Insulin degrading enzyme as a therapeutic target for Parkinson's disease

MARIANA GUARDA

A dissertation submitted in partial fulfillment of the requirements for the Degree of Masters in Biomedical Research

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Abstract

Parkinson's disease is the second most common neurodegenerative disorder, characterized by motor features that include tremor, bradykinesia and rigidity, possibly resultant from the loss of dopaminergic neurons in the *substantia nigra pars compacta*. A major pathognomonic feature is the neuronal accumulation of aggregated alpha-synuclein (aSyn), known to trigger neurodegeneration. Recent cohort association studies concluded that type-2 diabetes mellitus (T2DM) increases up to 380% the risk of PD development (young individuals with T2DM). Moreover, T2DM is also associated with faster motor progression and cognitive decline of PD patients. However, the molecular link between this association is not clear. We hypothesize that a possible molecular player underlying both conditions is the insulin degrading enzyme (IDE). IDE presents degradative capacity for different substrates including insulin or amyloid-beta peptide, but not aSyn. Interestingly, in pancreatic cells, IDE interacts with aSyn, preventing its aggregation and toxicity. Moreover, there is an inverse correlation between IDE and aSyn levels in the pancreas. Therefore, we hypothesize that diabetes may induce IDE impairment in the brain, therefore compromising its protective effects against aSyn pathogenicity. In this thesis we aimed to establish a relationship between IDE dysregulation and aSyn pathogenicity, using a two-pronged strategy: analysis of brain samples from animal models of (pre)diabetes; evaluation of IDE-aSyn interplay in a cellular model of Parkinson's disease. To that purpose, we analyzed protein extracts from brain tissue of WT mice under a control or a high-fat diet (HFD) for 12 weeks (pre-diabetic model). Remarkably, we showed that diabetes generally decreases the levels of IDE in all analyzed brain areas, at the exception of the *hippocampus*. In contrast, brain aSyn levels are increased in both *hippocampus* and *cerebellum* of these mice. Moreover, an inverse correlation between IDE and aSyn levels was observed (*cerebellum*). These findings support our hypothesis that diabetes may decrease IDE levels in the brain, and that aSyn levels inversely correlate with IDE levels. To better understand the interplay between IDE-aSyn, we used an established *in vitro* model of synucleinopathies, based on the overexpression of WT aSyn, or of an aggregation-prone variant of aSyn (SynT) in H4 cells. Notably, both catalytical active IDE-WT or catalytical inactive IDE-E111Q forms of IDE decrease the cytotoxicity of aSyn, but not of SynT. This confirms that IDE is able to reduce aSyn toxicity, and our findings suggest that it occurs in a catalytical-dependent manner. Intriguingly, both IDE-WT and IDE-E111Q increase the intracellular amount of aSyn. Since aSyn toxicity is suppressed, we hypothesize that IDE is diverting aSyn oligomeric/toxic species to more stabilized non-toxic species. In fact, we observed that IDE-E111Q is able to increase the solubility of SynT, and that both IDE-WT and IDE-E111Q favor the formation of larger inclusions of SynT, described to be less toxic. Altogether, we conclude that IDE may play an important role in the brain by suppressing aSyn pathogenesis, and that its potentiation could represent an appealing therapeutic target for Parkinson's disease. Moreover, a brain-IDE-targeted treatment could also have a great impact in T2DM individuals, to control their high risk to develop PD, particularly of individuals with higher impairment of IDE.

