striatum and back to the cortex by two pathways, this are the direct and indirect pathways (Calabresi et al., 2014; Wang et al., 2015).

The basal ganglia are a variety of five subcortical areas engaged primarily in motor control, together with a variety of roles such as motor learning, executive functions and behavior, and emotions. These five interconnected areas include, caudate nucleus, putamen, globus pallidus, SN and subthalamic nucleus (Lanciego et al., 2012).

Regarding the PD neuropathology, vulnerable DAergic neurons are mainly founded in SNpc of the midbrain, the progressive degeneration and loss of this neurons is one of the two criteria required to confirm the diagnosis of PD (Agid, 1991; Postuma et al., 2016). However, not all DAergic neurons are equally vulnerable. The DAergic neurons that project their axons to the putamen in the middle of the brain, an area associated with motor circuits, are more vulnerable than those that project their axons to cognitive areas. The second pathological

criterion is the presence of aSyn-positive inclusions accumulated in LB in neurons (Double, 2012). Although the main hallmarks of PD are well described, there are no techniques that allow an early diagnosis before the classic clinical features occur. In fact, the motor symptoms in PD patients are observed only when 70% of the striatal DAergic neurons are lost and half of the DAergic neurons in the whole brain have been degenerated (Fu, Hardy, and Duff 2018; Obeso et al. 2017).

Nigrostriatal neurons produce dopamine (DA) which is an important neurotransmitter that plays a critical role in controlling and coordination of movements. DA biosynthesis occurs from the amino acid tyrosine by action on the enzyme tyrosine hydroxylase (TH).

Progressive neurodegeneration of DAergic neurons, lead to a decrease in DA release disrupting the nigrostriatal pathway, impacting the regulation of movement and giving rise to the principal motor symptoms observed in patients. Following the loss of DAergic neurons and the subsequent decrease in DA levels, the brain attempts to compensate for this deficiency of neurotransmitter by increasing the density of postsynaptic DA receptors (Figure 1).

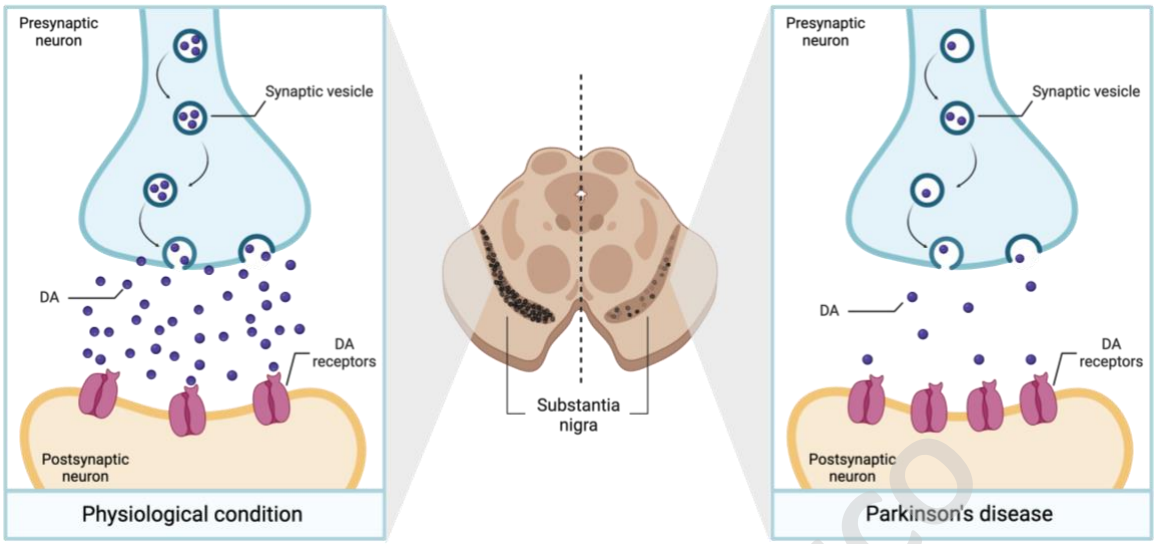


Figure 1: Loss of neurotransmitter DA in nigrostriatal system in PD pathology. representation of neurotransmitter dopamine release in neurons of the SN. Under physiological conditions (*left*) dopamine is transported into synaptic vesicles to the membrane. Vesicles are fused to the membrane leading to neurotransmitter release, which bind to DA receptors in postsynaptic neuron. In pathological conditions (*right*), degeneration of DAergic neurons result in a depletion of the neurotransmitter in the synaptic terminals leading to neuronal loss in PD.

1.2 Alpha-synuclein in Parkinson's disease

Polymeropoulos *et al.* in 1997 described for the first time that mutation in SNCA gene was linked to autosomal dominant form of PD in an Italian family and three unrelated Greek families (Polymeropoulos et al., 1997).

The implication of aSyn in both familial and idiopathic PD cases has made it the principal target to understand the mechanisms of PD and the elucidation of new therapeutic approaches.

aSyn was found to be the main component of the neuropathological hallmarks of PD in both familial and idiopathic PD cases and its accumulation is a key determinant of DAergic

neuronal degenerative susceptibility in PD. Most significantly, mutations in the SNCA gene, which encodes for aSyn protein, are responsible for PD in rare familial cases, and the aggregated protein is a major component of LB found in sporadic PD. Aggregation of misfolded proteins is a feature common to many neurodegenerative diseases and therapeutic approaches for these pathologies are based on treatments for reduced protein accumulation on brain patients (Ellis & Fell, 2017; Ross & Poirier, 2004).

The aSyn-positive deposits are present in brain patients affected by others neurodegenerative diseases such as dementia with LB, Multiple System Atrophy (MSA), and AD. The role of aSyn in all these disorders, referred as *Synucleopathies,* enhances the importance of aSyn accumulation in neurodegeneration (Baba et al., 1998; Ball et al., 2019b). Physiologically, aSyn has been found in neuronal cells mostly localized in the pre-synaptic terminals binding with high affinity to the membranes of synaptic vesicles, suggesting a function for aSyn in the synaptic homeostasis (Burré, 2015; Burré et al., 2010, 2018). Despite being a predominantly neuronal protein, aSyn is also found in glial cells and outside the central nervous system (CNS) (Booth et al., 2017). Studies demonstrates that aSyn knockout mice ($Snca^{-/-}$) are viable, fertile and their brain morphology is normal, which proves that aSyn is not essential to neuronal development suggesting that other proteins can compensate the absence of aSyn. However, mice lacking aSyn exhibit a significative reduction in striatal DA levels, demonstrating the implications of aSyn in regulation of synaptic pathways in nigrostriatal system (Abeliovich et al., 2000)

Physiological condition of monomeric aSyn can misfold into conformations rich in β-sheet structure that are proper to establish hydrogen bonds through specific side chains, generating oligomers and high-complexes structures as fibrils that lead to formation of intracellular inclusions, the LB and LN (Lewy neurites) (Cremades, Chen, and Dobson 2017).

Several studies described aSyn-mediated mechanisms involved in neuronal toxicity. Cellular mechanisms and organelles are affected by aSyn toxicity leading contributing to neurodegenerative environment. For example, impairment in protein degradation systems such as ubiquitin–proteasome system (UPS) and lysosomal systems including autophagy, synaptic and mitochondrial dysfunction, axonal transport disruption and nuclear dysfunction (Cookson and van der Brug 2008; Paumier et al. 2015; Wong and Krainc 2017).

Several genetic and idiopathic models have been described to study pathology of PD through aSyn toxicity, which provides valuable insights into the mechanisms underlying the disease progression. One established in vitro model involves the generation of aSyn PFFs, utilizing both murine and human species of aSyn to recapitulate the pathology. The feature of this protein that has attracted the most attention is its ability to aggregate *in vitro,* through a sequence of conversion from a natively unfolded monomeric form to an oligomeric prefibrillar form, also called proto-fibril, and finally to a fibrillar form (Figure 2) (Vasili et al., 2022a).

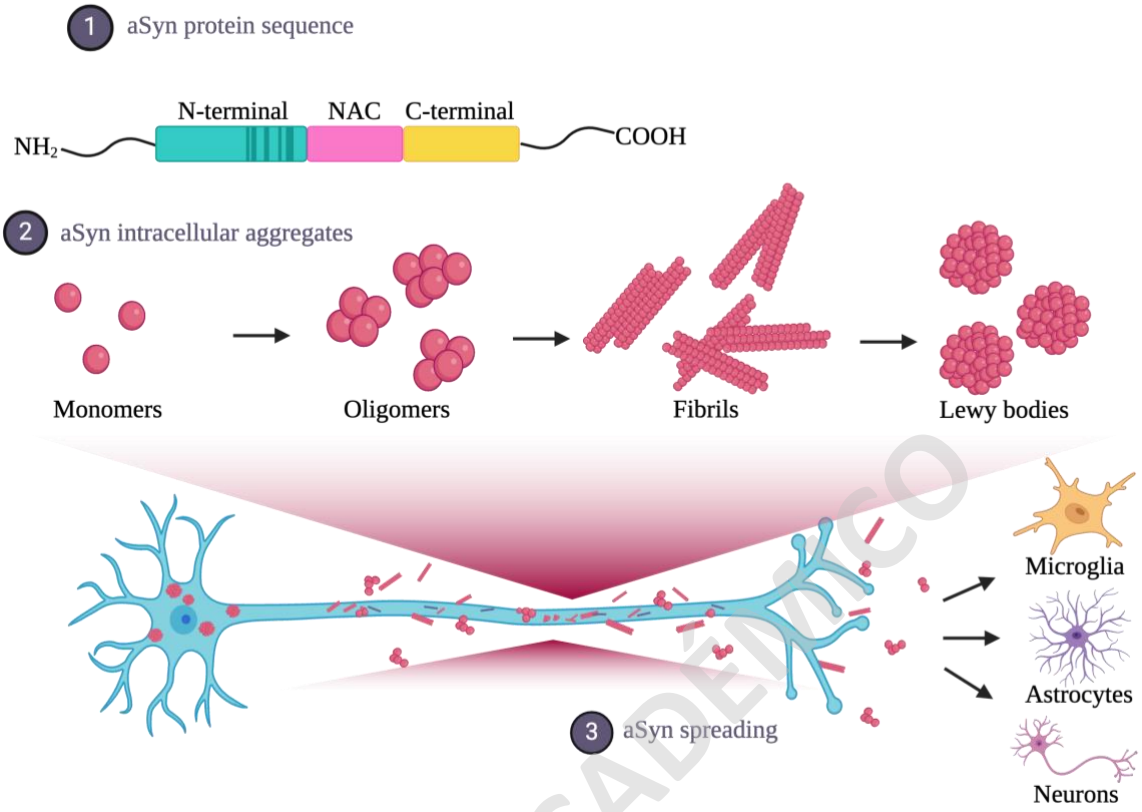


Figure 2: Physiological condition of aSyn and its intracellular aggregates formation. Schematic representation of native structure of aSyn protein which is composed by 140 aminoacids. (1) The N-terminal region, the non-amyloid-β component (NAC) region and the C-terminal region are represented in calypso, pink and yellow colors, respectively. (2) aSyn is founded in monomeric conformation that can misfold into more complex structures like oligomers and fibrils that deposit into larger aggregates, ultimately forming intracellular inclusions called LB. aSyn leads to toxicity and consecutive neurodegeneration when aggregated into pathological oligomers, fibrils, and LB. (3) aSyn PFFs are internalized by neighbor cells such us glia and neurons contributing to the spreading and pathology of aSyn.

Accumulating evidence from studies *in vitro* suggests that aSyn is secreted, allowing its spreading between cells (Brahic et al., 2016; Danzer et al., 2012a; Freundt et al., 2012). It has been demonstrated that aSyn propagates between neurons and can also spread from neurons to glial cells, such as astrocytes and microglia (Choi et al., 2018; Hansen et al.,

2011b; Lee et al., 2005). The astrocytes are able to internalize aSyn by endocytosis, and studies have shown that aSyn is founded within the lysosomes suggesting that astrocytes have an important role in aSyn removal and degradation, potentially maintaining a healthy environment (Gee & Keller, 2005).

Several mechanisms by which aSyn can spread between cells have been proposed. One pathway is by the aSyn secretion via nonclassical exocytosis to the extracellular space, followed by its internalization of neighboring cells (Fussi et al., 2018; Loria et al., 2017; Mahul-Mellier et al., 2015). Organelles like late endosomes participating in the release of misfolded proteins, such as aSyn, through exocytosis via exosomes have been identified (Lee et al., 2005).

In the field of PD investigation, researchers have developed cellular and animal models that mimic pathological features of PD, in order to expand the understanding of the biological basis and, consequently, develop therapies for treatment of PD.

It has been shown that exogenous aSyn PFFs promote the aggregation of endogenous aSyn, increasing its pathogenicity and affecting vulnerable neurons (Vasili et al., 2022b). Also, it has been demonstrated that in primary neuronal cultures from WT mice exposed to aSyn PFFs, exogenous aSyn induces the recruitment of endogenous monomeric aSyn into phosphorylated aggregates (paSyn), which subsequently spreads throughout the neurons, triggering neuronal degeneration and cell death. Also, Luck, *et al*. demonstrated the aggregates formation in axons and somatodendritic region by 10 days post aSyn PFFs incubation and consequent death by 14 days after incubation in primary cortical neurons (Volpicelli-Daley et al., 2014a).

Other important characteristic that contributes to aSyn toxicity in these models is the release and transmission of pathological aSyn species neuron to neuron *in vitro.* Using microfluidic devices in primary neurons cultures researchers has been demonstrating that fluorescent labeled aSyn PFFs are released into the medium (Brahic et al., 2016; Freundt et al., 2012). In addition, LAG3 (lymphocyte-activation gene 3) receptor has been identified as a receptor that binds to aSyn PFFs leading to endocytosis and transmission (Mao et al., 2016).

In mice primary cortical neurons and HEK (Human embryonic kidney) cell line, incubation with aSyn monomers and aSyn PFFs promote the formation of paSyn aggregates after treatment with aSyn PFFs. Importantly, incubation with aSyn monomers do not induce aggregates formation (Vasili et al., 2022a).

In addition, primary hippocampal cultures incubated with aSyn PFFs experiment a decrease in synapse formation and function. This phenomenon is before the declining in cell viability in cultures over time (Peelaerts et al., 2015a).

Regarding to *in vivo* models, inoculation of aSyn PFFs into the brain, recapitulates pathological features of PD. This model is widely used by several studies to emulate idiopathic PD, enabling the study of PD progression (Chia et al., 2020; Pingale & Gupta, 2020).

In addition, aSyn PFFs have the capacity to enter in to the brain tissue, promoting the seeding and propagation of aSyn pathology in both aSyn-overexpresing transgenic mice and non-transgenic (WT) mice, as well as in neuronal cultures, thereby inducing the progression of pathological characteristics (Luk et al., 2012a; Volpicelli-Daley et al., 2014a).

Is been described that PD models present molecular compatibility regarding to the specie, thus, sequence homology between the aSyn PFF seeds and endogenous aSyn monomers determine the efficiency of propagation in aSyn pathology (Polinski et al., 2018a). For example, unilateral injection of mouse aSyn PFFs into dorsal striatum induces aSyn pathology in multiple brain regions, including DAergic neurons of SNpc. However, the same regions in mice injected with human aSyn PFFs show an inefficient seeding of pathology compared to mouse aSyn PFFs mice (Luk et al., 2016).

Also, *in vivo* models have demonstrated that unilateral injection of 5 ug of recombinant aSyn PFFs in striatum leads to the appearance of LB like inclusions paSyn positive in striatum and SN. Analysis shows the appearance of intracellular inclusions at 30 days post injection (dpi) and dense perinuclear paSyn inclusions at 90 and 180 days followed injection. Additionally, administration of aSyn PFFs into the striatum mice induces an impairment in coordination and balance by a decrease in the motor performance in rotarod and wire hang test at 90 and 180 dpi (Luk et al., 2012a). Also, this study demonstrated, the appearance of aSyn inclusions in other brain regions, indicating that aSyn are capable of spreading through tissue to affect distant brain areas from the injection site, including amygdala, frontal cortex and SN (Luk et al., 2012a, 2016).

In addition, intrastriatal inoculation of mouse aSyn PFFs in rats, develop the intraneuronal accumulation of paSyn inclusions, observed in striatum and SN tissue. Also, aSyn propagates to other brain areas begin present in amygdala, cortex and thalamus tissue. PD pathology observed in these models is detectable from 30, 90 and 180 days followed aSyn PFFs injections. Interestingly, when unilateral injections were performed, the presence of aSyn aggregates were detected in ipsilateral side but not in contralateral in the begin pathology development. A decrease in striatal innervation and a reduction in DA levels

accompanied by DAergic neuronal loss, occurs significatively at 180 dpi in 2 sites injected mice, also, there are no significative loss of striatal neurons in this model (Paumier et al. 2015).

All this evidence demonstrates the ability of propagation of aSyn PFFs toward brain areas such as amygdala, dentate gyrus, fimbria, somatosensory area, motor cortex, visual cortex, hypothalamus and hippocampus contributing to development of pathology.

Based on this evidence, the aSyn PFFs model is described as a useful model to study the progression of this pathology from neurodegeneration to neuronal death. Currently, researchers are searching for treatments that delay the development of PD, decreasing the formation of aSyn protein aggregates.

Therefore, understand the underlying mechanisms of aSyn accumulation and its impact in neurodegeneration is focus of interest in PD research field. Researchers aim to elucidate mechanisms to prevent or reverse the accumulation of aSyn and the development of therapeutic approaches that can ameliorate pathological feature of PD. This research is crucial for the development of potential treatments that could delayed or stop the progression PD.

1.3 Insulin-growth factor 2, a neurotrophic factor in neurodegenerative diseases

Insulin-like growth factors (IGFs) are a family of peptide hormones that share a high similarity with insulin (Sakanosb et al., 1991). Insulin-like growth factor 1 (IGF1) is the serum neurotrophic factor (NTF) whose actions on the adult brain are best characterized, playing an important role in brain development, memory consolidation, neuroprotection after neuronal damage, and neurogenesis (Carro & Torres-Aleman, n.d.; D. Y. Chen et al.,

2011; Fernandez & Torres-Alemán, 2012; Torres-Aleman, 2010). For example, the administration of IGF1 after injury reduced neuronal loss against several stressors such as oxidative stress, excitotoxicity, hypoxia, hypoglycemia, and others (Suh et al., 2013). Recent evidence highlights the importance of Insulin-like growth factor 2 (IGF2) in neurodegenerative diseases. A role for IGF2 has been described in the clearance of amyloid-beta (Ab) peptides in AD models. Jarvis *et al.* demonstrates the potential effect of neurotrophic factors in Aβ-induced neurotoxicity was evaluated in primary cortical neurons. Results show that nerve growth factor (NGF), basic fibroblast growth factor (bFGF), and insulin-like growth factors (IGF1 and IGF2), reversing the neurotoxic effect Aβ-induced on neuron survival (Jarvis et al., 2007). In addition, Pascual-Lucas *et al*. demonstrated that overexpression of IGF1 or IGF2 in the hippocampus of aged mice enhances memory and promotes the formation of new dendritic spines (Pascual-Lucas et al., 2014). However, only IGF2 decreases the load of Aβ oligomers.