Resumo

A doença de Parkinson é a segunda doença neurodegenerativa mais comum. Esta doença apresenta várias manifestações motoras que incluem tremor, bradicinésia e rigidez, possivelmente resultantes da perda de neurónios dopaminérgicos na *substantia nigra pars compacta*. Uma característica subjacente à degeneração destes neurónios é a acumulação e a agregação de alfa-sinucleína (aSyn). Estudos epidemiológicos recentes concluíram que a diabetes mellitus tipo 2 (DM2) pode aumentar o risco de desenvolvimento de doença de Parkinson até 380% (em particular, caso a DM2 tenha sido desenvolvida entre os 25-45 anos de idade). Além disso, os doentes de Parkinson com diabetes apresentam uma progressão mais rápida dos problemas motores bem como um declínio cognitivo mais acentuado. No entanto, o mecanismo molecular subjacente a esta associação não é claro. Temos por hipótese que a enzima degradativa da insulina (IDE) possa ser responsável por esta associação. A IDE apresenta capacidade degradativa para diferentes substratos, incluindo a insulina ou o péptido beta-amilóide, mas não a aSyn. Curiosamente, nas células pancreáticas, a IDE interage com a aSyn, impedindo a sua agregação e toxicidade. Além disso, no pâncreas, os níveis de IDE correlacionam negativamente com os de aSyn. Portanto, acreditamos que a diabetes possa induzir uma redução dos níveis de IDE também no cérebro, comprometendo consequentemente os seus efeitos protetores contra a patogenicidade da aSyn. Nesta tese, o objetivo principal foi o de investigar se existe alguma relação entre a desregulação da IDE e a patogenicidade da aSyn no cérebro. Para tal, utilizámos duas estratégias. Por um lado, analisámos tecido cerebral provenientes de um modelo animal de (pré) diabetes. Por outro, investigámos a possível interação entre a IDE e a aSyn num modelo celular de Sinucleinopatias. Para tal, analisámos extratos proteicos provenientes de ratinhos C57BL/6J alimentados durante 12 semanas com uma dieta controlada ou com uma dieta hiperlipidica (modelo pré-diabético-HFD). Verificámos que com excepção do hipocampo, a diabetes diminui os níveis de IDE em todas as áreas cerebrais analisadas. Pelo contrário, os níveis cerebrais de aSyn aumentam quer no hipocampo quer no cerebelo destes ratinhos (pré) diabéticos. Além disso, constatámos que os níveis de IDE e de aSyn no cerebelo correlacionam-se inversamente. Estas evidências corroboram a nossa hipótese de que a diabetes pode diminuir os níveis cerebrais de IDE, aumentando potencialmente os níveis da aSyn. Para entender melhor a interação entre a IDE e a aSyn, usámos um modelo *in vitro* de sinucleinopatias estabelecido, que se baseia na sobreexpressão em células H4 da aSyn ou de uma variante de aSyn propensa a agregação (SynT). Observámos que quer a forma catalítica activa da IDE (IDE-WT) quer a forma inactiva da IDE (E111Q) têm capacidade de diminuir a citotoxicidade da aSyn, mas não da variante SynT. As nossas descobertas sugerem ainda que a capacidade protectora da IDE ocorre de uma forma dependente da sua actividade, já que a IDE-WT é mais eficaz do que a variante inactiva IDE-E111Q. Curiosamente, a IDE-WT e a IDE-E111Q aumentam a quantidade intracelular de aSyn. Dado que a toxicidade da aSyn é suprimida, pensamos que a IDE consiga converter espécies oligoméricas / tóxicas da aSyn em espécies não-tóxicas. De facto, observámos que a IDE-E111Q é capaz de aumentar a solubilidade da SynT e tanto a IDE-WT como a IDE-E111Q favorecem a formação de inclusões maiores de SynT, descritas como sendo menos tóxicas. Concluímos com este estudo que a IDE pode desempenhar um papel importante no cérebro, suprimindo a patogénese da aSyn. Propomos assim que a potenciação da IDE possa representar um alvo terapêutico relevante para o tratamento da doença de Parkinson. Além disso, um tratamento que vise aumentar a capacidade da IDE cerebral também pode ter um grande impacto no tratamento de doentes com DM2, de forma a reduzir o risco de desenvolverem a doença de Parkinson, principalmente em indivíduos que apresentem maior comprometimento da IDE.

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List of abbreviations

6-OHDA	6-hydroxydopamine
acetyl-CoA	Acetyl coenzyme A
AD	Alzheimer's disease
ADP	Adenosine diphosphate
aSyn	Alpha-synuclein
ATP	Adenosine triphosphate
AUC	Area under the curve
Aβ	Amyloid-beta
BBB	Blood brain barrier
BSA	Bovine serum albumine
Chx	Ciclyheximide
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
DBS	Deep brain stimulation
Dpp-4	Dipeptidyl peptidase-4
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ETC	Electron transport chain
EV	Empty-vector
fMRI	Functional magnetic resonance imaging
FoxO	Transcription factor
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GIP	Glucose-dependent insulinotropic peptide
GluTs	Glucose transporters
GSH	Glutathione
GSK-3B	Glycogen synthase kinase–3B
HFD	High fat diet
HK	Hexokinase
HSPs	Heat Shock Protein
HY	Hoehn and Yahr
IAPP	Islet amyloid polypeptide precursor
IDE	Insulin degrading enzyme

IDE-E111Q	Catalytic inactive form of IDE
IGF-1	Insulin-like growth factor
IR	Insulin receptor
LB	Luria Base
LDH	Lactate dehydrogenase
L-DOPA	Levodopa
LRRK2	Leucine-rich repeat kinase
MAO-B	Monoamine oxidase B
MAPK	Mitogen-activated protein kinase
MDS-UPDRS	Movement Disorders Society modified Unified PD Rating Scale
MGO	Methylglyoxal
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTOR	Mammalian target of rapamycin (mTOR)
NAC	Non-amyloidogenic component
NADPH	Nicotinamide adenine dinucleotide phosphate
NCD	Normal chow diet
NGS	Normal goat serum
PBS	Phosphate buffer saline
PD	Parkinson's disease
Pink1	PTEN-induced putative kinase 1
PPAR-γ	Peroxisome proliferator-activated receptor
PPP	Pentose phosphate pathway
PTMs	Post-translational modifications
R5P	Ribose-5-phosphate
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNpc	*Substantia nigra pars compacta*
SNPs	Single nucleotide polymorphisms
SOC medium	Super Optimal Broth with catabolite repression
SynT	C-terminally tagged aSyn variant more prone to aggregation
T1DM	Type 1 diabetes mellitus
TBS	Tris-HCl buffer saline
TBS-T	TBS supplemented with 10% tween-20 solution
TCA	Tricarboxylic acid cycle
NP-40	Tergitol-type NP-40

TH	Tyrosine hydroxylase
Tris-HCl	Tris hydrochloride
UPS	Ubiquitin–proteasome system
WT	Wild-type

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1. Introduction

Neurodegenerative disorders are age-related diseases that strongly affect the patients. Due to the absence of preventive and/ or restorative treatments, combined with the increase in the life expectancy, these diseases represent a major concern to our society [1]. The majority of neurodegenerative diseases are characterized by the misfolding, accumulation, seeding and aggregation of given proteins, and are therefore considered proteinopathies [2]. In particular, amyloid-beta (Aβ) peptide and tau in Alzheimer's disease (AD) and alpha-synuclein (aSyn) in Parkinson's disease (PD), are currently established as central proteins in the pathogenesis of these diseases [2]. These proteins become dysfunctional, aggregate and these species are believed to have the ability to spread throughout the brain in a prion-like manner [3]. The formation of these protein aggregates are thought to result from the misfolding of these proteins and consequent alteration of their conformation favoring their association with themselves and enter the so called oligomerization cascade forming high-order molecular structures [2]. Although protein aggregation plays a role in the disease pathogenesis, the mechanisms that trigger and control this process are still unclear. Aging is the major risk factor [4], genetics also play an important role, but there are also several environmental factors that may contribute for the development of these diseases, which are the focus of this thesis.

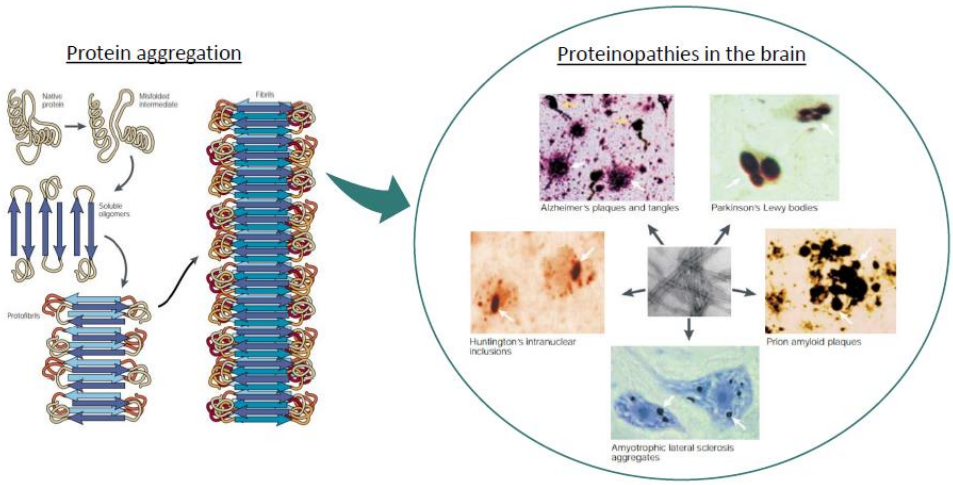


Figure 1. Schematic of Protein aggregation pathway and misfolding diseases

Upon synthesis, the native protein may undergo incorrect folding, forming misfolded intermediates that associate into higher magnitude species including oligomers, protofibrils and fibrils. Brain proteinopathies include Huntington's, Alzheimer's, Parkinson's and Prion diseases, as well as Amyotrophic lateral sclerosis, where huntingtin, Aβ and tau, α-synuclein, prions and superoxide dismutase 1 undergo protein misfolding and aggregation, correspondingly. Adapted from [2].

1.1 Parkinson's disease

PD is the most prevalent movement disorder and the second most common neurodegenerative disorder [5], following AD. PD affects more than 1% of the population over 60 years, a percentage that increases with

aging, reaching over 3% of incidence in people over 80 years old [6]. In Portugal, the most recent epidemiological studies estimate that there are approximately 20000 cases of PD [7].

The average age of PD onset is around 60 years [6], being more prevalent in men (1.5–2 times higher) that also present an earlier age of onset of about 2 years [8]. Although, women have a later onset of symptoms, they show a higher rate of tremor (67%) than men (48%) [8]. The reason why gender can affect disease onset and progression is still controversial. However, it has been postulated that gender-dependent features such as estrogens could have neuroprotective effects [9]. Additionally, the higher variability on the average age of onset of PD presented by different studies worldwide may suggest that geographical distribution affects the risk to develop PD, possibly changing the impact between genders [9].

The signs and symptoms of PD were firstly described by James Parkinson, in "An Essay on the Shaking Palsy" in 1817. He described the typical motor features that are still used for the clinical diagnosis of PD [10]. Later, Braak described that PD pathology starts at the peripheral nervous system and spreads to the central nervous system (CNS) [11]. PD symptoms usually begin unilaterally, in the anterior part of the body (hands and arms) spreading to the posterior (legs) [12]. The most common motor features include: resting tremor; gait impairment; postural instability; reduced step length and balance problems that accounts to a high risk for patients to fall and develop camptocormia (trunk flexion); muscle stiffness; bradykinesia; and rigidity [10]. However, PD is not only a motor disease. In fact, non-motor manifestations may precede the motor signs by decades and may represent the earlier manifestations of the disease. However, the early signs and symptoms of PD are unspecific and common to several other disorders. Approximately 90% of PD cases presented depression, anxiety and olfactory dysfunction. In some cases, along with motor control impairment, patients also develop deglutination and salivation problems. Although James Parkinson did not describe all the non-motor features, he explicitly defines evidences of hyposmia, constipation and sleep disorders, as rapid eye movement disorder , as well as dysfunction of the CNS to motor control [10].