Moreover, in ALS and spinal muscular atrophy (SMA), IGF2 has a protective role that opposes degeneration of motor neurons that were derived from fibroblasts of these patients (Allodi et al., 2016). This study further demonstrated that overexpression of IGF2 in muscles of mutant SOD^{G93A} mice promoted axonal regeneration and increased neuronal survival of the affected motoneurons (Terauchi et al., 2016). The protective effect of IGF2 is mediated by Glycogen synthase kinase-3β (GSK) inhibition through the activation of the PI3K-Akt (protein kinase B) pathway and by the upregulation of β-catenin expression, which plays a critical role in axonal and dendrite maintenance (Walsh et al., 2002.). IGF2 also protects human motor neurons from SMA-induced degeneration *in vitro*, improving neuronal survival and increasing the length of iPSC motor neurons. Additionally, IGF2 contributes to presynaptic stabilization; it is secreted from the presynaptic terminal, and it binds to IGF2

Receptor (IGF2R), which is also localized at the presynaptic terminal (Brown et al., 2009). The regenerative properties exerted by IGF2 on human motor neurons indicate that it could elicit beneficial effects on motor neuron survival that have lost connections with muscles.

A therapeutic approach, using a viral vector as a tool for gene delivery, demonstrates that intrastriatal administration of IGF2, reduce mHTT (mutant huntingtin) levels in three transgenic preclinical models of HD. Also, IGF2 decreases intracellular levels of mHTT and promotes its secretion to the extracellular media through microvesicles and exosomes (García-Huerta et al., 2020a). Despite the accumulate evidence, the molecular mechanism triggered by IGF2 is still unknown.

Since PD is characterized by the selective degeneration of DAergic neurons in the SN, approaches aim to reverse this loss to delay the disease progression. Several studies propose therapies based on the delivery of genes encoding enzymes required for DA synthesis. In addition, researchers attempt to develop therapies based on the down-regulation of aSyn, which prevents its accumulation in the SN and significantly protects DAergic neurons from degeneration in PD (Hayashita-Kinoh et al., 2006).

In this context, gene delivery using adeno-associated virus (AAVs) demonstrate being safe and tolerable for PD patients. Also, the efficacy of AAVs vectors as gene therapy persisted up to 2 years with a significant improvement in the UPDRS motor scores in clinical trials (Muramatsu et al., 2010).

A reduction in the bioavailability of NTFs during aging in the nervous system is reported, suggesting a role of these factors in neurodegenerative diseases such as PD. Recently, therapeutic approaches aim to restore NTFs deficiency in the brain to decrease the neurodegeneration process in PD. Thus, gene therapy to increase IGF2 levels in the brain

has been shown to have a favorable impact in a murine model of HD (García-Huerta et al., 2020b).

Taken together, IGF2 becomes an interesting molecule for the development of therapies that delay the pathology progression of PD in patients.

3. HYPOTHESIS AND AIMS

Hypothesis:

Neurotrophic factor IGF2 treatment has a neuroprotective effect in aSyn pathology decreasing aSyn accumulation and preventing neuronal loss in nigrostriatal system in Parkinson's disease preclinical model.

General Aim:

To investigate the neuroprotective effect of IGF2 treatment in aSyn-induced pathology of in vitro and *in vivo models of PD.*

Specific Aims:

Specific aim 1: To determine the protective effect of IGF2 in aSyn-induced cell death in PD cellular models.

Specific aim 2: To assess the effect triggered by IGF2 in pathological accumulation of aSyn in PD cellular models.

Specific aim 3: To evaluate the contribution of IGF2 expression as a treatment in aSyn pathology of *in vivo* PD model.

4. MATHERIALS AND METHODS

To investigate the impact of IGF2 in PD, we propose employ a combination of different complementary approaches, using *in vivo* and *in vitro* PD models, to determine the effect of IGF2 on aSyn-induced toxicity and DAergic neuronal loss. To address this question, we performed different cell biology and biochemical studies to define how IGF2 regulates neuronal damage, aSyn secretion and cell death *in vitro*. To further validate the effects of IGF2 *in vivo* models, we used C57BL/6 WT mice performing aSyn PFFs intrastriatal injections to model aSyn pathology. In these animals, we evaluated motor performance, aSyn aggregates levels, DAergic neuronal loss and dendritic spines changes. Finally, employing our PD mice model, we evaluated neuronal loss in the context of IGF2 ablation using the *Igf2*$^{-/-}$ knockout transgenic mice (IGF2 KO).

4.1. Generation of aSyn PFFs from recombinant aSyn monomers

The purified monomeric recombinant aSyn was obtained through collaboration with Dr. Rodrigo Díaz and Dr. Esteban Nova from the University of Chile. The aSyn PFFs was generated following the protocols established in the literature (Luk et al., 2012b; Patterson et al., 2019; Volpicelli-Daley et al., 2014c)

Briefly, aSyn monomers were diluted with PBS 1X to a 2 mg/ml final concentration and centrifugated at 15,000 x g for 10 min at 4 °C. The monomers were then incubated at 37 ° C with shaking at 1,000 RPM for 7 days. Then, the samples were sonicated for 60 pulses (1s on; 1s off) setting amplitude to 30% to produce aSyn fibrils of suitable lengths before quality control and storage. Fibrilization of aSyn could be assessed using Thioflavin S (ThS) assay, electron microscopy, and/or Western blot aggregates. To assess the fibrillation of our

generated fibrils, we stained with ThS. This assay has been used in research field to stain amyloid structures measured by fluorescence (Gade Malmos et al., 2017). ThS exhibits increased fluorescence when it binds to amyloid conformations. Therefore, the increased fluorescent signal from samples indicates the presence of amyloid structures. After 7 days of incubation, aSyn monomers produced an increase in ThS signal measured by Relative Fluorescence Units (RFU). Generated aSyn fibrils were aliquoted and stored at -80 °C (Figure 3). We perform this assay to validate the protocol to generate aSyn PFFs, and consequently use the same protocol every time we prepared fibrils (Gade Malmos et al., 2017).

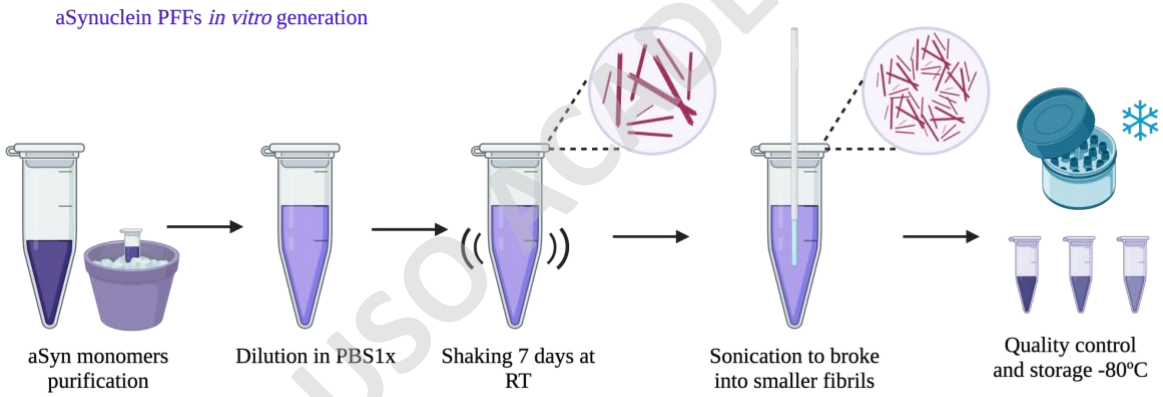


Figure 3: Schematic description of the protocol to generate *in vitro* aSyn PFFs from recombinant aSyn monomeric protein. Briefly, the protocol for generating aSyn PFFs start with the purification and dilution in PBS 1x to desired concentrations of aSyn monomers (recommended concentrations and volumes from literature). After dilution, to generate aSyn PFFs, the solution is centrifugated to discard the sediment and incubated at 37°C shaking for 7 days. Then, fibrils were sonicated, to obtain less size of a Syn PFFs which were capable to enter into the cells, before quality control and storage.

4.2. Neuroblastoma cell line culture

Human neuroblastoma cell line SHSY5Y, obtained from a metastatic cell found in the bone marrow, were purchase from ATCC (American Type Culture Collection). Cell line were cultured in DMEM medium supplemented with 5% Fetal bovine serum (FBS) and 1% penicillin/streptomycin. For cell viability assays and aSyn intra and extracellular levels measurements, cells were plate and incubated with plate and with extracellular monomeric, aSyn PFFs or a mixture of monomeric and aSyn PFFs. Mixture conditions were composed by aSyn PFFs and monomers in a proportion of (1:9), respectively. aSyn species concentration was determined as previously have been described (Mahul-Mellier et al., 2015).

4.3. Primary cortical neuron cultures

Primary cortical neurons were prepared from embryonic day 16 (E16) of C57BL/j6 WT mouse brains. Briefly, after dissection, the tissue was washed three times with Hank's Balanced Salt Solution (HBSS Gibco) and digested by Trypsin 1x (Gibco) for 10 min at 37 °C. Cortices were then disaggregated by mechanical dissociation with glass Pasteur pipettes. Dissociated neurons were transferred in fresh DMEM 5% SFB medium, and 1% penicillin/streptomycin. After 24 hrs, DMEM medium was completely removed and replaced by neurobasal medium containing B27 supplement and L-glutamine (Conc).

For IGF2 overexpression experiments, primary neurons were transduced with AAVs particles ($1x10^9$) at 1DIV. To induce aSyn cell toxicity, neuron cultures were exposed to aSyn PFFs for 72 h. As a control, aSyn monomers were used.

4.4. Cell viability assays

We evaluated the effect triggered by IGF2 treatments in cell toxicity-induced by aSyn species. The neuroblastoma cell line SHSY5Y and primary cortical neurons were stimulated during 1 hr with recombinant (human or mouse) IGF2 or transfected with a plasmid-containing the genetic construct to express aSyn. After IGF2 treatment, cultures were incubated with aSyn species at indicated concentrations (PFFs, monomers or mixture). Total cell number were analyzed 72 h after aSyn PFFs incubation and cell death were detected by propidium iodide (PI) permeability. Hoechst 33342/PI double staining was performed and cells were fixed with PFA and placed in mounting medium.

Images were acquired with two excitation/emission filter sets; 380/535 nm for Hoechst and 555/645 nm for PI. Images from each condition were captured using a 20x/NA 0.75 dry objective using Leica DMi8 microscope. The percentage of dead cells was calculated from the total number of cells/PI-positive cells. Cell nuclei emitting fluorescence signal from both Hoechst and PI (fluorescence co-localization) were considered as dead cells, while cells emitting only Hoechst signal were counted as live cells (Ciancio et al., 1988; Lema et al., 2011; Pei et al., 2020).

Cell damage was quantitatively assessed by the measurement of lactate dehydrogenase (LDH) released from damage or destroyed cells in the extracellular media 72 h after the aSyn

PFFs incubation. Cells were seeding into a 96 well plate and LDH was detected using Cytotoxicity Detection Kit (Cat #C20300). Absorbance (490-680 nm) was measured using a fluorescence microplate reader (Kumar et al., 2018; Lobner, 2000).

In addition, the metabolic activity of cell cultures was measured by the 3-(4,5-dimethylthiazole2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were seeding into a 96 well plate (10^4 cells/well) and viability assay was performed using MTT cell viability assay (Cat #V13154). Absorbance (570 nm) was measured using a fluorescence microplate reader (Ghasemi et al., 2021).

Crystal violet (CV) staining were performed to quantify the percentage of cell viability through the number of cells per condition. Briefly, cells were seeded in a 96 well plate. After 24h cells were exposed to aSyn species during 72h. CV staining solution were added to each condition and incubated for 20 min at room temperature. Cells were washed with water and images were obtained by microscopy. The number of cells were counted and cell percentage were calculated using ImageJ software.

4.5. Immunohistology analysis

For tissue immunofluorescence analysis, mice were anesthetized and subjected to transcardial perfusion with ice cold PBS (pH 7.4). Brains were removed and fixed in 4% PFA for 24 h and then stored in a 30% sucrose solution. Fixed hemispheres were cut using a cryostat (Leica CM1520) and 20µm of thickness coronal slices were obtained from the striatum. Free floating slices were preserved in PBS azide 0.02% at 4 °C. For immunofluorescence staining, brain slices were washed in PBS, blocked in 0.1% Triton-X

and 10% fetal bovine serum (FBS) solution for 2 h at room temperature. Slices were incubated overnight at 4°C with the primary antibody in blocking solution. Then, the secondary antibody was incubated for 2 h at room temperature. Slices were washed and mounted on glass slides using Entellant mounting solution. Primary antibodies used in this study were anti- tyrosine hydroxylase (TH) (rabbit, 1:250, Cell Signaling #58844), anti-tyrosine hydroxylase (TH) (sheep, 1:250, Thermo Ficher #PA1-4679), anti-paSyn (mouse, 1:250, Biologend #825701), and anti-synaptophysin (rabbit, 1:250, Cell Signaling #36406). Secondary antibodies used in this study were Alexa Fluor 488 anti-mouse IgG (donkey, 1:500, Invitrogen #A-11005), Alexa Fluor 488 anti-Rabbit IgG (donkey, 1:500, Invitrogen #A-11011) Images were acquired using confocal microscopy 880 Zeiss (40x/1.1 NA Air).

4.6. Behavioral motor test in PD mice model

To evaluate the effect of IGF2 treatment in motor performance of aSyn mice model, we performed Beam test and Cylinder test in experimental groups of animals. The Beam balance test and Cylinder test are used to assess fine motor coordination and balance in rodents, especially in experimental models associated with an alteration of the motor skills (Huang et al., 2022; Taylor et al., 2010). The goal of this test is for the mouse to stay upright and walk across an elevated narrow beam to a safe platform. This test takes place over 3 consecutive days: 2 days of training and 1 day of testing (Luong et al., 2011). In addition, we evaluate motor impairment in PD mice model by Cylinder test. In this test, mice were placed in a glass cylinder and number of touches to the cylinder wall using forelimbs was measured (Magno et al. 2019).

Motor performance was evaluated at three different times during the experiment: Time 0, when intrastriatal injections were performed to aSyn PFFs inoculation; at 6 weeks, when AAV particles were injected in SN, and finally, at 12 weeks, at the end of the experiment.

4.7. Stereotaxis injections

C57bL/j6 wild type mice were housed in a 12 h light/dark cycle, with *ad libitum* access to food and water, in accordance of ethic commit for the manipulation animals in the laboratory. For stereotactic injection, the animals were deeply anesthetized with constant isoflurane prior to placement in a stereotaxic frame. To model aSyn pathology, 5ug (2 uL) of aSyn PFFs, previously generated *in vitro*, were injected in the right brain hemisphere of striatum using a 10 µl Hamilton syringe with a 1 µl/min infusion rate.

To trigger IGF2 overexpression in SN, animals were injected with AAV carrying IGF2 (AVV-IGF2) or empty plasmid carrying GFP as a control (AAV-GFP) in the right brain hemisphere of SN using a 10 µl Hamilton syringe with a 1 µl/min infusion rate. The titer virus used was 1 x 10^9 viral genomes/ul (VGs) for each of them.

The striatum was targeted at the following coordinates: AP: +0,07 cm, ML: -0.17 cm relative to bregma, DV: -0.31 cm (according to the atlas of Franklin and Paxinos, Second Edition, 2001) relative to skull surface. The SN was targeted at the following coordinates: AP: -0,29 cm, ML: -0.13 cm relative to bregma, DV: -0.42 cm (according to the atlas of Franklin and Paxinos, Second Edition, 2001) relative to skull surface.

4.8 Dendritic spines analysis

Synaptic terminals in SN were labeled by performing immunofluorescence with the use of an anti-synaptophysin antibody (Cell Signaling #36406) as a presynaptic marker. Following acquisition, images were subjected to confocal microscopy. The obtained images were then processed using the Huygens Professional software (Scientific Volume Imaging, The Netherlands, version 23.10), applying the Classic Maximum Likelihood Estimation (CMLE) algorithm for image deconvolution. The deconvolved images were exported in the Huygens H4 format for subsequent analysis.

In the next step, we utilized Imaris software (Bitplane, Switzerland, version 10.1) for neuron and synapse identification. The Filament Tracer tool was employed to recognize and measure neurite lengths, with the parameter of diameter being meticulously adjusted to match our samples. The Spot Detector tool was subsequently used for synaptic termini identification, with an estimated diameter for spots detection set at 0.9 μm.

The spots were selected based on their distance from the filament, employing a custom-made function within the Imaris software. Only the spots located within a maximum distance of 2.5 μm from the filament's center were deemed positive and included in further analysis. This procedure was conducted in three-dimensional space to better represent the complex architecture of the dendritic trees.

In the final stage, data were normalized by the total length of dendrites, and are presented as the number of spines per 10 microns of dendrite. We processed approximately 2,800 filaments for the control condition (AAV-GFP) and around 9,400 filaments for the IGF2-treated condition (AAV-IGF2).

All data obtained from the Imaris software were exported for further analysis. Custom macros in Microsoft Excel were utilized to compute all necessary measurements and perform the statistical analysis, the details of which are described in the subsequent section of the paper.

This methodology provides a thorough and robust approach to dendritic spine quantification, allowing for an accurate representation of changes in spine density in relation to dendrite length under various experimental conditions.

4.9. Statistical analysis

The statistical analysis for viability assays was performed using ANOVA followed by a Tukey post-hoc test. Statistical analysis of DAergic innervation in striatum and number quantification of the nigral TH-positive neurons loss also was performed using ANOVA statistical analysis. Neuronal loss percentage comparing injected hemisphere of experimental groups and behavioral data was performed by Student's *t-test*. Finally, for the motor trials two-way ANOVA test followed by Tukey`s multiple comparisons post-hoc test was performed. All data results are expressed as average ± standard error of the mean (SEM). A p value of < 0.05 was considered significant (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

5. RESULTS

aSyn accumulation is an important hallmark in the pathology of sporadic and genetics cases of PD, highly used in research field. aSyn forms multiple conformations, including monomers, oligomers, and fibrils. Last tridimensional conformation is highly insoluble, characterized by β-sheet conformation accumulation. This aSyn accumulation generates cell toxicity in vulnerable neurons leading to degeneration and neuron death in mouse models (Hansen et al., 2011b; Pieri et al., 2012; Wong & Krainc, 2017b). Several studies demonstrate that overexpression of aSyn or incubation with exogenous aSyn PPFs induce pathological phosphorylation and accumulation of endogenous paSyn in neuronal cultures. This produces cellular toxicity leading to neurodegeneration and neuronal death (MacIsaac et al., 2020; Volpicelli-Daley et al., 2014c).