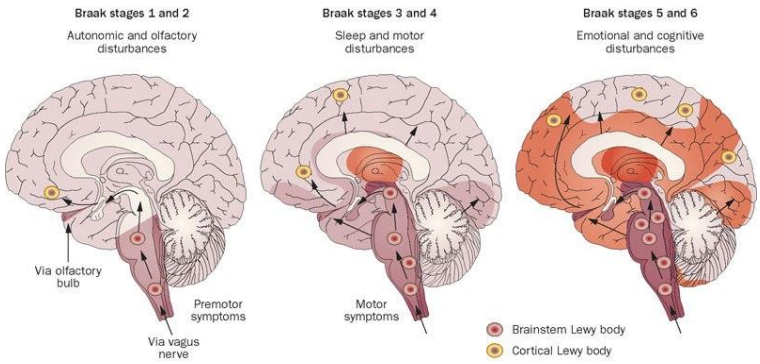


Figure 2. PD stages and progression.

According to Braak, PD initiates in the olfactory bulb and the *medulla oblongata*. aSyn related pathology is possibly initiated in the periphery entering the brain through the brain stem, where it forms Lewy bodies, until cortical regions, where it also aggregates and forms Lewy bodies. The red shading represents the pattern of PD progression according to the staging. Adapted from [13].

A major pathognomonic feature of PD is the loss of dopaminergic neurons in the *substantia nigra pars compacta (SNpc)*, which is currently accepted as be the major cause of the typical motor impairment observed in PD [12]. Moreover, patients also exhibit in the surviving neurons accumulated aSyn in the form of protein inclusions, named Lewy bodies [14]. Dopaminergic neurons represent 1% of the total number of brain neurons. Nevertheless, as the name implies, they are the main source of dopamine in the mammalian brain [15]. Dopamine controls the brain reward system [16,17] and it also plays an important role in regulation of movement activation, motivation and cognitive functions [18], upon binding to dopamine receptors [15]. Dopaminergic neurons are found in diencephalon, mesencephalon and the olfactory bulb [15]. They are involved in several systems, being the most characterized the nigrostriatal pathway, which originates in the *SNpc* (midbrain) and extends into the dorsal *striatum* [15]. This system plays an essential role in the control of voluntary movement [15]. Moreover, the dopaminergic neurons which arises from ventral tegmental area are also involved in the mesocorticolimbic system, in which they innervate the *nucleus accumbens*, *olfactory tubercle*, *septum*, *amygdala*, *hippocampus*, and also the prefrontal, cingulate and perirhinal cortex [15]. This pathway is also known to modulate emotional behaviors as motivation and reward [19].

The cellular mechanisms underlying dopaminergic neurons degeneration [20] are thought to be associated with increased sensitivity to mitochondrial disfunction [21], and proteostasis impairment [22]. One possible explanation for this impairment could be the high rates of oxygen metabolism, the reduced levels of antioxidants and the high content of iron that these neurons require [23]. The degeneration process can in fact be triggered by oxidative stress [24], aging [25] and neuroinflammation [20,26,27]. Due to their distribution and function, the progressive loss of dopaminergic neurons in PD follows a specific pattern, being the *SNpc* the more susceptible area [17]. This area is known to house the vast majority of dopaminergic cell bodies which projects to the *striatum* [17]. When cell bodies are impaired, a severe dopamine depletion in *striatum* is observed, triggering several motor symptoms associated with PD [28,29].

Etiology

There are both familial and sporadic cases of PD, representing 10% and 90%, respectively [6]. Genetic cases are considered monogenetic forms of PD, which are defined by mutations in inheritable known single genes, that can be classified in autosomal dominant PD [30] and autosomal recessive juvenile parkinsonism [31].

The first gene that has been identified in autosomal dominant PD was *SNCA*, which encodes for aSyn [32]. Mutations in *SNCA* gene lead to alterations in aSyn production and aggregation pattern [33]. Moreover, *SNCA* gene multiplications, in particular gene duplication [34] and triplication [35] may result in a severe phenotype and faster disease progression. The most common mutated gene in autosomal dominant PD is leucine-rich repeat kinase-2 (*LRRK2*) [12]. The phenotype of the disease caused by mutated *LRRK2* is indistinguishable from sporadic forms [36]. LRRK2 mutations induce a gain of its abnormal function, ultimately damaging mitochondria, increasing mitophagy and consequently drive to the degeneration of dopaminergic nigrostriatal neurons [37].