Therefore, we the purpose to induce aSyn toxicity in neuronal cultures and aSyn pathology *in vivo,* we generated *in vitro* aSyn PFFs from human and mouse aSyn recombinant protein (Figure 4). The monomers of this protein have no native secondary structure. However, a common property of amyloid proteins is the ability to catalyze the assembly of soluble monomers into fibrils at micromolar concentrations under specific conditions. The purified monomeric recombinant aSyn was obtained through collaboration with Dr. Rodrigo Díaz and Dr. Esteban Nova from the University of Chile. Capacity to form b-sheet structures was validated by Thioflavin T staining (ThT), measured by fluorescence intensity (Supplementary Figure 1) (Gade Malmos et al., 2017; Polinski et al., 2018b; Volpicelli-Daley et al., 2014c).

In order to validate cell damage induced by aSyn PFFs in neuronal cultures, we carry out viability assays in cellular culture exposed to aSyn PFFs (Figure 4). Specifically, to modelate

PD pathology in cellular cultures, we employed neuroblastoma cell line SHSY5Y and developed primary cultures of cortical neurons, which were exposed to aSyn monomers, aSyn PFFs and mixture of monomers plus aSyn PFFs. After 72h of aSyn species incubation in SHSY5Y cells, MTT and LDH assay were performed (Figure 5A and 5B, respectively). These results were validated by the literature, which stablished the concentrations of aSyn species to induce aSyn toxicity and cell death (Mahul-Mellier et al., 2015).

The first results demonstrate that incubation with aSyn PFFs and mixture increases LDH release to extracellular media, indicating cellular impairment. Also, MTT assay results demonstrate that incubation with aSyn PFFs decrease cell viability in cell culture.
Specifically, we observed a decrease in viability of 50% and 80%, in cultures treated with 1uM and 2uM mixture (aSyn PPFs plus monomers), respectively (Figure 4A). Similar results were observed in LDH assay measurement, with an increase in cell damage by 50% and 60% in both conditions, respectively (Figure 4B).

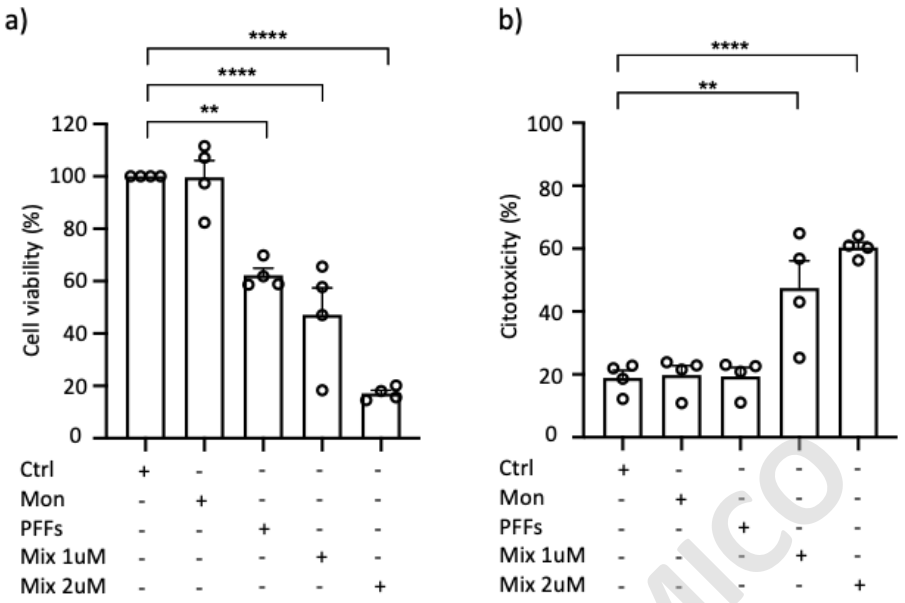


Figure 4: Validation of *in vitro* generated aSyn PFFs in neuroblastoma cell line. SHSY5Y cells were seeded and then aSyn species were added to media cultures to induce cellular toxicity at different concentrations. Cells were incubated with human aSyn monomers (Mon), human aSyn PFFs (PFFs) and two aSyn mixture conditions. PBS 1X was used as a control. After 72 hrs, to evaluate the effect of aSyn toxicity, MTT and LDH assay was performed a) and b), respectively. Data shown represent the means of four independent experiments performed in triplicate for each condition (bars are means ± SEM.). One-way ANOVA test followed by Sidak's multiple comparisons post-hoc test was performed; p-value, **$p < 0.01$, ****$p < 0.0001$.

Moreover, to validate the capability of aSyn PFFs to promote the formation of protein inclusions *in vivo*, we performed stereotaxic injections to inoculate aSyn PFFs into the dorsal striatum of WT mice. Given that aggregation of aSyn protein leads to formation of intracellular protein inclusions which is a hallmark of PD pathology, we utilized ThS staining to identify the presence of protein aggregates structures in the striatal tissue of injected mice (Figure 5A and 5B). Furthermore, we evaluated the aSyn PFFs spreading potential from the injection site by ThS staining in SN tissue of injected mice (Figure 5C). Fluorescence intensity (ThS burden) of ThS staining measurement shows significative differences between ipsilateral hemisphere compared to contralateral in both analyzed tissues of injected mice.

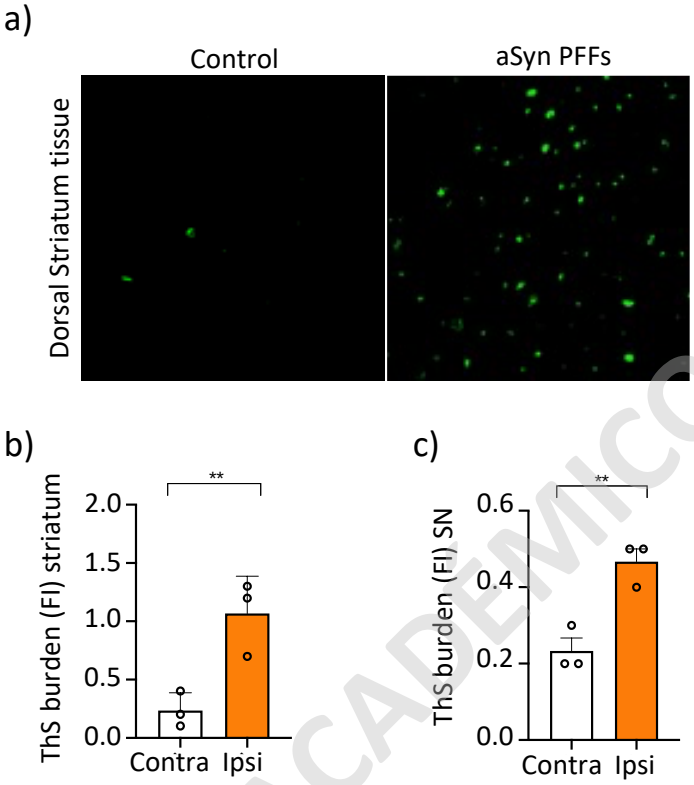


Figure 5: aSyn PFFs induces the formation of aggregates structures *in vivo*. In order to evaluate aSyn-induced pathology *in vivo*, unilateral stereotaxis to inject aSyn PFFs (5ug/2uL) in dorsal striatum WT mice were performed. Over 12 weeks, aSyn aggregates was detected by ThS staining in striatum and SN tissue. a) ThS staining in ipsilateral dorsal striatum of aSyn PFFs injected mice present ThS-positive aggregates compared to control injected mice. Fluorescence intensity (FI) was measures for ThS burden quantification in striatum and SN tissue of aSyn PFFs injected mice, b) and c), respectively. Results show an increase of ThS burden of ipsilateral compared to contralateral in both analyzed tissue. Data shown represent the means of three animals per group (bars are means ± SEM.). *t-test one tail* was performed to statistical analysis. p-value, **p < 0.01.

These findings demonstrate aSyn PFFs capability to induce toxicity in neuronal culture, leading to a decrease in cell viability and promoting cell damage. Moreover, aSyn PFFs generated *in vitro* exhibit the capacity to induce the formation of protein aggregates in the striatal region of *in vivo* model. Also, we observed aSyn ThS-positive inclusions in SN region, which indicates the capacity of aSyn to propagate from the injection site to different brain areas, such us the SN. These results provide the validation of pathogenicity of *in vitro* aSyn PFFs generation from aSyn monomers.

Next, to evaluate the neuroprotective effect of IGF2 on neuronal toxicity mediated by aSyn, we assessed the effect of IGF2 using human recombinant protein (rhIGF2) to stimulate cells (100 ng/ml) 1h prior to aSyn PFFs incubation (Figure 6).

Important, for rhIGF2 treated control condition we showed similar results compared to not treated condition (without rhIGF2 stimulation) indicating that recombinant protein concentration do not induce a significant decrease in cell viability (Figure 6B).

Cell death was measured by the percentage of PI-positive cells. Briefly, cells with intact plasma membranes, PI is unable to enter the cells, while cells undergo damage or death, their plasma membranes become compromised, allowing PI to enter the cell. Then, PI staining is detected by fluorescence microscopy, indicating loss of membrane integrity and cell death.

Results show that cells incubated with aSyn species, at concentrations of mixture 1 uM of aSyn PFFs, we observed 20% of PI-positive cells, while increasing the concentrations to mixture 2 uM aSyn PFFs, we obtained 50% of PI-positive cells. After rhIGF2 treatment, PI-positive cells percentage decreased significatively in mixture 2 uM aSyn PFFs compared to NT condition (Figure 6B).

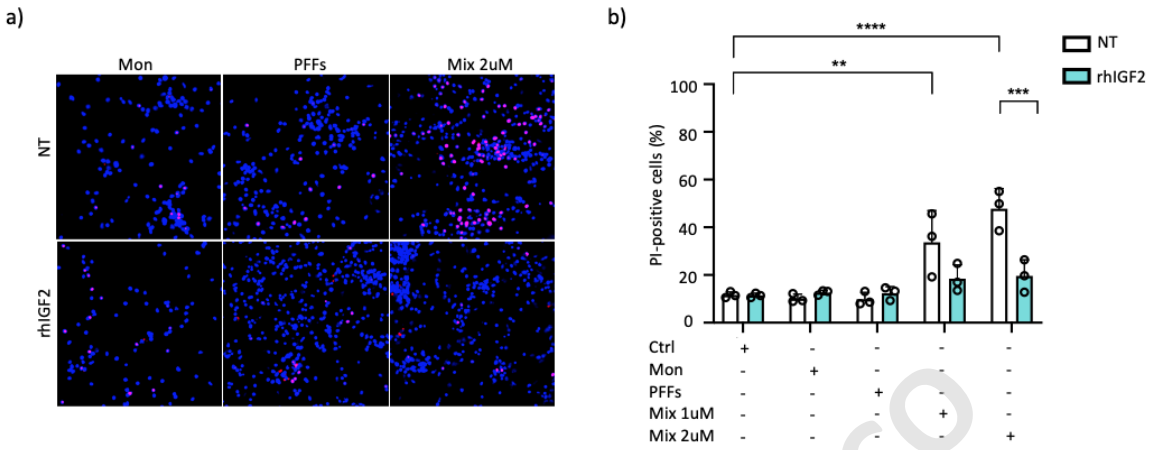


Figure 6: rhIGF2 stimulation decreases aSyn-induced cell death in SHSY5Y neuroblastoma cell line. Neuroblastoma cultures were stimulated with rhIGF2 (100 ng/ml) 1 hr before human aSyn species treatment. PBS 1X was used as a control. After 72 h, cell death was measured by quantification of PI-positive cells. a) Representative fluorescence images were obtained after Hoechst/PI double staining in SHSY5Y cells. Hoechst (blue) was used to stain nuclei and PI (magenta) dye, which enter only in cells with damaged plasma membranes, used to detect die cells. b) Quantification of cell death expressed as the percentage of cells with loss of plasma membrane integrity (PI-positive cells) to the total cell number analyzed by microscopy from a) panel. Data shown represent the means of three independent experiments performed in triplicate for each condition (bars are means ± SEM.). Two-way ANOVA test followed by Sidak`s multiple comparisons post-hoc test was performed; p-value, **p < 0.01, ****p < 0.0001.

An alternative approach to assess the impact of IGF2 involves employing molecular techniques to induce overexpression of the gene. The overexpression of a gene offers advantages in terms of a longer and more consistent expression time of the gene of interest. Thus, the effect could be evaluated through the time compared to the recombinant ligand, which decreases its bioavailability over the course of the experiment.

Due to rhIGF2 shows a positive effect in aSyn-induced cell death, we decided transiently transfect SHSY5Y cell line with a plasmid vector to generate IGF2 overexpression (IGF2 OE).

Then, cell cultures were transfected with a plasmid for the expression of IGF2 or GFP 24h before aSyn PFFs incubation. After 72 h, to evaluate cell death, PI-positive cells were quantified and CV staining were performed to detect cell number.

We observed that IGF2 OE in SHSY5Y, decreases significatively PI-positive cell percentage and prevents cell loss aSyn-induced in both mixture conditions (Figure 7B and 7D).

These results indicate that increase expression levels of IGF2 exerts a protective effect against aSyn-induced cell death in SH-SY5Y cells, evidenced by PI and CV staining techniques (Figure 7).

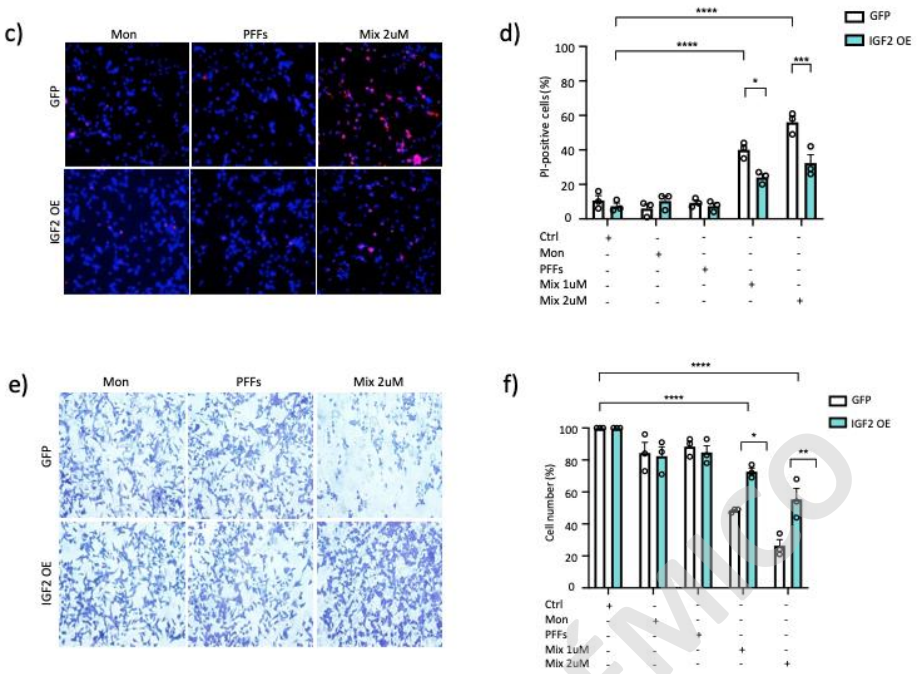


Figure 7: IGF2 overexpression prevent neuronal death in SHSY5Y cultures exposed to aSyn species. Cell cultures were transiently transfected with a plasmid for the expression of GFP or IGF2. After 24h, cells were exposed to aSyn species and PI-positive cells were analyzed. a) Double stain Hoechst/PI in transfected cells. b) Percentage of PI-positive cells quantification from a) panel. c) CV stain was performed in IGF2 and GFP transfected cells. d) Cell percentage quantification from CV stained cells in c). Data shown represent the means of three independent experiments performed in triplicate for each condition (bars are means ± SEM.). Two-way ANOVA test followed by Sidak`s multiple comparisons post-hoc test was performed; p-value, *p < 0.05, **p < 0.01, ****p < 0.0001.

It is known that IGF2 system is regulated by three structurally similar ligands. These are IGF1, IGF2, and insulin that lead to the activation of signaling pathways mediated by IGFs receptors (Blyth et al., 2020; Okuyama et al., 2021). Specifically, IGF2 binds and consequently activates their receptor (IGF2R) and also is capable to binds to IGF1 receptor (IGF1R) and Insulin receptor (IR) activating related signaling pathways (Brown et al., 2009). Then, in order to investigate whether the IGF2R is involved in the protective effect of IGF2 in aSyn-induced cell death, we decided to evaluate its effect by blocking IGF2R using a specific IgG antibody against to IGF2R.

We employed neuroblastoma cell line SHSY5Y which were previously transfected with the plasmid to increase IGF2 expression. After 24 hrs of transfection, cell culture was incubated with IgG antibody to block IGF2R by 2 hrs and then incubated with aSyn species (Figure 8). As control transfection, cells were transfected with GFP vector plasmid.

Cell death was evaluated by quantification of PI-positive cells related to total cell number, represented by PI-positive cells percentage. Conditions incubated with aSyn mixtures, 1uM and 2uM, generates around 40% and 56% of PI-positive cells, respectively. The % of PI-positive cells were significatively reduced by IGF2 OE in cells incubated with aSyn mixture 1uM and 2 uM showing around 20% and 30% of PI-positive cells, respectively. After antibody incubation for blocking IGF2R, the % of PI positive cells was restored, becoming like the % of cell death in the control condition. No significative differences were observed in monomers and aSyn PFFs conditions (Figure 8B and 8C). These results demonstrate the neuroprotective effect of IGF2 on aSyn-induced neuronal death in SHSY5Y neuroblastoma cells are reverted by IGF2R blocking, suggesting the positive effect on cell survival of IGF2 is triggered by IGF2R activation (Figure 8D and 8E).

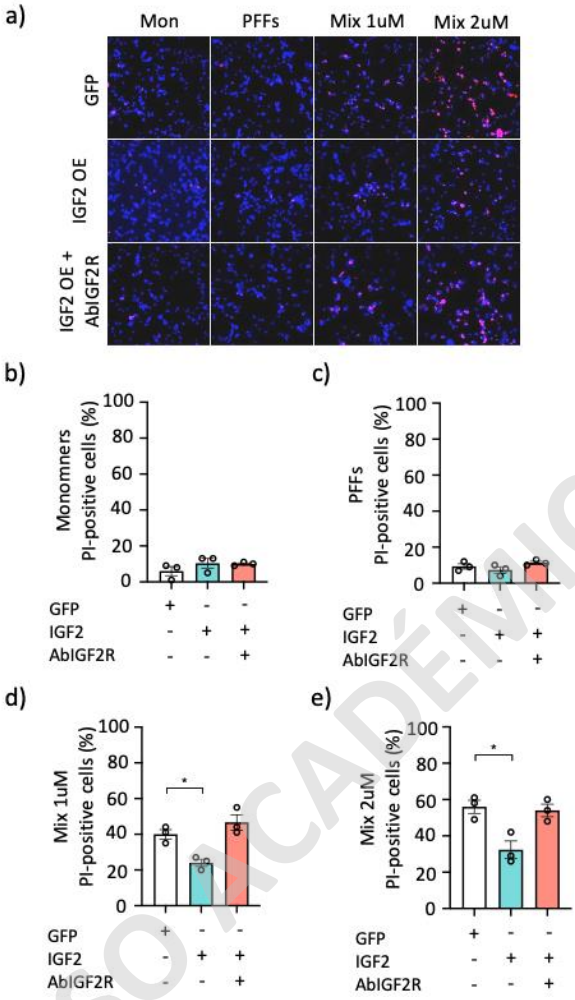


Figure 8: The neuroprotective effect of IGF2 is through IGF2 receptor. SHSY5Y culture were transfected with plasmid for the expression of GFP as a transfection control and plasmid to increase IGF2 expression. After 24h cells were incubated with IGF2R antibody by 2hrs and then exposed to human aSyn monomers, aSyn PFFs, and aSyn mixture. After 72 hrs of incubation, cell death was measured by quantification of the PI-positive cells %. a) Representative images of cell cultures double staining with Hoechst/PI. b) Pi-positive cell % in monomers treated conditions, c) aSyn PFFs, d) mixture 1uM and e) mixture 2uM condition. Data shown represent the means of three independent experiments performed in triplicate for each condition (bars are means ± SEM.). One-way ANOVA test followed by Tukey`s multiple comparisons post-hoc test was performed; p-value, *$p < 0.05$.

Next, we propose to determine the cellular mechanism involved in neuroprotective effect trigger by IGF2 treatment. Considering previous results which demonstrated that IGF2

treatment decreases the load of intracellular aggregates of mHtt in cellular models of HD (García-Huerta et al., 2020b)**,** we aim to evaluate the effect of IGF2 in aSyn accumulation in SHSY5Y cell line. Cells were transiently transfected using a plasmid vector carrying IGF2 or GFP-containing plasmid as a control. After 24h cells were incubated with aSyn PFFs and mixture or PBS 1x as a control. Intracellular load of aSyn aggregates were detected by westerblot (WB) technique using anti-aSyn antibody (Figure 9A). No significative results are observed in PFFs condition between IGF2 OE or GFP transfected cells (figure 9B). However, IGF2 OE decreases significatively aSyn levels in aSyn mixture exposed condition (Figure 9C).

Additionally, secreted fraction was evaluated using Dot blot technique (Figure 9D). Results show that IGF2 OE promotes aSyn secretion in monomers (Figure 9E), aSyn PFFs (Figure 9F) and mixture condition (Figure 9G), significatively compared to control GFP.

These results suggest the effect at the cellular mechanism level triggered by IGF2 treatment is related to the regulation of intra and extracellular aSyn levels, indicating as a significant effect, the decrease of intracellular aSyn aggregates and promoting the release of aSyn into the extracellular media.

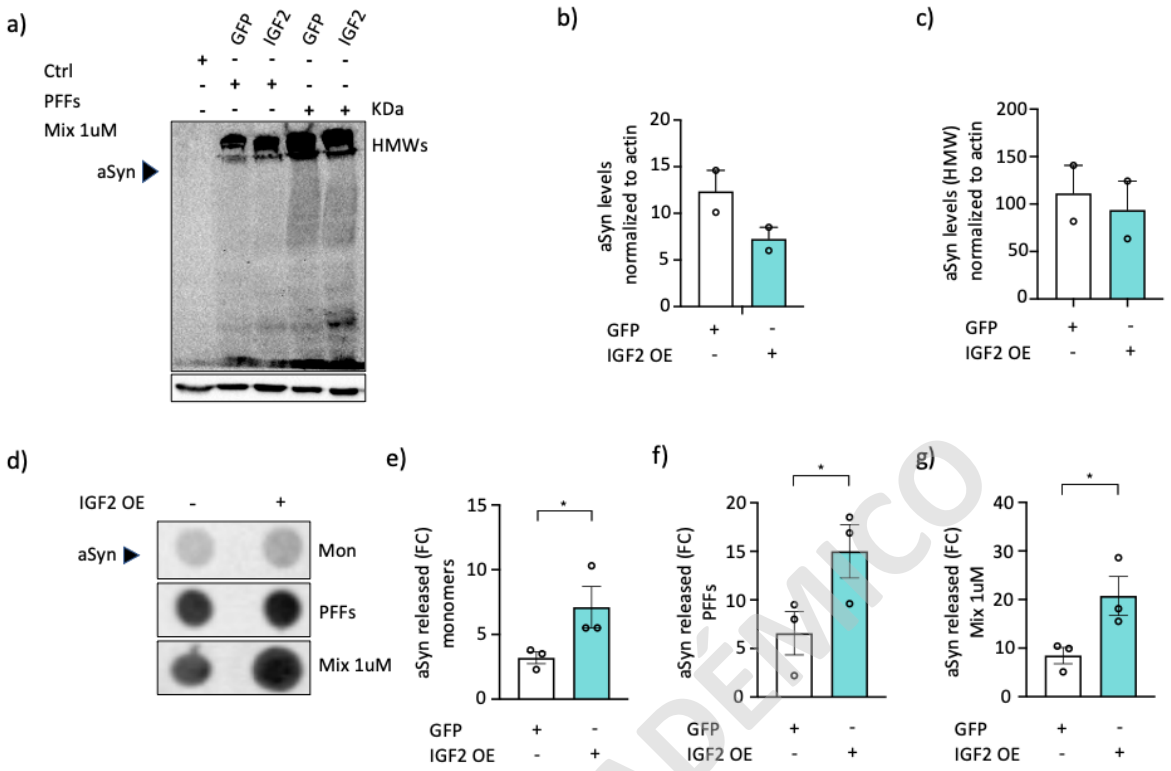


Figure 9: IGF2 overexpression enhances aSyn secretion in neuroblastoma cultures exposed to aSyn PFFs. a) SHSY5Y cells were transfected to GFP or IGF2 plasmid to increase expression of IGF2. After 24h, cells were exposed to aSyn species during 72h. a) Intracellular levels of aSyn were analyzed by WB technique. b) Intracellular aSyn aggregates between GFP or IGF2 transfected cells in aSyn PFFs and c) aSyn mixture exposed condition. d) Extracellular levels of aSyn were measured by dot blot technique. Dot blot quantification from cultures exposed to e) monomers, f) aSyn PFFs and g) mixture conditions. Data shown represent the means of three independent experiments performed in triplicate for each condition (bars are means ± SEM.). *t-test one tail* was performed to statistical analysis. p-value, *P<0.05.

Several studies demonstrate different neuron types have the intrinsic potential to be differentially affected by aSyn. DAergic neurons have been described as the neuronal type most affected in PD (Dagra et al., 2021; Praschberger et al., 2023). In this context, primary neuronal cultures have been extensively used to investigate the mechanisms of aSyn cell toxicity (Vasili et al., 2022b). Primary neuronal models are capable of internalizing PFFs leading to aSyn intracellular accumulation. The presence of intracellular aSyn inclusions promotes a progressive cellular impairment leading to cell death (Polinski et al., 2018a; Vasili et al., 2022c; Volpicelli-Daley et al., 2011, 2014c).

In order to investigate the impact of IGF2 treatment, we attempt to use a neuronal culture model employing primary cortical neurons from E16 WT mice (Figure 10). For this purpose, cortical neurons were incubated with aSyn PFFs to generate aSyn toxicity in neuron cultures. At 6DIV, primary neurons were incubated with recombinant mouse (rmIGF2) or PBS 1x as a control. Following this, neuronal cultures were exposed to aSyn PFFs, to generate aSyn toxicity, or aSyn monomers as control. The effect of rmIGF2 on aSyn-induced toxicity was evaluated through LDH and MTT assays, as well as PI staining (Figure 10).

These results demonstrate that primary cortical neurons exposed to aSyn PFFs show a significative decrease in cell viability and increase in cell toxicity. A significant release of LDH was observed in primary neurons treated with aSyn PFFs, accompanied by a decrease in viability assessed by the MTT assay. Additionally, a significant increase in the percentage of PI-positive cells was observed in the condition treated with aSyn mixture 2uM. Then, the results demonstrate that the incubation with aSyn PFFs promotes cellular damage and induces cell death in primary cortical cultures (Figure 10).

Interestingly, we observed that treatment with rmIGF2 reduces the levels of LDH released into the medium and the percentage of PI-positive cells, indicating that IGF2 decreases the

cellular damage caused by the incubation with both aSyn PFFs and monomers. Interestingly, the most significantive effect on preventing cell death induced by aSyn is observed in the condition of high concentration of aSyn PFFs and monomers (Mix 2uM). This could be explained by the fact that in the other evaluated conditions, there is no significant increase in percentage of PI-positive cells, hence no significant differences in cell death compared to control cultures are observed (Figure 10C). No significant differences were observed in the primary neurons treated with rmIGF2 compared to the control in MTT assay (Figure 10B).

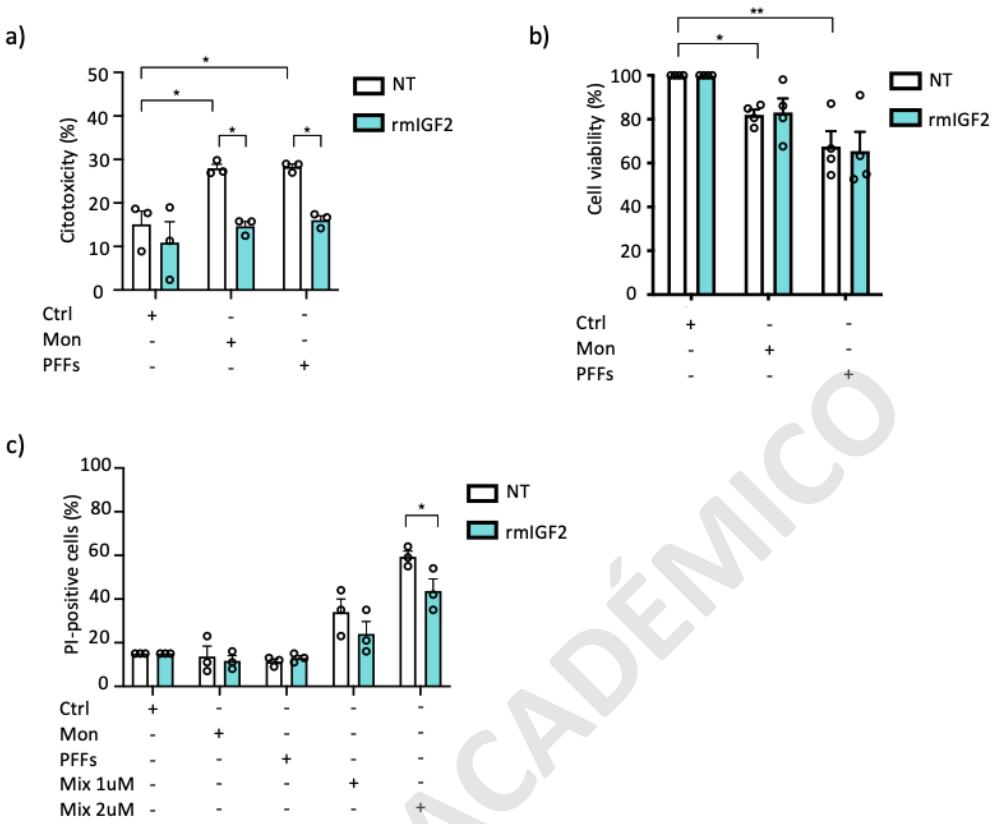


Figure 10: rmIGF2 stimulation decreases aSyn-induced toxicity in primary cortical neurons. Cortical neurons were treated at 6DIV with rmIGF2 (100 ng/mL) 1 hr previously to incubation with mouse aSyn species. PBS 1X was used as control of rmIGF2. aSyn monomers were used as aSyn PFFs control. Then, 72h after aSyn incubation, cellular damage was evaluated by LDH (a) and cell viability was determined through MTT assay (b). c) Additionally, PI-positive cells were quantified by Hoeschst/PI double stain to obtain cell death percentage. Data shown represent the means of three (LDH and PI assay) and four (MTT assay) independent experiments performed in triplicate for each condition (bars are means ± SEM.). Two-way ANOVA test followed by Tukey`s multiple comparisons post-hoc test was performed. p-value, *$p < 0.05$, **$p < 0.01$.

Our previous results demonstrated that primary cortical neurons are vulnerable to aSyn PFFs exposure, as evidenced by a decrease in cell viability in primary neurons (Figure 10). Furthermore, studies have indicated that primary neurons are capable of internalizing exogenous aSyn PFFs, which subsequently induce the aggregation of endogenous aSyn showing their ability to replicate aSyn pathology. Because of this, we wondered if IGF2 had an effect on intracellular accumulation of aSyn in this cellular model. Previously, our results showed that IGF2 promotes aSyn secretion in human SHSY5Y cultures (Figure 9). Then, we also aimed to evaluate whether IGF2 promotes the release of aSyn into the extracellular media in murine cellular model (Figure 11).

We demonstrated that aSyn PFFs are internalized by the cortical neurons, and after the stimulation with rmIGF2, we observed a significant increase in extracellular aSyn levels compared to control condition. It is noteworthy at this point that the presence of intracellular protein aggregates was observed in cultures incubated with aSyn PFFs compared to control condition of primary neurons exposed to aSyn monomers.

These results suggests that aSyn release is a mechanism triggered by IGF2 across different cell types (Figure 9 and 11).

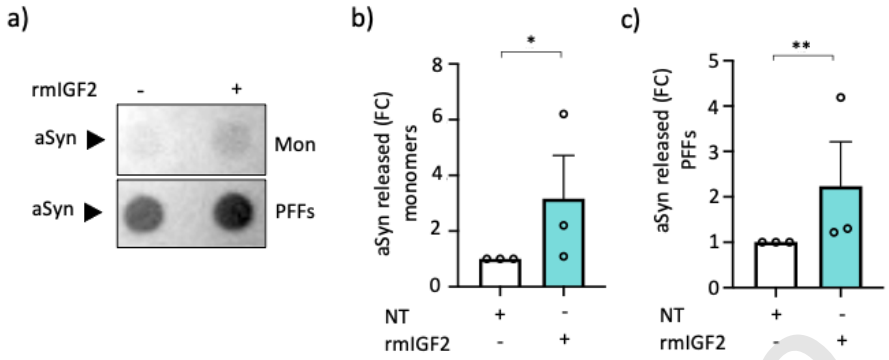


Figure 11: rmIGF2 promotes aSyn secretion in primary cortical neurons exposed to aSyn PFF. Primary cortical neurons were treated at 6DIV with rmIGF2 (100 ng/mL) 1 hr previously to incubation with mouse aSyn monomers and PFFs. PBS 1X was used as a treatment control and monomers were used as aSyn PFFs control. After 72 hrs of incubation, aSyn extracellular levels were measured using dot blot technique showed in a). Fold change respect to control of aSyn released levels is showed in b) for monomers incubated condition, and in c) for PFFs incubated cells. Quantification shows a significative increase in aSyn secretion for monomers and PFFs in neurons treated with rmIGF2 compared to control. Data represent the means±SEM of three independent experiments performed. *t-test one tail* was performed. p-value, *P<0.05, **P<0.001.

Based on that, the effect of rmIGF2 promotes aSyn release in two cellular models, and additionally, the overexpression of IGF2 in the human SHSY5Y cell line significantly prevents cell death induced by aSyn PFFs, we decided to evaluate whether IGF2 overexpression modulate aSyn levels in primary cortical neurons. For this purpose, we employed a viral vector for gene delivery, AAVs, that carry the sequence to increase the expression levels of IGF2 or empty plasmid in primary cortical neurons (Naso et al., 2017). Primary neurons from E16 WT mice were transduced at 1DIV with AAVs containing IGF2-HA or empty. In addition, at 7DIV, primary neurons were exposed to aSyn PFFs and aSyn monomers by 72 h. Then, we evaluate aSyn aggregates by western blot. Therefore, these results demonstrate that aSyn PFF are internalized by primary cortical neurons (Figure 12). In addition, aSyn released to extracellular media was evaluated using dot blot technique. Results demonstrate that transduced cortical neurons with AAV-IGF2-HA present a significative decrease in intracellular inclusions of aSyn load compared to AAV-empty transduced neurons (Figure 12A and 12B). aSyn release do not show significative differences observed between AAV-empty and AAV-IGF2-HA neurons incubated with aSyn monomers and aSyn PFFs. However, we observed a significative increase of aSyn released in AAV-IGF2-HA transduced neurons incubated with mixture condition compared to AAV-empty as a control (Figure 12C-F).