Autosomal recessive juvenile parkinsonism is a hereditary form of the disorder associated with an early onset of PD, usually before 40 years old [31]. Parkin, encoded by the *PARK2* gene, may display E3 ligase-like activity, that triggers mitophagy of dysfunctional mitochondria via the ubiquitin proteasome system (UPS). PD patients expressing a mutant variant of parkin display decreased mitochondrial function [38] and peripheral blood analysis showed a decrease in leukocyte complex I activity in comparison to aged-matched controls [39]. In mice, loss of parkin function cause nigral degeneration possibly via an exacerbated accumulation of its substrates [40]. These labile lipid peroxides may damage and reduce dopamine transporter (DAT) activity leading to a cyclic process in which oxidative stress may result in the decreased parkin activity found in PD [40]. PTEN-induced kinase 1 (*PINK1*) mutations are also known to cause autosomal recessive juvenile parkinsonism [31]. PINK1 is involved in the initiation of mitophagy process by recruitment of parkin from the cytoplasm to the mitochondria [38,41]. Mutations on *PARK7*, that encodes for DJ-1, causes recessive forms of PD [42]. DJ-1 displays chaperone function [43], in response to oxidative stress [44], but it also presents glyoxalase activity [45], and has been described for its deglycase properties (capacity to remove advanced glycation end-products from proteins) [46]. Parkinsonism caused by *PARK7* deletion may be triggered by a higher sensitization of cells to oxidative stress or to carbonylic stress (glycation) [47]. Moreover, the down regulation of DJ-1 induces cell death via oxidative stress, *endoplasmic reticulum* (ER) stress, and proteasome inhibition [48]. In contrast, DJ-1 overexpression showed protective effects in *in vitro* models of PD [44,49,50].

There are several other genes (*PARK1-24*) identified as familial-associated genes. This set of genetic mutations have been functional characterized to unveil molecular mechanisms that could be commonly dysregulated in sporadic PD. Sporadic PD has been defined as a multifactorial disorder, in which genetic network and environmental factors may interplay and result in a PD pathological condition [6]. Studies in twins diagnosed with sporadic and with late onset PD support the hypothesis that the genetic background also impact in sporadic cases [51]. Moreover, genetic studies observed that some genetic variants may influence the risk for PD [52]. For example, single nucleotide polymorphisms (SNPs) in *SNCA* have been established to modify the susceptibility to PD [53].

In fact, several factors may influence disease onset and progression, being aging the best recognized [6]. As previously mentioned, the risk of developing PD is 3 times higher in individuals over 80 years in comparison to 60 years old individuals, meaning that age is a critical feature in disease progression [54]. Genetic studies have found that aging impacts the regulation of 170 PD-related genes, including *PRKN and PARK7*. These genes exhibit age related changes that are concurrent with the promotion of the disease [54].

Behavioral and environmental factors have also been associated with the development of the disease. In particular, environmental factors as rural living, well water consumption and agricultural chemicals have been identified to increase the risk of develop PD [55,56]. For example, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) a toxic agent discovered in 1983 that was commonly used in pesticides, was found to induce dopaminergic neuronal loss causing Parkinsonism [57]. Life-style behaviors were also associated with PD risk. In particular, smoking and caffeine intake decrease PD risk [58]. Nicotine may impact PD development, as smokers present half the risk to develop PD compared to non-smokers [59]. Caffeine consumption present more evident effects in men than in women, reducing the risk of a man developing PD [58]. Physical activity, specific diets and the use of certain drugs, such as anti-inflammatory agents and calcium channel blockers, showed to decrease the risk of PD [10,58].

Additionally, diabetes has been established as an important risk factor for PD [60]. This risk is more severe for patients over 65 years old [60] that developed diabetes for longer duration [61,62] prior to PD [63]. Strikingly, younger diabetic individuals (between 25-45 years old) have 380% higher risk to develop PD [61]. Concurrence of diabetes and PD lead to more severe PD symptoms and resistance to levodopa therapy [63]. In particular, to an accelerated progression of both motor and cognitive symptoms of PD [64]. Furthermore, studies revealed that 50 to 80% of PD patients have abnormal glucose tolerance, supporting the association between diabetes and sporadic forms of PD [65]. Moreover, the onset of T2DM prior to PD is associated with severe PD symptoms, supporting the idea that T2DM may also impact PD development and progression [63].

PD diagnosis

PD diagnosis is mainly based on the previously described symptomatologic features of the patients. However, when individuals do not present clear symptoms, imaging techniques may allow a better diagnosis. In particular, DATscan can be used to visualize dopaminergic signaling impairment in the *substantia nigra - striatum* [66]. In some cases, functional magnetic resonance imaging (fMRI) have proved to be efficient to correlate behavior characteristics and brain connectivity [67]. Changes in functional connectivity in anterior

brain regions also seem to be related to executive dysfunction, whereas changes in posterior regions may be related to dementia development [10].

To evaluate disease progression, evaluation rating scales have been developed. The mostly used is the Movement Disorders Society modified Unified PD Rating Scale (MDS-UPDRS) [68] that can be divided in four parts. Part I and II reports to non-motor and motor experiences of every day. Part III consists in the physical evaluation of the patient's motor performance. Part IV evaluate motor complications. Alternatively, disease severity can also be assessed by the Hoehn and Yahr (HY) [69] rating scale which evaluates the motor impairment progression of the disease.