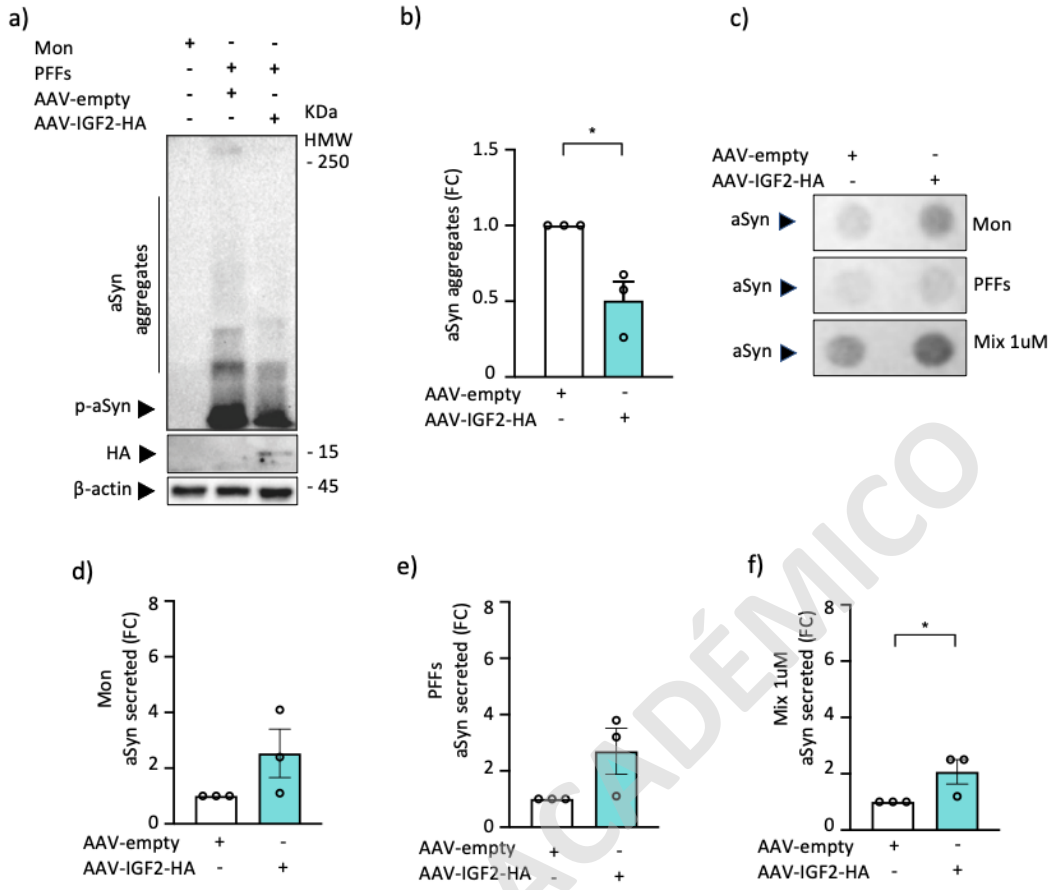


Figure 12: IGF2 overexpression decreases intracellular aSyn aggregates in primary cortical neurons exposed to aSyn PFFs. Primary cortical neurons were transduced with AAV-IGF2-HA or AAV-empty vector at 1DIV. At 7DIV, cortical neurons were exposed to aSyn species during 72h. a) Intracellular aSyn aggregates were evaluated by western blot technique. b) aSyn intracellular aggregates quantification fold change (FC) normalized to b-actin. c) Culture media was obtained from primary neuron cultures and aSyn released was detected by dot blot technique. Quantification of aSyn released to extracellular media from primary neurons incubated with d) aSyn monomers, e) aSyn PFFs and f) mixture conditions. Data shown represent the means of three independent experiments performed (bars are means ± SEM). *t-test one tail* was performed. p-value, *P<0.05. Statistical analysis for AAV-IGF2-HA compared to AAV-empty present a p-value 0.0761 (aSyn monomers exposed) and 0.0532 (aSyn PFFs exposed).

Taken together, in vitro results obtained in two cellular models, SHSY5Y cell line and primary culture of cortical neurons, which were exposed to aSyn PFFs to promote toxicity and cell death, demonstrate that IGF2 treatment has a neuroprotective effect in both human and murine PD cellular models. Therefore, our results suggest that treatment with IGF2 has a beneficial effect on aSyn pathology in the proposed cellular models.

Given our previous obtained *in vitro* results, where the effect of IGF2 overexpression has been demonstrated to have a beneficial effect in a Syn pathology, we asked whether IGF2 treatment impacts in aSyn pathology in a *in vivo* model. To answer this question, we propose model idiopathic PD in a murine model (Figure 5).

For this, is known that a pathological hallmark of PD is the loss of the nigrostriatal DAergic neurons and the presence of intraneuronal cytoplasmic inclusions predominantly composed by aSyn protein. The progressive degeneration of DAergic neurons results in an imbalance of the nigra-striatal circuit leading to clinical features of PD.

A variety of animal models have been developed for aSyn pathology research. To model aSyn pathology in mice, stereotaxic injections of aSyn PFFs has been used due to aSyn propagates trough the brain, affecting different areas leading to neurodegeneration (Luk et al., 2012c; Patterson et al., 2019; Peelaerts et al., 2015b). Idiopathic PD models based on aSyn PFFs administration in mice enhanced aSyn intracellular accumulation, which are paSyn-positive staining, neuronal loss of DAergic neurons, and motor behavior impairment (Chung et al., 2019).

In order to modulate aSyn pathology *in vivo*, we perform unilateral stereotaxis injections in the striatum of 3 months old WT C57bL/6j mice. Additionally, with the aim of increase IGF2 expression in SN, we employed a genetic tool of gene delivery using a

recombinant viral vector AAV (rAAVs) containing the genetic sequence to increase IGF2 levels (AAV-IGF2) (S.-H. Chen et al., 2019; Y. H. Chen et al., 2018).

First, unilateral stereotaxic injections were performed to inoculate 5ug/2uL of aSyn PFFs into right hemisphere of striatum WT mice. Contralateral hemisphere was injected with PBS 1x as injected control hemisphere (Figure 13). In addition, to evaluate the motor performance in experimental groups we performed motor test at three time points during the experiement. At this point, when aSyn PFFs were inoculated into the mice brain, we performed the first evaluation of motor performance in experimental groups (Figure 19).

Past 6 weeks, AAV-IGF2 stereotaxis injections were performed in right hemisphere to overexpression of IGF2 in SN. As a control, a GFP plasmid vector (AAV-GFP) was injected. At this point of the experiment, when AAV-IGF2 or AAV-GFP was injected, the second evaluation of motor performance were assessed (Figure 19).

After 12 weeks from the aSyn PFFs injections, mice were euthanatized, and brain tissue was collected for analysis. We evaluated the impact of IGF2 injection in paSyn-positive intracellular inclusions, DAergic neuronal loss, changes in number of dendritic spines and motor performance.

Several studies demonstrates that aSyn PFFs mice model not only seed the aggregation of endogenous mouse Syn in WT mice, but also propagates to other brain regions including SN (Luk et al., 2012c). To validate our *in vivo* PD mice model, we evaluated the presence of aSyn aggregates in striatum tissue of both experimental groups detecting paSyn-positive striatal neurons by immunohistochemical analysis. GABAergic (gamma-aminobutyric acid) medium spiny neurons (MSN) represent more than 95% of neurons in striatum and can be identify by immunofluorescence using an antibody against DARPP-32 (DA and cyclic AMP- regulated phospho protein, 32 kDa), begin the most

commonly used MSN markers in rodent models (Avanes et al., 2019; Matamales et al., 2009; Ouimet et al., 1998).

Three random images per hemisphere in the dorsal striatum were obtained and quantified the total number of DARPP-32 and paSyn-positive cells in both hemispheres by cell count (Figure 13). Then we calculate the average of the striatal neurons from three animals analyzed and calculate the percentage of paSyn/DARPP-32-positive neurons. To analyze these images, we used ImageJ software.

Total number of DARPP-32-positive neurons in both hemispheres were quantified. Non significative differences were observed between both experimental groups (Figure 13B). Similar, DARPP-32 positive neurons quantified in ipsilateral hemisphere do not show differences between both experimental groups (Figure 13C). Then, we quantified paSyn/DARPP-32-positive neurons resulting in a significative decrease of paSyn-positive cells in AAV-IGF2 injected mice compared to AAV-GFP mice (Figure 13D and 13E).

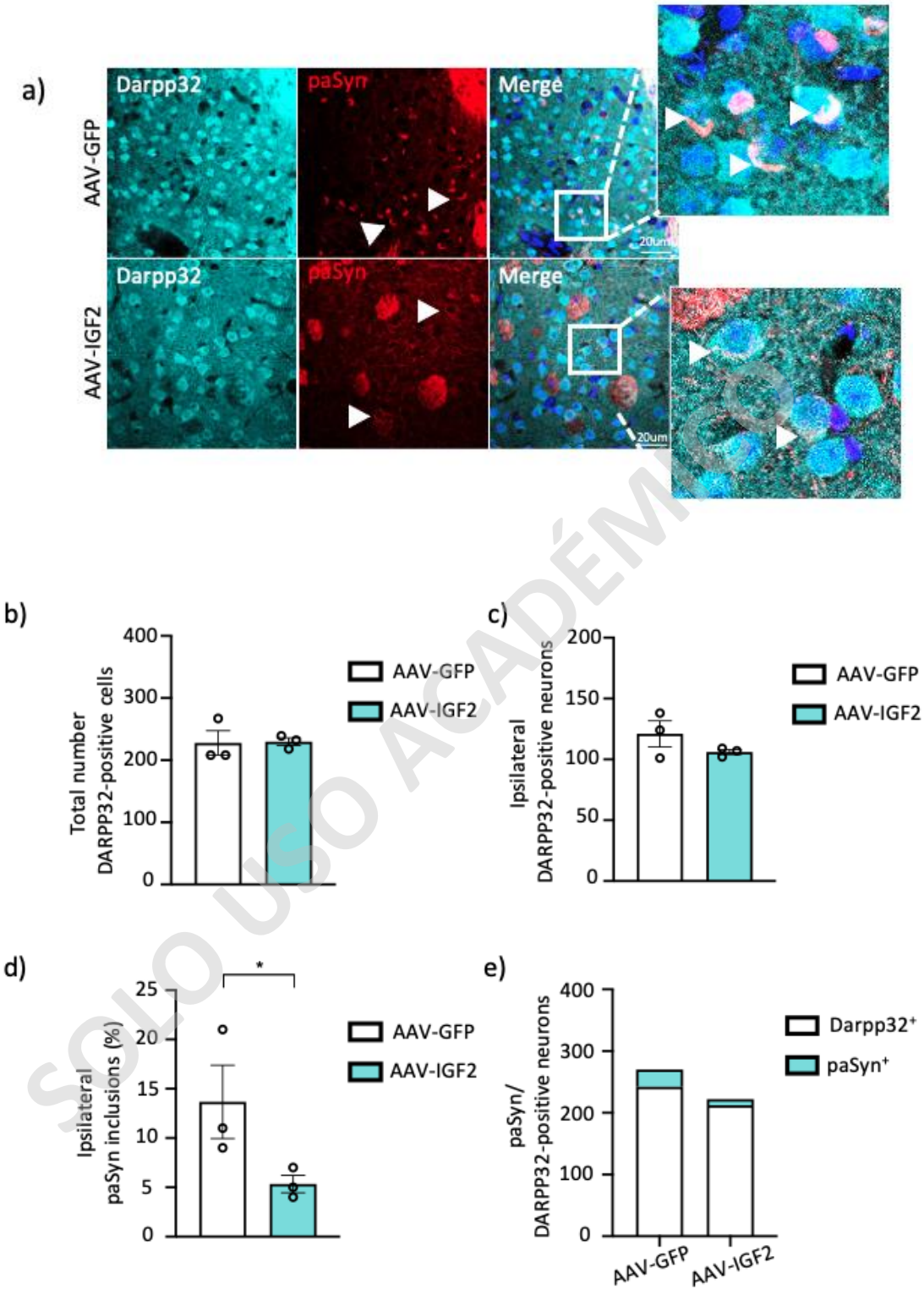


Figure 13: IGF2 overexpression decreases the number of paSyn-positive neurons in striatum PD mice model. Unilateral stereotaxis injections were performed in mice to inoculate aSyn PFFs (5ug/2uL) into the striatum of WT mice. After 6 weeks, unilateral injections to inject AAV-IGF2 or AAV-GFP in SN. a) Immunolabeling in ipsilateral hemisphere of injected mice were performed to detect paSyn-positive inclusions in MSN of striatum tissue. MSN were detected using anti-Darpp32, aSyn inclusions were detected using anti-paSyn. b) total number of DARPP-32-positive neurons. c) DARPP-32-positive neurons in ipsilateral hemisphere. d) paSyn/DARPP-32-positive inclusions in ipsilateral hemisphere. e) paSyn/DARPP-32-positive inclusions in neurons from both hemispheres. Data shown represent the means of 3 animals (n=3) analyzed per group (bars are means ± SEM). *t-test one tail* was performed; statistical significance differences was observed in paSyn/DARPP-32-positive neurons in ipsilateral hemisphere of AAV-IGF2 injected mice compared to control. p-value, *$p < 0.05$.

Therefore, our results showed that intrastriatal inoculation of aSyn PFFs develops paSyn-positive inclusions into striatum mice 12 weeks post-injection. In addition, AAV-IGF2 administration into the brain decreases the number of paSyn-positive neurons in striatum of AAV-IGF2 injected mice compared to control (Figure 13).

Next, we plan to evaluate the effect of IGF2 in DAergic innervation loss from SN to the striatum (Figure 14). For this purpose, 40x images were taken in confocal microscope in striatum of both experimental groups (AAV-GFP and AAV-IGF2). Three images per hemisphere were taken in dorsal striatum (Figure 14A). To evaluate DAergic innervation, striatum slides were stained by immunofluorescence using anti-TH antibody to detect DAergic projections (Figure14A). Fluorescence intensity of TH labeling was measured to quantify DAergic innervation in striatum tissue of both animal groups. These results indicate that there is no significant difference in TH levels in the striatum of animals injected with AAV-IGF2 or AAV-GFP at 90 dpi (Figure 14C).

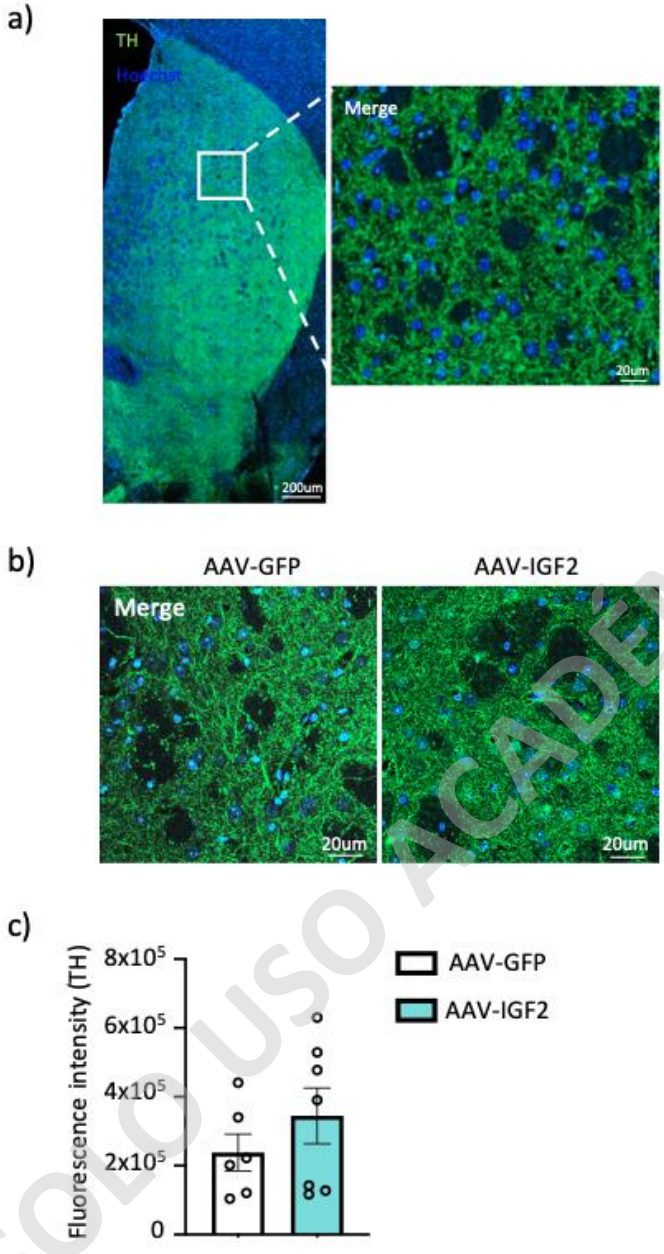


Figure 14: IGF2 overexpression do not show significative differences in DAergic innervation compared to control in aSyn PFFs mice model. Stereotaxis injections were performed in WT mice to inject aSyn PFFs (5ug/2uL) into dorsal striatum. After 6 weeks, AAV-IGF2 or AAV-GFP were injected into SN of aSyn PFFs model. a) Overview of injected hemisphere showing DAergic innervation (*green*) in striatum tissue, three random images were taken in three different sites. b) DAergic innervation was detected using anti-TH antibody in striatum tissue of brain slides. Hoechst was used to detect cellular nuclei. c) TH fluorescence intensity quantification. Images are representative of AAV-GFP (n=6) and

AAV-IGF2 (n=7) animals analyzed per group. Scale bar = 20 um. Confocal 880 Zeiss 40x/1.1 NA Air. Data shown represent the means analyzed animals (bars are means ± SEM). *t-test one tail* was performed; no statistical significance differences was observed between AAV-GFP and AAV-IGF2.

Stereotaxic injections of aSyn PFFs target the dorsal striatum mice, which is interconnected with multiple brain areas including DAergic neurons of SN. Additionally, several studies demonstrates that aSyn PFFs inoculation seed aggregation of soluble endogenous aSyn observing aSyn aggregates in SN. For this reason, we aim to evaluate the impact of AAV-IGF2 treatment in paSyn-positive inclusions in SN. For that, we performed immunostaining in SN tissue of ipsilateral hemisphere using an antibody against paSyn for both experimental groups (Figure 15). Integrated density of paSyn in SN was measured, showing that AAV-IGF2 injected mice decreases significatively paSyn levels compared to control AAV-GFP *in vivo* (Figure 15B).

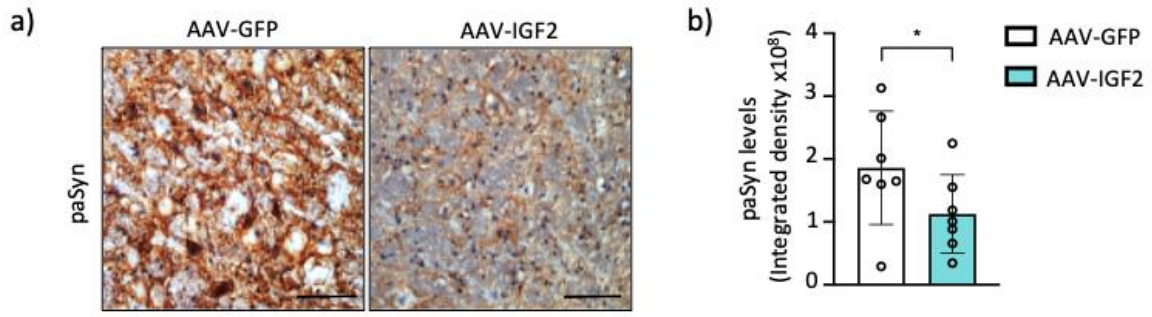


Figure 15: IGF2 administration decreases paSyn levels in SNpc of PD mice model. Unilateral stereotaxis injections were performed in WT mice to inject aSyn PFFs (5ug/2uL) into dorsal striatum. After 6 weeks, AAV-IGF2 or AAV-GFP were injected into SN of aSyn PFFs model. Contralateral hemisphere was used as a control. a) IHC using anti-paSyn antibody to detect the aSyn accumulation in SNpc from AAV-GFP of AAV-IGF2 injected mice. d) Integrated density was measured to quantification of paSyn levels from b). Data shown represent the means of (n=7) animals per group (bars are means ± SEM). *t-test one tail* was performed; statistical significative differences was observed in paSyn levels between AAV-GFP and AAV-IGF2. Scale bar = 50 µm.

Additionally, due to aSyn pathology is related to neurodegeneration and DAergic neuronal loss in SN, we evaluated TH levels performing immunostaining using anti-TH antibody in SN of both experimental groups. TH levels was measured by integrated density of TH staining (Figure 16). We observed significative differences between experimental groups, demonstrating that AAV-IGF2 treatment prevents the decline in TH levels in comparison with control injected mice (Figure 16B). Thus, results show that AAV-IGF2 administration in SN of PD mice model, decreases paSyn-positive levels, effect that could be related to the results observed in TH levels, suggesting that IGF2 treatment has a protector effect in the loss of TH levels by the decrease in aSyn accumulation in SN of PD mice model (Figure 15 and 16).

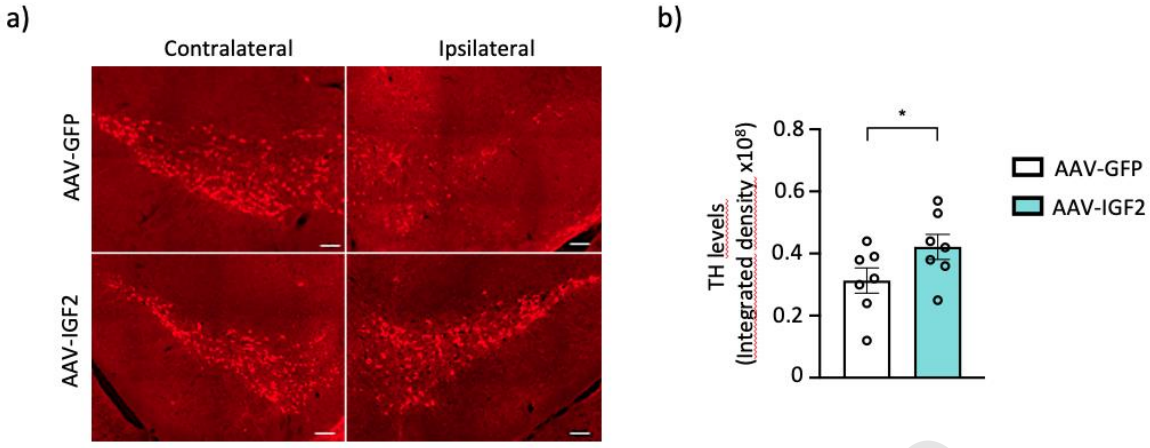
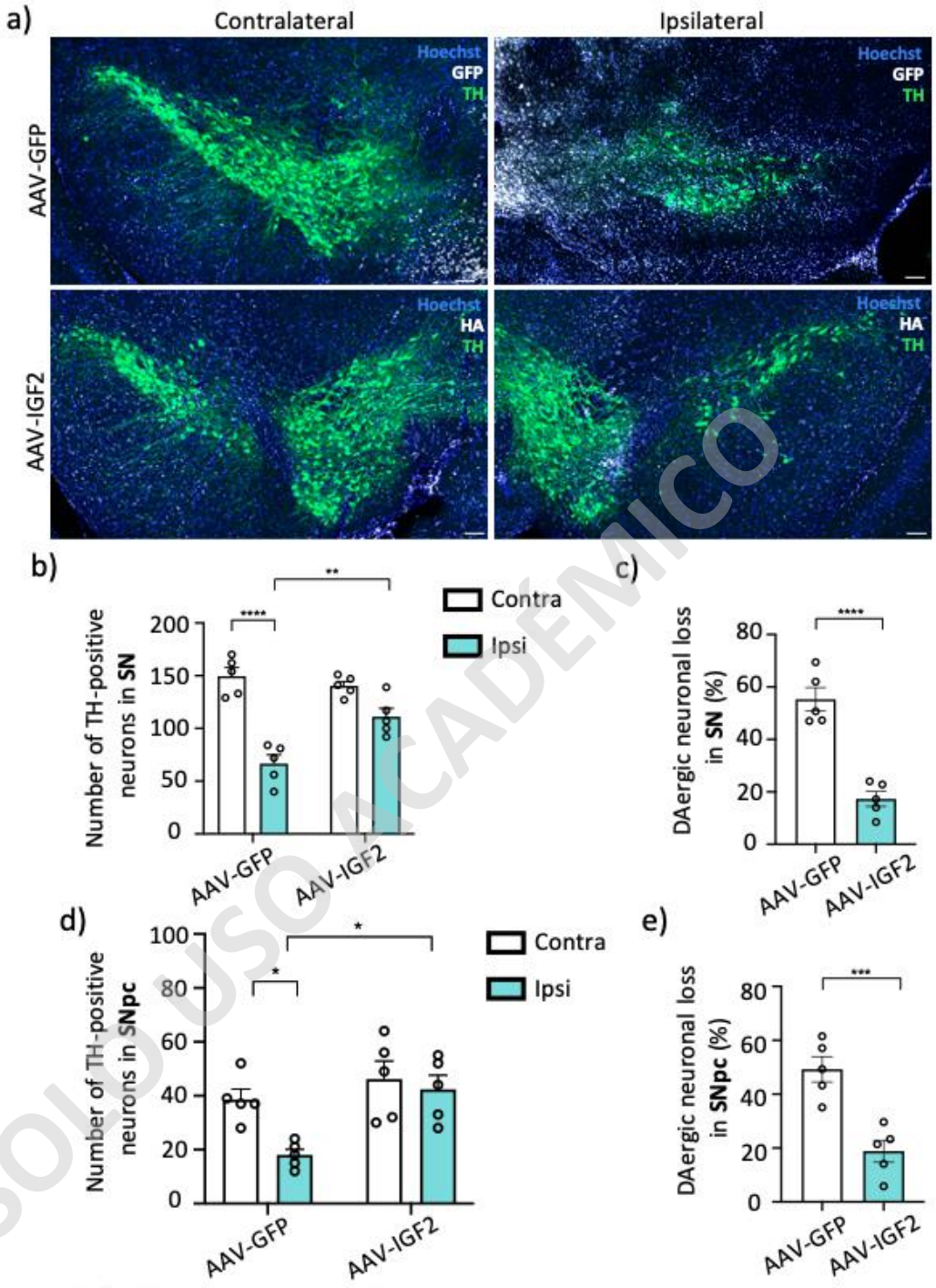


Figure 16: IGF2 treatment prevents decline of TH levels in SN tissue of PD mice model. a) Immunolabeling of TH-positive neurons in SN tissue of AAV-GFP and AAV-IGF2 mice. b) TH levels in SN tissue was quantified by integrated density in both hemispheres of PD mice model. Barr scale = 50 um. Data shown represent the means of (n=7) animals per group (bars are means ± SEM). *t-test one tail* was performed; statistical significative differences was observed in paSyn levels between AAV-GFP and AAV-IGF2.

Due to administration of IGF2 using AAVs vector into the brain of PD model (AAV-IGF2), reduce paSyn accumulation, which is involved to trigger degeneration and neuronal death in PD, we aim to evaluate the number and loss percentage of DAergic neurons in SN in both experimental groups (Figure 17). For this purpose, we perform TH staining of DAergic neurons using anti-TH antibody in SN slides tissue. To demonstrate the effect of IGF2, we quantify TH-positive neurons in SN of AAV-empty and AAV-IGF2 injected mice. We determined the number and loss percentage of DAergic neurons in ipsi and contralateral hemisphere (Figure 17C, 17E, 17G and 17I). Also, we assess the number of DAergic neurons in other regions of SN, such as, SNpc, SNpr and VTA (Figure 17B, 17D, 17F and 17H).

A significative DAergic neuronal loss in SN is observed in injected hemisphere (ipsilateral) compared to non-injected hemisphere (contralateral) in aSyn PFFs injected mice (Figure 17B and 17C). In addition, our results demonstrate a significative prevention of TH-positive neuronal loss in SN and SNpc region observed in IGF2 injected mice compared to control mice (Figure 17B-17E). No significative differences were observed for neuronal number and loss percentage of SNpr and VTA (Figure 17F-17I).

Then, a significant loss of DAergic neurons in SNpc at 90 dpi is observed in mice inoculated with aSyn PFFs. These findings demonstrate that the effect of IGF2 administration using a viral vector therapy in PD models rescues aSyn-induced neuronal death. Specifically, we evidence a positive contribution of IGF2 in the loss of DAergic neurons in the SNpc region, which are vulnerable neurons in the disease.



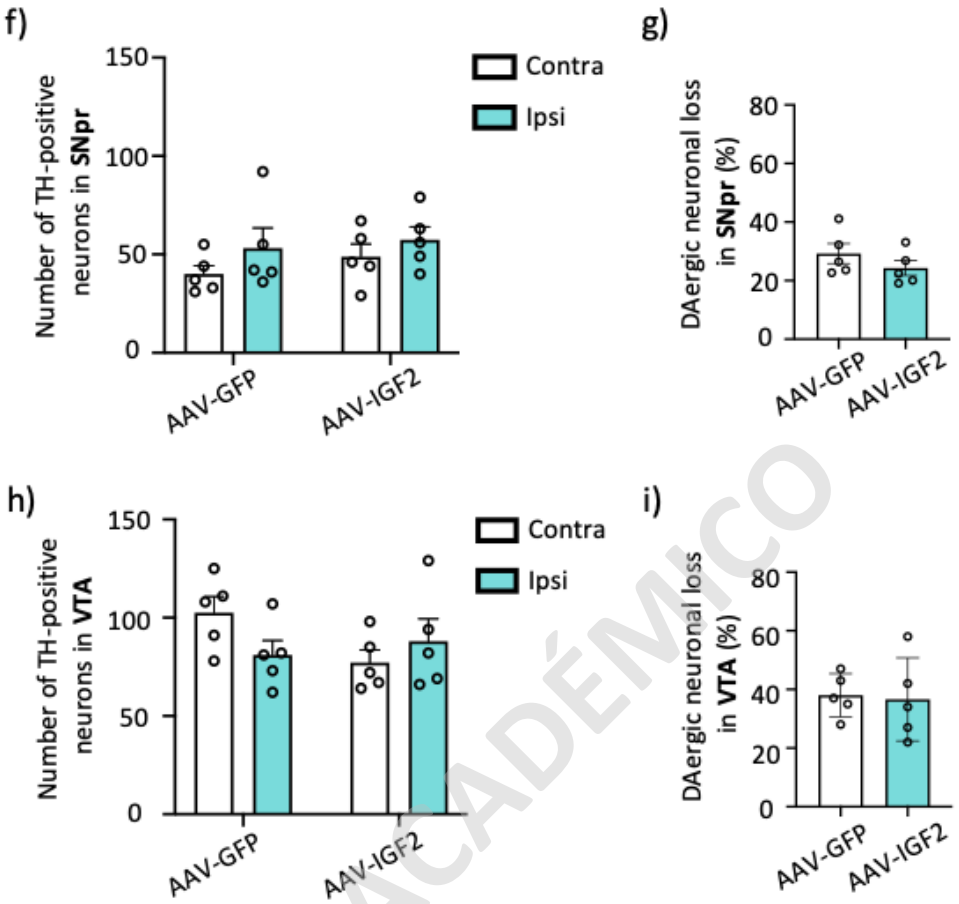


Figure 17: Gene therapy to deliver IGF2 into SN rescues DAergic neuronal death in PD mice model. C57BL/j6 mice were injected in the right hemisphere of striatum to inoculate aSyn PFFs (5ug/2uL). Over 6 weeks, we used gene therapy to deliver AAV-IGF2 or AAV-GFP into ipsilateral SN of PD mice model. a) Immunolabeling of DAergic neurons in SN was detected using anti-TH antibody (1:250). Image shows the loss of DAergic neurons in injected side of aSyn PFFs injected mice. Number of TH-positive neurons were quantified in total b) SN, d) SNpc, f) SNpr and h) VTA. Additionally, neuronal loss percentage in ipsilateral hemisphere was calculated in same regions of c) SN, e) SNpc, g) SNpr and i) VTA. IGF2 administration reduces significatively the loss of DAergic neurons in SNpc. Other regions of SN, VTA and SNpr do not present significative differences between both experimental groups. 3 MIP comprising images of coronal sections of 200 um thickness. 2 tiles images. Confocal 880 Zeiss 10x/0.45 NA Air. Data shown represent the means (n=5) animals per group (bars are means ± SEM). Two-way ANOVA test followed by Tukey`s multiple comparisons post-hoc test was performed to compare number of TH-

positive neurons in both experimental groups; p-value, *p < 0.05, **p < 0.01, ***p < 0.001; *t-test one tail* was performed to compare neuronal loss percentage.

It has been described that neurons undergo structural changes in the density, morphology and ultrastructural features of their dendritic spines in PD animal models (Calabresi et al., 2023). In this context, there is a lack of understanding about the precise synaptic level changes that are associated in this neurodegenerative pathology. Furthermore, we asked whether AAV-IGF2 administration has an effect in synaptic changes in mice model of PD.

To investigate the effect of IGF2 at synaptic level in aSyn PFFs mice model, we analyzed the spots number to detect dendritic spines in both AAV-IGF2 and AAV-GFP experimental groups of PD mice model (Figure 18). In order to evaluate morphological changes in synaptic connections, we decided to detect a synaptic marker in SN tissue of our experimental group mice. Synaptophysin is a calcium-binding glycoprotein and the most abundant integral membrane protein of small synaptic vesicles. It is present in presynaptic region of neurons, suggesting an involvement in synaptic vesicle endocytosis and begin one of the most common synaptic markers used for the study of synaptic structures in CNS research field (Bai and Strong 2014; Hobson et al. 2022; Kolos, Grigoriyev, and Korzhevskyi 2015; Pyeon and Lee 2012; Sortwell et al. 1998).

Then, brain slides were immunolabeled to detect DAergic neurons using anti-TH antibody, synaptophysin as a presynaptic marker and DAPI to detect nuclei in SN (Figure 18A). Images were taken at 40x magnification in confocal microscope, deconvolved in Huygens software, and analyzed in Imaris software.

We analyzed a large number of neuronal TH-positive filaments and the presence of spots at 2.5 um of distance from the center of the neuronal filament. We analyzed ~ 2.800 neuronal filaments for AAV-GFP condition and ~ 9.400 neuronal filaments analyzed for AAV-IGF2 animal groups (Figure 18B). In order to simplify the obtained data, the spots present in

fragments of 10 um of neuronal filaments were measured. Results show that IGF2 injection in SN increases significatively the spots number in DAergic neurons compared to the control group (Figure 18C).

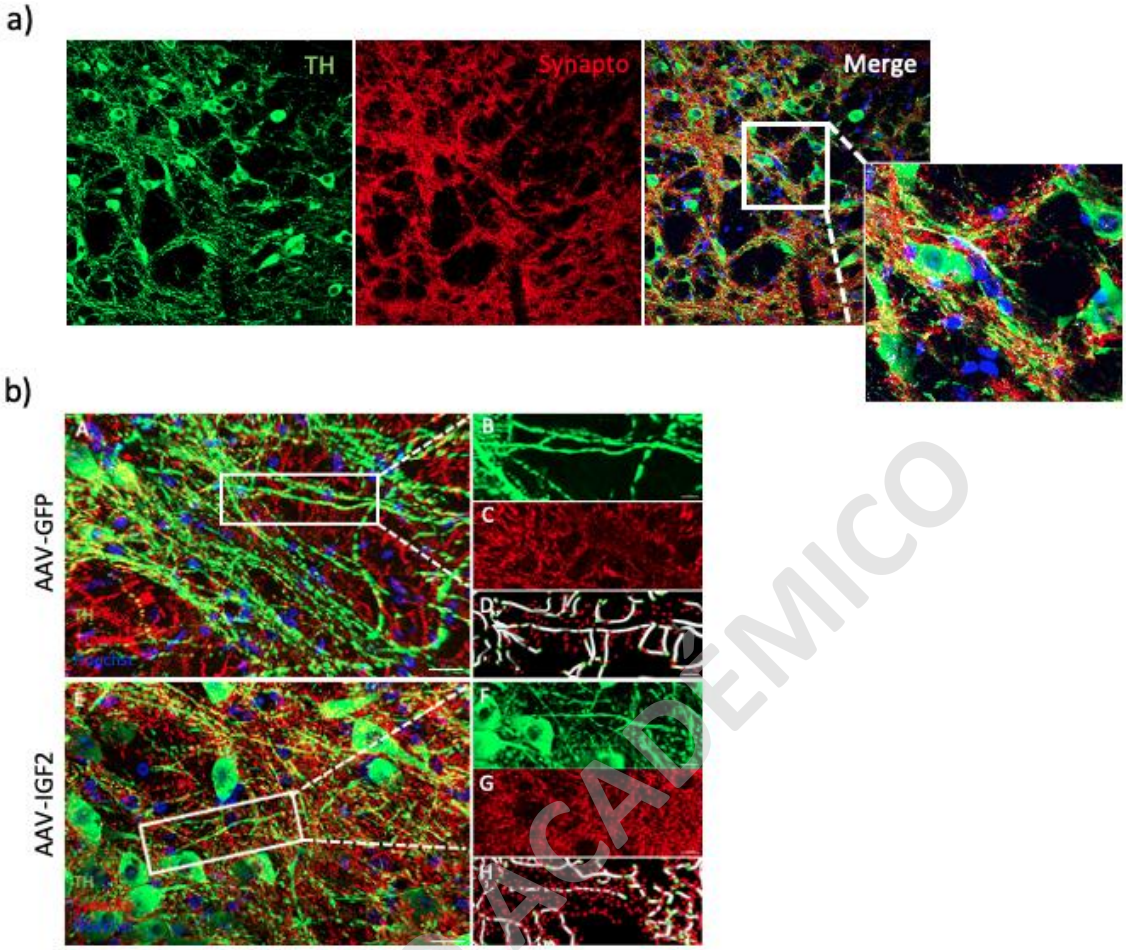


Figure 18: IGF2 treatment rescues synaptophysin levels loss in aSyn PFFs mice model. C57BL/j6 mice were injected in the right hemisphere of striatum to inoculate aSyn PFFs (5ug/2uL). Over 6 weeks, we used gene therapy to deliver AAV-IGF2 or AAV-GFP into ipsilateral SN of PD mice model. a) Representative images of immunolabeling in brain slices detecting TH-positive neurons in SN of PD mice model. DAergic neurons were detected using anti-TH antibody. anti-synaptophysin was used as a presynaptic marker and Hoechst to detect cellular nuclei. b) Representative images show the selection of analyzed neuronal filaments (grey dendrite) and spots (red spots) in both experimental groups. c) High magnification of neuronal filaments and spots analyzed. Comparison between AAV-empty and AAV-IGF2 conditions. d) Graph represent quantification of spots number present in 10um of neuronal filaments. Spots present at a distance of 2.5 um from the center of the neuronal filaments were analyzed. Data represents ~ 2.800 neuronal filaments analyzed for AAV-GFP condition and ~ 9.400 filaments analyzed for AAV-IGF2. *t-test one-tail* unpaired test was performed; p-value, *$p < 0.05$. Images were analyzed using *Imaris* software. Scale bar = 20 um. Confocal 880 Zeiss 40x/1.1 NA Air.