PD treatment

PD is characterized by the progressive loss of dopaminergic neurons in the *SNpc* that leads to loss of dopamine transmission from the *striatum* to the motor cortex, triggering PD motor impairment [12]. There are no curative treatments for PD, or disease-modifying therapeutics. The currently used pharmacological treatments for PD seek to compensate the loss of dopamine by inducing/stimulating/replacing its production to ameliorate associated motor deficits.

The use of dopamine precursors approaches including, levodopa (L-DOPA) which is a natural precursor for the metabolism of dopamine, has been used as a treatment for PD. It can replace the loss of dopamine function in affected midbrain due to its capacity to cross the blood brain barrier (BBB) and to be mainly taken up by the dopaminergic neurons that can convert it to dopamine via aromatic L-amino acid decarboxylase (DOPA decarboxylase). In fact, the intake of L-DOPA alleviates PD symptoms severity. L-DOPA is not only the best current medicine/treatment but also facilitates PD diagnosis. After treatment with L-DOPA, the positive patient's response to the treatment allows to clarify if the individual has PD [10]. Although L-DOPA can ameliorate PD symptoms, it is not able to prevent neurodegeneration. Nevertheless, L-DOPA therapy is associated with an increase of the average age at death by approximately 5 years [70,71]. Although L-DOPA ameliorates PD motor performance, it causes several side effects including nausea, vomiting, altered blood pressure, and, interestingly, it may induce both hyperglycemia and hyperinsulinemia [72]. Carbidopa is usually given as a complementary treatment with L-DOPA, since it does not cross the BBB and is able to prevent peripheral L-DOPA degradation by inhibiting peripheral DOPA-decarboxylase. Carbidopa thus allows for an L-DOPA treatment with lower doses [73].

Besides compensating the dopamine loss with L-DOPA, dopamine agonists are also used to manage PD symptoms, which includes apomorphine, bromocriptine, ropinirole, pramipexole, and rotigotine. Dopamine agonists act on dopamine receptors to amplify and sustain the effects of dopamine [73].

Other strategies aiming to increase dopamine levels include the inhibition of Monoamine oxidase B (MAO-B) and Catechol-O-methyl transferase (COMT), which are enzymes involved in the catabolism of dopamine. Approved MAO-B irreversible inhibitors include selegiline and rasagiline, while entacapone and tolcapone are approved COMT-inhibitors [73].

Alternatively, to pharmacological treatments, Deep Brain Stimulation (DBS) has been used for the management of PD, presenting the advantage of minimizing medication needs [74]. DBS is a surgical strategy, which consists in placing electrodes into one or both sides of the brain in specific areas, which can be *subthalamic nucleus* or the *globus pallidus interna* to ameliorate different motor problems such as stiffness, slowness and tremor. Although it does not have beneficial effects in imbalance, gait problems and non-motor symptoms. Moreover, this strategy is only applicable to a specific subset of PD patients. Patients have to be diagnosed with PD for at least four years and should present responsiveness to PD medications although with significant "off" time, the period of time when symptoms are present because medications is not working properly and patients present severe dyskinesia. Moreover, this strategy is not recommended for patients with dementia [74].

Since these therapeutic options do not target neurodegeneration, there is a strong pressure to develop disease-modulating therapeutic options. To that purpose, there are some clinical trials currently evaluating the potential of immunotherapeutic approaches to target toxic aSyn [73]. Moreover, experimental transplantation of neural stem cells from fetal tissue or from mid-brain neurons derived from induced pluripotent stem cells into the striatum of PD patients seems to be a promising approach, since these cells seem to survive and replace the function of damaged dopaminergic neurons [75].

1.2 Alpha-synuclein

Notably, both sporadic and familial cases of PD have alterations in aSyn levels and pathology [6]. aSyn accumulation and aggregation is therefore a hallmark of PD and this protein is considered to play a central role in the pathogenesis of the disease. By still unclear mechanisms, aSyn may accumulate, aggregate and cause neurotoxicity.

aSyn structure

aSyn belongs to a family of synucleins also comprising β-, and γ-synuclein [76]. These are small soluble proteins known to bind to phospholipid membranes [77]. aSyn gene (*SNCA*) is located in chromosome 4. aSyn protein is composed by 140 amino acids (14 kDa), which primary level of structure can be subdivided into the basic N-terminus (residues 1-60), the hydrophobic core named NAC (non-amyloid-β component) domain (residues

61-95), and the acidic C-terminus (residues 96-140) [76]. The N-terminus of aSyn is highly conserved between species and predicts the alpha helix secondary structure and is known for mediating its interaction with phospholipids and membranes [76,78]. aSyn central domain is thought to be underlying the aggregate prone nature of the protein [76]. The C-terminal is the protein region with higher variability between species and within synuclein family [76]. This region may play a role in the solubility of aSyn, as well as in regulating aSyn conformation and function since it is highly enriched in positively charged residues [76]. The C-teminal may also mediate the interaction of aSyn with other proteins [79].

aSyn is considered a "natively unfolded" protein due to its labile secondary level of structure that induces conformational changes depending on the environment [80,81]. For this reason, aSyn lacks a defined tertiary level of structure [82], that requires the presence of other molecular interacting partners for the stabilization of its conformation. Beyond interaction with other proteins, aSyn can self-associate with several monomeric units, entering an amyloidogenic cascade, forming oligomers up to insoluble fibers and aggregates [83 84]. There is still controversy in the field about the role of aSyn oligomerization, given that there are evidences that the formation of a stable tetramer may favor the physiological non-toxic function of aSyn [85]. However, it is also well established that the formation of oligomers and aggregates triggers aSyn pathological activity [86]. How this process is regulated is still unclear.

aSyn function

Although it is present in non-neuronal tissues such as red blood cells [87] and pancreas [88], aSyn is considered a neuronal protein and as the name suggests 'synuclein' results from the conjugation between "SYNapse" and "NUCLEus" [89]. This denomination characterizes aSyn location within the neuron, which is highly present in the presynaptic terminal of neurons, but it can also be found on the nuclear envelope, while in dendrites and axons it appears to be present in low levels [90]. aSyn may play a role in synaptic transmission, supported by its high expression in neuronal tissues within both peripheral and central nervous systems [91,92]. In particular, aSyn is believed to act as a negative regulator of synaptic transmission. In presynaptic terminals, aSyn interacts with synaptic vesicles [89] and SNARE complex proteins [79,92], regulating vesicular trafficking [93] and vesicles docking to the presynaptic membrane [94], consequently mediating neurotransmitter release [95-97]. aSyn may have a specialized role in the dopaminergic synapses, due to its interaction with proteins involved in dopamine biosynthesis and homeostasis [98,99]. Specifically, aSyn interacts with tyrosine hydroxylase (TH) [99-101] and with DAT [98,102]. As verified by a previous study, aSyn knockout mice present decreased dopamine stores and decreased synaptic vesicle endocytosis [103,104].

In pathological conditions, aSyn initiates an aggregation process [105] which is believed to be irreversible due to the high stability of aSyn aggregated species [83]. This process is a feed-forward mechanism, in which

oligomers or protofibrils can act as seeds promoting/accelerating the formation of further aSyn aggregates [106,107]. This mechanism is a hallmark underlying synucleinopathies, including PD [108,109], dementia with Lewy bodies [14], multiple system atrophy [110], pure autonomic failure [111], Lewy body variant of AD [112], and neurodegeneration with brain iron accumulation [113]. In PD, aggregated aSyn is mostly localized within neuronal soma and neurites [14].

It is believed that aSyn pathological function in PD may be associated with the aSyn loss of function theory [114]. First, dopaminergic neurons from *SNpc* present low levels of soluble aSyn, possibly due to aggregation of aSyn into Lewy bodies [14]. Second, according to aSyn function as a regulatory protein that can bind and inhibit TH, the rate-limiting enzyme in dopamine synthesis, it is predictable that the loss of functional aSyn may result in dopamine alterations [100]. Specifically, it may cause dysregulation of TH and of dopamine transport and storage that outcome in excessive cytosolic dopamine concentrations. Unpackaged dopamine and its metabolites are highly toxic reactive molecules that may contribute to PD neurotoxicity[24].

Factors influencing the pathological state of aSyn

Although it is known that particular conditions may favor aSyn aggregation process, the precise mechanisms are still not well defined. In particular, with aging, there is a pathological increase in the levels of monomeric and oligomeric forms of aSyn [54]. aSyn genetic mutations [84,115,116] are also suggested to be underlying the pathological state of aSyn. Moreover, the initial steps of aSyn aggregation process could be favored by the high concentrations of free macromolecules (macromolecular crowding) [81,117], the increased levels of aSyn [84,109,118], its post-translational modifications (PTMs) [119,120] and, low pH [121], oxidative conditions [122] and temperature [121].

aSyn structural mutations may influence its accumulation and aggregation processes [118]. Within the primary level of structure, intramolecular interactions between the N and C termini are relevant in the refolding process to maintain aSyn in a monomeric state [118]. An efficient folding can prevent aggregation, although when it becomes defective, the structural shift to an unfolded or partially folded state is sufficient to trigger aSyn aggregation [118]. Mutations in the hydrophobic core are associated with disruption of the folding state by reducing the stability of alpha helix structure, which also exacerbates aggregation [123]. Furthermore, defective folding can lead to C-terminus truncation [124] and increased exposure of amyloidogenic NAC core, can initiate the feed-forward aggregation process [125]. PTMs can induce changes in protein charge and structure, consequently modifying the binding affinities with other molecules (proteins and lipids) and the overall protein hydrophobicity [120,126]. aSyn is a target for several PTMs, including phosphorylation, oxidation, acetylation, ubiquitination, glycation, glycosylation, nitration [126-128].