Based on our results, which demonstrate the beneficial impact of administration of AAV-IGF2 gene therapy into the brain of aSyn PFFs PD mice models, we achieve to understand the consequences of the absence of *Igf2* gene in mice PD mice model.

In order to evaluate the development of aSyn pathology in a *Igf2* lacking condition, we employ a *Ifg2*$^{-/-}$ knockout mice, IGF2KO, which was obtained from Jackson Laboratory (materials and methods). Briefly, transgenic mice were generated by electroporation, in embryonic stem cells, inserting a replace vector in first encoding exon of *Igf2* gene containing a neomycin resistant cassette. Phenotype of transgenic mice is viable and fertile, however show a significative decrease in body weight (DeChiara et al., 1990).

Then, we perform unilateral stereotaxis injections of *in vitro* generated aSyn PFFs (5ug/2uL) in dorsal striatum of C57BL/j6 WT and IGF2KO mice (Figure19). Contralateral hemisphere was used as aSyn PFFs control injection.

Both groups, WT and IGF2KO, shows a reduction in number of TH-positive neurons of SN in ipsilateral hemisphere compared with contralateral (Figure 19A and 19B). Additionally, we calculate DAergic neuronal loss percentage in SN of ipsilateral hemisphere (Figure 19C). The results obtained do not reveal significant differences in the number of TH-positive neurons in the SN tissue of WT mice compared to IGF2KO mice. Taken together, results indicates that the absence of IGF2 does not exacerbate aSyn-induced neuronal death in the SN of PD mice model (Figure 19).

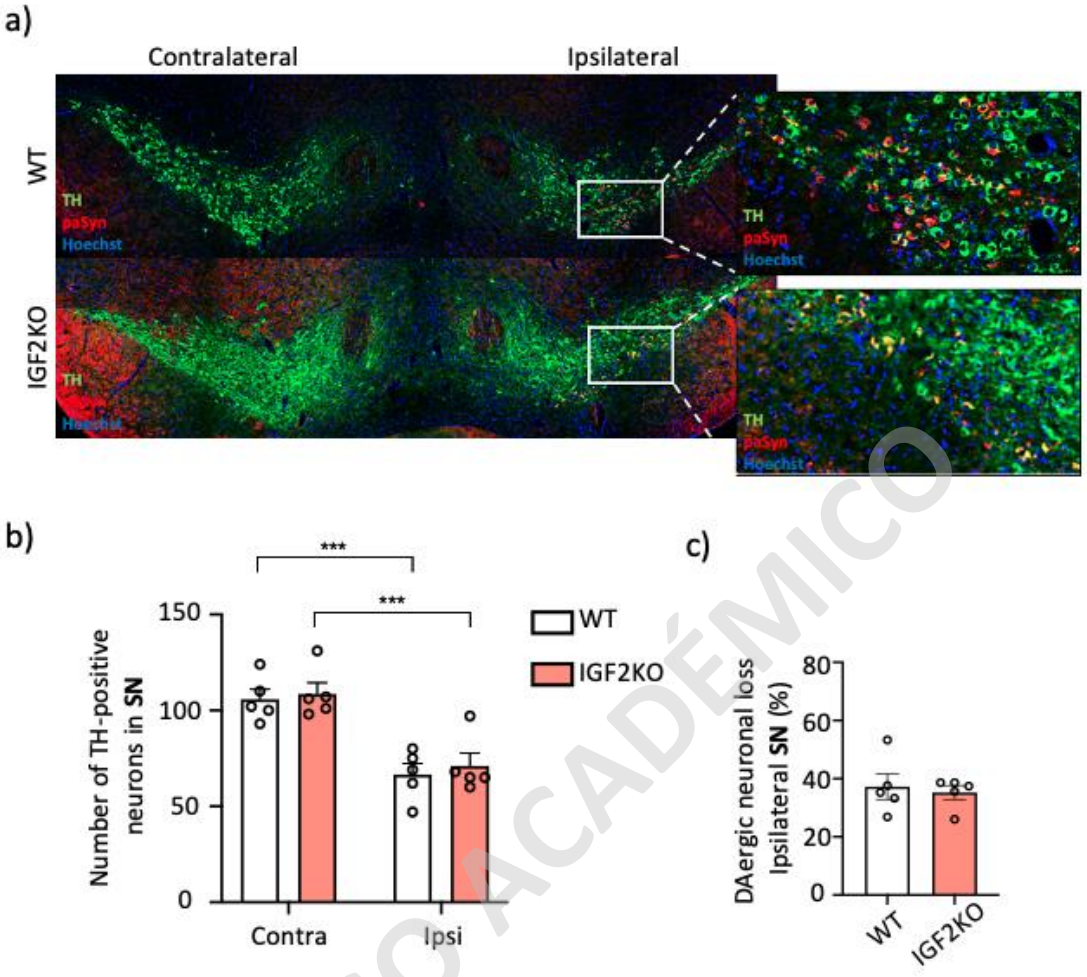


Figure 19: IGF2KO mice do not show significative differences in neuronal loss induced by aSyn pathology in mice model. Unilateral stereotaxis injections were performed to inoculate aSyn PFFs (5ug/2uL) into striatum mice of WT mice and IGF2KO transgenic mice. After 12 weeks samples were collected and DAergic neuronal loss was assesed. a) Immunostaining of brain sections to label DAergic neurons using anti-TH antibody *(green)*, paSyn to detect aSyn inclusions *(red)* and Hoechst to label nuclei *(blue).* b) Quantification of TH-positive neurons in SN of WT and IGF2KO mice. c) Graph represents percentage of neuronal loss in ipsilateral SN of both experimental groups. Data shown represent the means of (n=5) animals per group (bars are means ± SEM). Two-way ANOVA test followed by Tukey`s multiple comparisons post-hoc test was performed to compare number of DAergic neurons; p-value, ***p < 0.001; *t-test one tail* was performed to compare neuronal loss percentage.

Since clinical features in PD patients are often characterized by motor symptoms that lead to insufficiency coordinate and execute movements, and current therapies approaches aim to alleviate motor symptoms, we decided to evaluate whether IGF2 administration improves motor disfunction in aSyn PFFs PD mice model (Mazzoni et al., 2012; Moustafa et al., 2016b).

Since several studies in PD models demonstrates that PD mice models develop neurodegeneration leading to appearance of motor symptoms observed, we aim to evaluate motor performance in our experimental groups (Dovonou et al., 2023; Han et al., 2020; Luk et al., 2012c).

Finally, motor coordination and balance were assessed by the Beam walking and Cylinder test. The goal of this test is for the mouse to stay upright and walk across an elevated narrow beam to a safe platform. Thus, motor performance was carried out on Beam and Cylinder test by three times; first, before aSyn PFFs injections were performed (time 0), before AAV-GFP and AAV-IGF2 injections (6 weeks), and finally at the end of the experiment, 12 weeks from aSyn PFFs injections (Figure 19). At 12 weeks, we observed significative differences between AAV-GFP and AAV-IGF2 injected mice showing that AAV-IGF2 decreases the error number mice compared to control injected mice (Figure 20A). In addition, no significative differences between both groups were observed in Cylinder test (Figure 20B). Thus, our results demonstrates that intracerebral IGF2 administration into SN of PD preclinical model, improves motor performance induced by aSyn pathology evaluated by beam motor test.

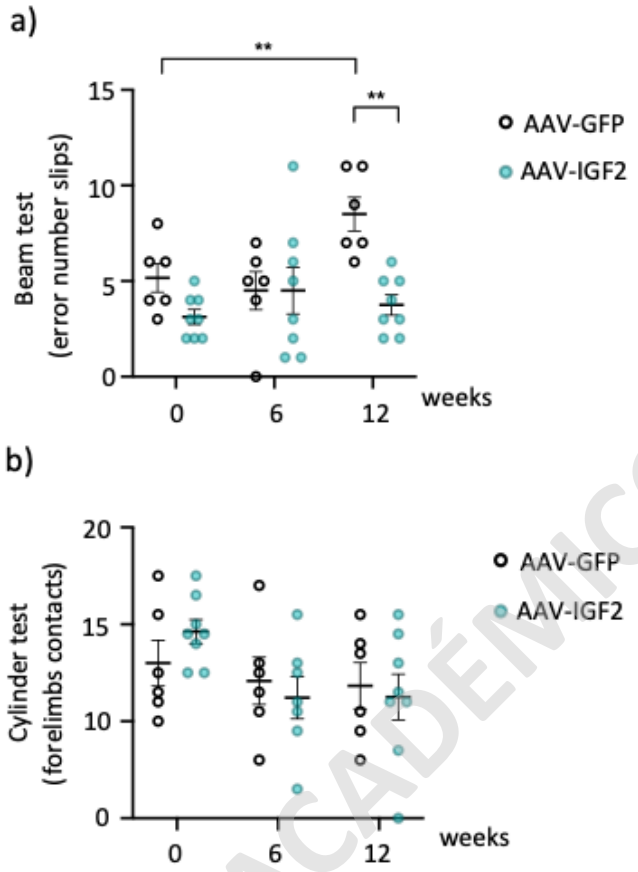


Figure 20: Motor performance is improved by IGF2 gene therapy in PD mice model. Unilateral stereotaxis injections were performed in striatum mice to inoculate aSyn PFFs (5ug/2uL) to model aSyn pathology. After 6 weeks AAV-IGF2 or AAV-GFP injections were performed in ipsilateral SN. Motor performance was evaluated at 0, 6 and 12 weeks by a) Beam and b) Cylinder test. Significative differences are observed in Beam test, indicating that aSyn PFFs injected mice increase the error numbers, which is recovered by IGF2 administration at 12 weeks. Data shown represent the means of AAV-GFP (n=6) and AAV-IGF2 (n=8) animals (bars are means ± SEM.). Two-way ANOVA test followed by Tukey`s multiple comparisons post-hoc test was performed; p-value, *$p < 0.05$, **$p < 0.001$.

6. DISCUSION

aSyn protein is implicated in a spectrum of neurodegenerative disorders, including MSA, LB dementia, and genetic and idiopathic cases of PD. These diseases share common neuropathological features, notably the formation of aSyn inclusions observed in post-mortem tissue of affected patients.

aSyn plays a relevant role in the pathogenesis of PD, leading to the impairment physiological processes, such us, disturbances in mitochondrial function, dysfunction of the autophagy-lysosome system, impairment in axonal transport, synaptic dysfunction, neurotransmitter release abnormalities, and a disbalance in the proteostasis system have been described in PD pathology. The collective contribution of these processes is intricately linked to the initiation of neurodegeneration and subsequent neuronal death in PD (Braak et al., 2004; Surmeier et al., 2017).

Currently, new preclinical model based on aSyn misfolded has been reported and recapitulated several symptoms associated to idiopathic PD, such as aSyn propagation, motor dysfunction and DAergic neuronal loss. The inoculation of aSyn PFFs into the mice striatum region induces intracellular LB-like inclusions in neurons and disseminates through other brain areas (Chung et al., 2019; Hansen et al., 2011a, 2011b; Luk et al., 2012c; Ulusoy et al., 2015).

Dopaminergic neuron viability.

Also, several studies have demonstrated that cell lines and primary neuronal cultures exposed to aSyn PFFs initiate a degenerative process through a proteostasis imbalance leading to cell death (Vasili et al., 2022a; Volpicelli-Daley et al., 2014c).

We demonstrate that our *in vitro* and *in vivo* PD model recapitulates the neuropathology associated with aSyn in PD. SHSY5Y cell exposed to aSyn PFFs exhibited a significant reduction in cellular viability in a dose-dependent manner (Figure 10). In addition, the murine PD model inoculated with aSyn PFF in dorsal striatum replicates pathological features, such as, intracellular accumulation of aSyn, DAergic neuronal loss in SN, and motor impairment (Figure 13, 15 and 20) (Chung et al., 2019; Volpicelli-Daley et al., 2014b). In this study, to determine the effect of IGF2 in cellular models of PD, we evaluate two different approaches as IGF2 treatments. rmIGF2 and rhIGF2 treatment to stimulate IGF2 receptor and the overexpression of IGF2 for transient transfection with IGF2-HA vector to increase IGF2 levels in cells. Both IGF2 treatment provides beneficial effect protecting neurons from aSyn-cytotoxicity, due to mechanism mentioned above (Figure 7, 9 and 12).

Others peptide such as neurotrophin-4, a member of a family of neurotrophic factors, trigger neuroprotective effect against several insults that lead to neuronal death in SHSY5Y cell line. They demonstrate that evaluated protective compounds by LDH released and MTT reduction show significative differences against evaluated insults suggesting that effect of this compounds act downstream of mitochondrial impairment. However, neurotrophin-4 do not show differences in MTT reduction, suggesting that protective effect of this neurotrophic factor acts upstream of mitochondrial dysfunction. Since IGF2 treatment do not show significant differences by the MTT assay, when mitochondria are damaged observed by a decrease in MTT reduction in cultures treated with a Syn PFFs, suggest that IGF2 also can act upstream mitochondrial dysfunction. Supporting this, a study indicate aSyn-induced cell death is mediated via caspase activation in LUHMES cells, a human dopaminergic cell line,

also, indicate that caspase activation occurs upstream of mitochondrial pathways of aSyn toxicity these dopaminergic neurons (Höllerhage et al., 2017).

While several articles have suggested the occurrence of different types of neuronal death in neurodegenerative environment, the most commonly associated type of cell death in PD is apoptosis (Hirsch et al., 1999; Mochizuki et al., 1996; Yasuda et al., 2007).

Moreover, the incubation of primary neurons with aSyn PFFs resulted in the activation of both caspase 8, an initiator caspase in the extrinsic pathway, and caspase 9, an initiator caspase in the intrinsic pathway. This suggests that aSyn PFFs initiate apoptotic mechanisms leading to DAergic neuronal death in PD model (Mahul-Mellier et al., 2015).

Our results demonstrated that IGF2OE reduces the number of PI-positive neurons in neuronal cultures incubated with aSyn PFFs, indicating a protective effect against aSyn-induced toxicity. Based on these findings we can speculate that the positive effect of IGF2 is linked to apoptotic processes triggered by aSyn (Figure 6 and 7). However, it is important to assess the protective effect of IGF2 on apoptotic neuronal death induced by aSyn complementing our results with specific techniques that evaluate, for example, morphological characteristics of apoptosis and caspase activation.

aSyn misfolded protein

Physiologically, aSyn is founded in equilibrium between a soluble cytosolic form and a partially alpha-helical membrane-bound fraction. Emerging evidence from in vitro studies indicates that aSyn is secreted into the extracellular space through nonclassical exocytosis pathway (Danzer et al., 2012b; Lee et al., 2005; Mochizuki et al., 2018; Wong & Krainc, 2017c). Recent findings highlight involved organelles such us late endosomes in release of

misfolded proteins, including aSyn, through exocytosis via exosomes into the extracellular space (Alvarez-Erviti et al., 2011; Fussi et al., 2018).

We reported that IGF2 treatment result in a decrease in intracellular levels of aSyn in in vivo and in vitro settings (Figure 12, 13 and 15). We could attribute the decrease of intracellular aSyn inclusions may be related to a reduction in protein synthesis or an increase in degradation such us autophagy mechanism.

In this context, our recent study evidence that IGF2 decreases the accumulation of intracellular mHtt aggregates in different cell cultures models of HD, demonstrating that this phenome was independent of the activity of autophagy and the proteasome pathways. Interestingly, this study demonstrates that IGF2 signaling enhance the secretion of soluble mHtt through exosomes and microvesicles. (García-Huerta et al., 2020b).

Furthermore, we demonstrate that IGF2 also promotes aSyn release to the extracellular media in neuroblastoma cell line and primary cortical neurons, suggesting that the mechanism by which IGF2 decrease intracellular load of aSyn is by promoting its secretion into the extracellular environment. Based on this evidence, and since several studies demonstrate that aSyn is secreted via the exosomal pathway, it is important to assess aSyn secretion employing techniques that allow for the modulation of protein secretion and extracellular vesicle biogenesis in IGF2 context. Researchers have demonstrated the use of genetic and pharmacological tools enables the inhibition of secretion mechanisms through extracellular vesicles, such exosomes and microvesicles.

Additionally, this study evidence that AAV-IGF2 administration into the brain reduce paSyn levels and prevents significatively the loss of vulnerable neurons in the SNpc.

Furthermore, the results obtained from in vivo and in vitro models of PD allow us to establish a link between the reduction in intracellular aSyn load and its impact on cell viability. This

can be attributed to the decrease in intracellular aSyn burden, resulting in reduced cellular damage and preventing the loss of dopaminergic neurons.

Afterwards, IGF2 is a member of the IGFs family, sharing structural similarities with IGF1 and insulin. These polypeptides exhibit mitogenic properties, enhancing cell proliferation, growth, survival and differentiation of neurons in the nervous system. While IGF2 ligand exhibits a stronger affinity for IGF2R, it can also form associations with lower affinity to IGF1R an IR (Hawkes et al., 2006; Sakanosb et al., 1991; Torres-Aleman, 2010;Hawkes et al., 2006; Hawkes & Kar, 2004a).

Due to IGF1R and IR present a tyrosine kinase domain, several studies have been demonstrating that IGFs signaling are involved in neurogenesis, metabolism, cell growing, cell differentiation, through the activation of this receptors triggering cellular signaling pathways that play a role in diverse biological processes in cell response (Alvino et al., 2011). (O'Kusky & Ye, 2012; Okuyama et al., 2021; Manning & Toker, 2017; Okuyama et al., 2021; Trejo et al., 2004; Yang et al., 2018). For example, IR activation, predominantly stimulates mitogenic signaling providing protection from antiapoptotic signals (Sciacca et al., 2003). Also, IGFs activate signaling pathways involved in cell survival, AKT/PI3K signaling has been described as the major regulator of cell survival and growth (Manning & Toker, 2017).

Regarding to IGF2R, is been described that plays a crucial role in the intracellular trafficking of lysosomal enzymes from the trans-Golgi network to the lysosomes for degradation. Also, IGF2R regulate the extracellular levels of IGF2 ligand promoting its endocytosis and leading to lysosomal degradation (Hawkes & Kar, 2004b).

However, IGF2R can initiate specific cellular responses possibly involving the activation of heteromeric G proteins and downstream calcium signaling. Additionally, it has been implicated in activating PKC and MAP kinases, contributing to a complex network of intracellular signaling pathways (Hawkes & Kar, 2004a).

Taken together, we suggest that the neuroprotective impact of IGF2 is mediated through the activation of IGFs receptors triggering signaling pathways associated with neuronal survival. Based on our results, we demonstrate that IGF2 effect is related to the activation of IGF2R, then, we can speculate that IGF2 activation can be involved in protein secretion mechanism to decrease loading of neurotoxic species observed in neurodegenerative diseases as PD (Figure 8). However, in this study, we did not demonstrate the activation of the IGF1R or IR signaling pathways stimulated by IGF2 ligand. Thus, we cannot discard the involvement of these receptors in the protective effect of IGF2. Therefore, it is important to elucidate the signaling pathway through which IGF2 is exerting its neuroprotective effect, through the activation of its own receptor or through the activation of the others IGFRs.

Moreover, dendritic spines are neuronal protrusions extensions to form a specialized structure that constitute synaptic connectivity and signaling in nervous system. Morphological spine types are characterized in mushroom, stubby, tinny, and filopodia (Pchitskaya & Bezprozvanny, 2020). Spines present constant structural changes, in their morphology like size and shape, number and density that are essential for synaptic and neuronal plasticity in response to neuronal activity (Gipson & Olive, 2017; Nimchinsky et al., 2002; Yoshihara et al., 2009). There are many mechanisms and molecules involved in regulations that underly plasticity of neural function (Nimchinsky et al., 2002; Yoshihara et al., 2009). Studies suggest that signalling pathways that govern cellular functions are

required for the assembly of synaptic structures, such as PKA, JNK and PI3K/AKT pathways (Jiang et al., 2021).

Specifically, dendritic spines are vulnerable in many neuropathological conditions. Synaptic degeneration has been involved in many neurological disorders including, Dementia, motor neurons diseases, AD, HD and PD (Honer et al., 2019; Morigaki & Goto, 2017).

Since synaptic dysfunction is known in vulnerable DAergic neurons in PD, it is important to study changes in synaptic connections within a neuronal pathology, focusing on the morphological, functional and density levels characteristics of the dendritic spines, which seems to be a key structure in the development of these neurodegenerative pathologies.

Several techniques to identify synaptic proteins using antibodies are used to target presynaptic and postsynaptic regions of the synapses. Presynaptic markers are widely used to evaluate synaptic density, morphology and functions. Many proteins that participate in several synaptic process such proteins of SNARE complex that play a crucial role in exocytosis and intracellular vesicle trafficking are principal targets of synaptic markers (Burré et al., 2010; Kolos et al., 2015).

One of the most commonly utilized markers for presynaptic proteins is synaptophysin, a major membrane protein found in the membrane of synaptic vesicles representing a □90-95% localization on synaptic terminals. Its ubiquity at the synapse has led to the use of synaptophysin immunostaining for synaptic quantification. Also, is usefully for studies of mice and rat models and human samples (Calhoun et al., 1996).

Studies have been evidenced an important loss of synaptophysin in neuronal pathologies such, dementia with LB, AD, PD and other neurodegenerative diseases (Bate et al., 2010)

In addition, in vitro studies shows that human wild type aSyn, accumulation in PD, MSA and Dementia with LB, triggers synaptophysin loss in primary mouse cortical neurons and adult mouse hippocampal neurons (Lowder & Liddle, 1975). Additionally, cell culture models of PD show the relevance to study the role of synaptophysin in the pathogenesis of PD, characterizing the expression of synaptophysin in different DAergic cell lines such us SHSY5Y neuroblastoma cell line (Bai & Strong, 2014).

In this study, we observed that IGF2 administration in to the brain, significatively prevents the loss of synaptophysin levels in SN of PD mice model. We quantified spots/filaments from synaptophysin labeling representing dendritic spines in SN filaments, thus, we can speculate that IGF2 expression significantly prevents the loss of the number of dendritic spines in SN region.

IGF2 neuroprotective effect in PD preclinical model

Moreover, aSyn has been shown to associate with synaptic vesicles as a function of neuronal activity. Synaptic dysfunction is related to phenomena where presynaptic proteins are recruited by aSyn aggregates leading to neurotransmitter release impairment which decreases synaptic connections (Burré et al., 2018; Day et al., 2006). We demonstrate that IGF2 overexpression decreases intracellular aSyn levels in vitro (Figure 18), therefore, we could suggest that the protective effect of IGF2 on the loss of dendritic spines could be due to the fact that a reduction in the intracellular load of aSyn leads to a diminished in the recruitment of synaptic proteins and with this, a delay in synaptic damage by preventing the loss of dendritic spines.

Importantly, studies provide evidence of the involvement of nuclear factor k-B (NF-kB) signaling in regulation of synaptic plasticity through structural changes, formation and

maturation. In addition, have been demonstrate the participation of IGF2 in this signaling, where IGF2 is directly targeted by NF-kB in vitro and in vivo and identified NF-kB–Igf2–Igf2R signaling axis tightly controlling the number of synaptic connections in hippocampal cultures and remodeling of synaptic connections. Also, gene therapy to increase IGF2 levels in hippocampus induces synaptic formation of WT mice and IGF2 restores spine density and excitatory synaptic transmission in AD Tg2576 mice model (Kaliman et al., 1999; Pascual-Lucas et al., 2014).

Given that DAergic neurons in the SN form synapses with other neuron types from different regions, it is crucial to consider future research to determine whether the beneficial effect of IGF2 on synaptophysin loss is specific to a particular type of synapse in the SN. Additionally, it is important to assess the postsynaptic terminals located on the dendrites to elucidate the effect of IGF2 specifically on these postsynaptic terminals of DAergic neurons. Furthermore, we speculated that the absence of IGF2 could increase vulnerability in DAergic neurons in aSyn pathology. However, we demonstrated that under IGF2 ablation conditions using IGF2KO transgenic mice, no significant differences in neuronal loss were observed in aSyn pathology, indicating that the lack of IGF2 does not increase susceptibility and, consequently, does not lead to significant neuronal death in the context of aSyn pathology. This could be explained by a possible compensatory effect of other neurotrophic factors such as insulin-like factors. Therefore, we can suggest that the lack of IGF2 does not increase the vulnerability of DAergic neurons in the SN in PD model.

Based on these results, we can argue that the absence of this neurotrophic factor might be supplied by compensatory mechanisms that maintain homeostasis involving other members of the IGFs family. On the other hand, it is crucial to emphasize that the consequences of the absence of a molecule within a biological system can generate an effect related to the

intrinsic function of the molecule in question, which could be supplemented by these compensatory mechanisms. Nevertheless, our study demonstrates that the neuroprotective effect of IGF2 in this pathology is observed due to an increase in the expression levels of this factor, which could be linked to the activation of other potential protective mechanisms not directly related to its own function.

Finally, we monitored the motor performance of PD mice model injected with AAV-IGF2 or AAV-GFP using the beam and cylinder test (Figure 20). The beam and cylinder test are both experimental assessments commonly used in preclinical research to evaluate motor function in rodents, particularly in the context of neurodegenerative diseases. Basically, beam test indicates deficits related to balance and coordination, while the cylinder test is better suited to detect asymmetrical motor deficits (Luong et al., 2011; Magno et al., 2019). Considering that motor symptoms start asymmetrically in PD and we do not observe significant differences in the cylinder test, we can suggest that the motor impairment in mice injected with aSyn PFFs is related to an impairment of motor coordination and not the presence of asymmetrical symptoms in the limbs.

In this context, future research IGF2 gene therapy for long time, allowing the observation of its effects in a model exhibiting severe motor symptoms. Thus, by extending the duration of the aSyn pathology or using a more aggressive model of PD, we can assess the protective effect of IGF2 on a functional aspect of the disease.

Neuropathologically, PD is characterized by a reduction in DA levels in the striatum. This DA deficiency results in the motor symptoms commonly associated with PD, such as tremors, rigidity, bradykinesia, and postural instability. Our model recapitulates the progression of the disease, as evidenced by mild motor deterioration, particularly in balance

and coordination, observed in the beam test. Additionally, we have observed a decrease in tyrosine hydroxylase (TH) levels in the striatum, indicating axonal degeneration, which was not rescued by treatment with IGF2.

In addition, several findings in the field suggest that the degeneration of the nigrostriatal pathway initiates in axons. Human brain imaging conducted in vivo indicates that presynaptic abnormalities precede the onset of symptoms in PD. These studies indicate the likelihood of presynaptic dopaminergic dysfunction in individuals affected by PD before its onset.

Based on our results, we propose future investigations employing a model that recapitulates acutely the motor symptoms of PD. Furthermore, we suggest evaluating longer durations of IGF2 expression in our model to observe advanced motor dysfunction. This approach will allow us to assess the functional effect of IGF2 within the context of symptom onset.

Finally, our current study proposes that IGF2 plays a significant role in aSyn pathology, enhancing its secretion in two distinct cellular models of different species. This underscores the relevance of the IGF2 effect in both human and mouse neuronal cultures, proposing the IGF2-promoted secretion of aSyn as a common mechanism across different cell types.

Due to the IGF2 treatment promotes aSyn secretion in cellular models, we hypothesize that, *in vivo*, aSyn may potentially degraded by glial cells and avoided its propagation of neighbor's neurons. Additionally, recent studies have proposed that the degradation of aSyn occurs at a faster rate in astrocytes compared to neurons (Gee & Keller, 2005; Joe et al., 2018; Lim et al., 2018; Loria et al., 2017; Pitt et al., 2017).

Interestingly, a recent study from our laboratory, evidence a significative reduction of IGF2 plasma levels in PD patients compared to healthy individuals. Additionally, examination of

blood samples from HD and PD patients revealed a significative decrease of IGF2 levels (García-Huerta et al., 2020b; Sepúlveda et al., 2022). Therefore, evidence demonstrates the deficiency of IGF2 related to pathological contexts of HD and PD, enhancing the relevance to understand the role of IGF2 in the development of neurodegenerative pathologies.

In summary, our research position IGF2 as a promising candidate molecule for PD treatment. Given that IGF2 is a soluble factor, the exploration of gene transfer strategies to enhance IGF2 levels in the brain may emerge as an attractive approach for future therapeutic development.

7. CONCLUSIONS AND PERSPECTIVES

Our study proposes IGF2 as an interesting candidate for the treatment of PD pathology using gene therapy. The model proposed in this research is based on the accumulation of aSyn in brain tissue, which has an important role in the development of the genetic and idiopathic cases of PD. We could suggest that IGF2 gene therapy have beneficial effects in PD mice models and is possible apply this therapeutic approach in PD patients in the future.

To understand the underlying mechanisms of aSyn accumulation and its impact on neurodegeneration is an active area of research in the field of PD. Interestingly, it must be emphasized that aSyn has been described as a prion-like protein involved in a range of neurodegenerative diseases, known as synucleinopathies. Moreover, in PD, it is implicated in both genetic and sporadic cases, making it a focal point of interest for the study to develop treatment in these pathologies.

The goal behind the use of neurotrophic factors in treating these diseases is to prevent neurodegeneration of damaged neurons, which could potentially slow down or even reverse the degenerative process. However, the practical application of this approach has proven to be a significant challenge. The direct administration of neurotrophic factors through injections or infusions can present side effects.

In this context, several approaches, such as gene therapy and stem cell therapy, have been investigated to sustainably release neurotrophic factors in the brain. Despite promising advances in preclinical research, there are still technical and safety challenges to address before these approaches become efficient treatments (Troncoso-Escudero et al., 2020).

In summary, neurotrophic factors have been the focus of scientific interest for decades due to their potential in treating neurodegenerative diseases. However, translating these findings into effective therapies for patients remains an active and developing area of research, with technical and safety challenges still to be overcome.

8. SUPPLEMENTARY FIGURES

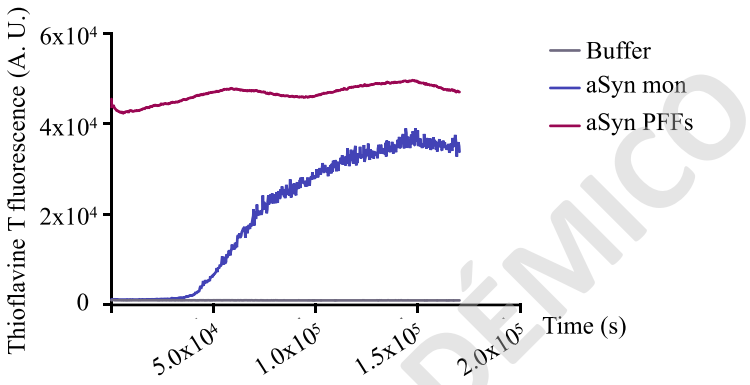


Figure 1: Validation of amyloid structures by thioflavin T assay of aSyn PFFs generated from recombinant monomers of aSyn. Measurement of fluorescent signal from mouse aSyn monomer and aSyn PFFs simples. A PBS 1x buffer was used as a negative control. All measurements are expressed as relative fluorescent units (A.U). Data shown represent the triplicate by three independent experiments.

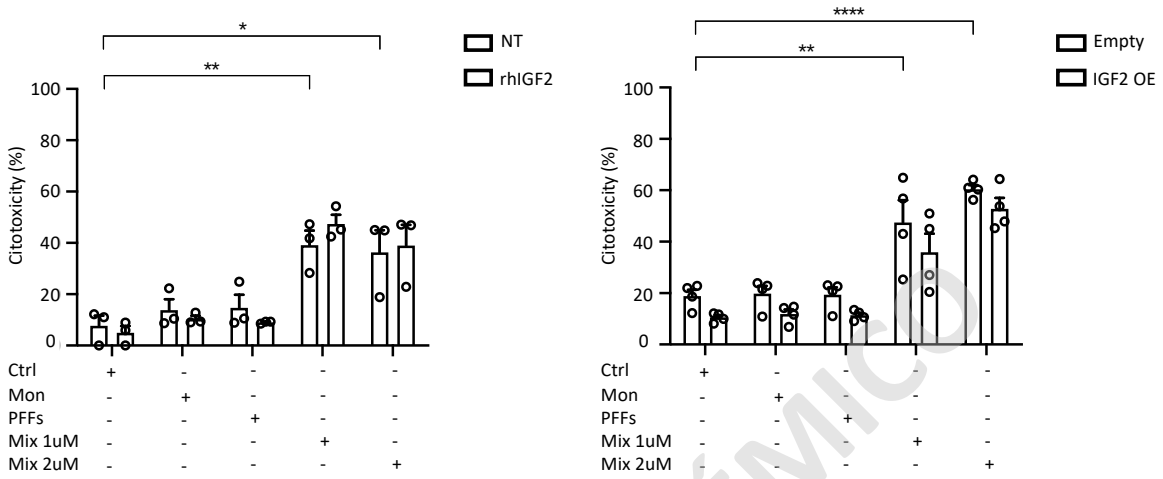


Figure 2: Effect of rhIGF2 and IGF2OE in neuroblastoma cell line exposed to aSyn PFFs in cell damage evaluated by LDH assay. SHSY5Y cells were exposed to human aSyn monomers (Mon), human aSyn PFFs (PFFs) and aSyn mixture. To evaluate the effect of IGF2, cells were stimulated with rhIGF2 1h previously to aSyn exposure. In addition, we increase expression level of IGF2 by transient transfection (IGF2OE). After 72 hrs, to evaluate cell damage we performed LDH assay. Data shown represent the means of three (rhIGF2) and four (IGF2OE) independent experiments performed in triplicate for each condition (bars are means ± SEM.). Two-way ANOVA test followed by Tukey`s multiple comparisons post-hoc test was performed. p-value, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

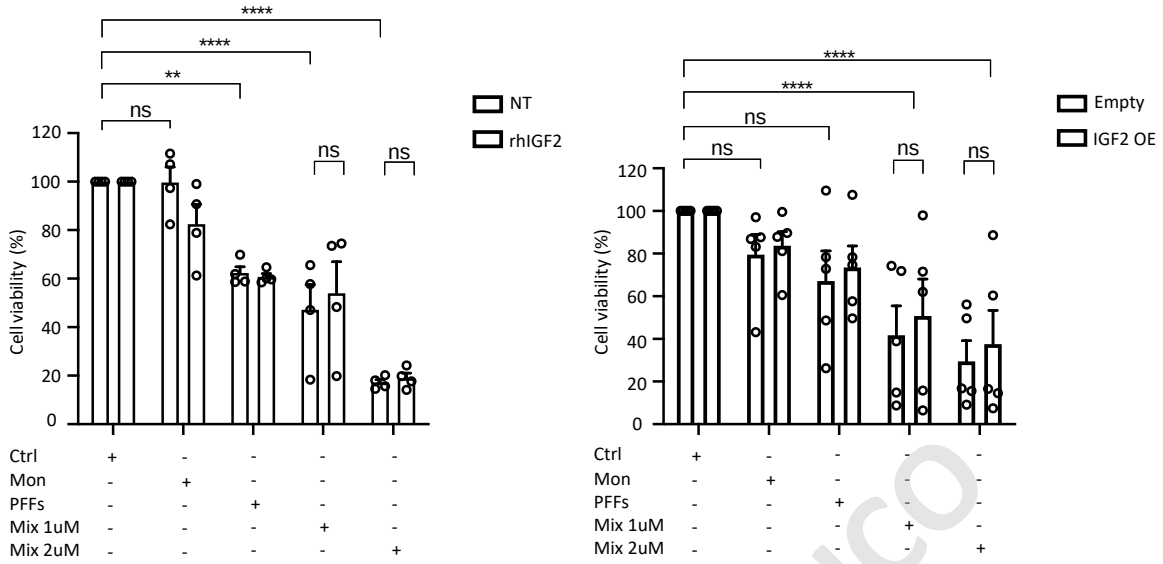


Figure 3: Cell viability in neuroblastomas cell cultures exposed to aSyn PFFs in rmIGF2 and IGF2OE evaluated by MTT assay. SHSY5Y cells were exposed to human aSyn monomers (Mon), human aSyn PFFs (PFFs) and aSyn mixture. To evaluate the effect of IGF2, cells were stimulated with rhIGF2 1h previously to aSyn exposure. In addition, we increase expression level of IGF2 by transient transfection (IGF2OE). After 72 hrs, to evaluate cell viability we performed MTT assay. Data shown represent the means of four independent experiments performed in triplicate for each condition (bars are means ± SEM.). Two-way ANOVA test followed by Tukey`s multiple comparisons post-hoc test was performed. p-value, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

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