1.3 Diabetes

Currently considered a relevant risk factor for PD, type-II diabetes mellitus (T2DM) is a chronic / metabolic disorder characterized by a relative or absolute lack of insulin possibly due to the insufficient insulin production by the pancreatic cells or due to the unproper response of cells to the produced insulin [129]. The absence of insulin may result in increased body sugar levels for an extended period of time (hyperglycemia) [129]. Chronic hyperglycemia can lead to a variety of complications such as neuropathy, nephropathy, retinopathy and increased risk of cardiovascular disease [130]. Importantly, diabetic patients present at least the double risk of death than healthy individuals [129]. The worldwide prevalence of diabetes over 18 years old was around 8.5%, representing 422 million people affected by the disease in 2014 [226]. There are several different classifications of diabetes, being the most common type 1 diabetes mellitus (T1DM) and T2DM [129].

T1DM affects around 2-5% of worldwide [129] and is also known as ‘‘insulin-dependent diabetes mellitus’’ or ‘‘juvenile diabetes’’. As the name implies, T1DM is an autoimmune disease leading to the destruction of the insulin-producing pancreatic beta cells in the islets of Langerhans, resulting in insufficient insulin production [129]. Underlying the onset of this disease it is believed that both genetic and environmental factors may play a role in disease development [131].

The impact of non-genetic factors in disease onset seem to be even more relevant in T2DM, which is the most common form of this disease, corresponding to 90% of the cases, with equal prevalence both in women and in men [129]. Both the environment and behavior habits also have high impact in the onset of this pathology, with obesity significantly contributing to the development of T2DM [132,133]. The hallmarks of T2DM are the disruption of glucose sensing, insulin signaling impairment and defective hypothalamic circuits [134,135], that could result from insulin resistance. Also known as ‘‘adult-onset diabetes’’, the pathology begins with insulin resistance, in which cells do not respond properly to insulin, in particular the intracellular processing of insulin becomes compromised. The excessive insulin demand leads to β-cells failure, which is compensated by an increase in insulin production by the remaining cells. Upon disease progression, it may result in lack of insulin and also, this compensatory mechanism may be extremely demanding to the β-cells ER protein folding system [136]. Upon ER collapse, protein accumulation and aggregation may occur [136]. Therefore, T2DM may also be considered a proteinopathy, since aggregated islet amyloid polypeptide precursor (IAPP) is found in pancreatic islets [137]. The primary cause of T2DM is associated with high sugar and calorie intake, genetic susceptibility and lifestyle, including the reduced practice of physical exercise [129].

Symptoms and Treatments

The symptoms observed in diabetic patients include frequent urination, increased thirst, hunger, diabetic ketoacidosis , and hyperosmolar coma [138], that can lead to long-term complications as kidney failure, diabetic heart disease, stroke, foot ulcers, and damage in vision [129]. To prevent and treat diabetes, a healthier lifestyle is required, where a proper diet with controlled sugar intake is vital for people with this disease, together with control of blood pressure [129]. Moreover, physical exercise and no tabaco consumption are also required [129]. Specifically, T1DM patients produce minor quantities of endogenous insulin throughout lifetime. Regular subcutaneous injections may be applied to replace the failure in insulin production and blood glucose levels may be frequently monitored to manage the risk of hypoglycemia [129].

Pathologically, T2DM demonstrates insulin resistance that can be improved by lifestyle interventions, as weight reduction and exercise. If this approach fails, there are a variety of drugs available to manage blood sugar levels diabetes, with different mechanisms of action [139]. 1) Sulphonylureas, are able to stimulate insulin secretion from the already compromised β-cells. However, this stimulation at low glucose levels may lead to side effects, as hypoglycemia. 2) Biguanides, are able to reduce hepatic glucose production, being metformin the most widely used. 3) α-glucosidase inhibitors are able to delay of carbohydrate uptake in the gut. 4) Thiazolidinediones are synthetic agonists of a nuclear peroxisome proliferator-activated receptor (PPAR-γ). Its activation can improve insulin sensitivity. 5) Drugs that target dipeptidyl peptidase-4 (Dpp-4) can also be used. Dpp-4 is a ubiquitous cell surface enzyme that degrades glucagon-like peptide-1 amide and glucose-dependent insulinotropic peptide (GIP). Dpp-4 action removes insulinotropic activity. In fact, Dpp-4 inhibitors such as exenatide are currently being explored as a putative therapeutic strategy for PD (under clinical trials) [140].

Importantly, weight loss surgery in people with T2D who are obese demonstrates beneficial effects [141,142].

1.4 Commonly dysregulated pathways between T2DM and PD

Genetic association between PD and Diabetes

Altered homeostasis of sugar metabolism is a common feature underlying both diseases. This relationship was explored by genetic associations between both diseases, where common genetic alterations have been identified by genome-wide transcriptome profiling. These studies found that T2DM individuals present mutations in *PARK7* gene, which is a PD related gene. This gene encodes for DJ-1 protein, which was found reduced in pancreatic islets of diabetic patients [143]. Similarly, *AKT* gene mutations may be also commonly

found in both diseases. This gene encodes for the kinase Akt that regulates cell survival and metabolism and these protein levels were found altered in both pathologies [144]. Akt signaling can modulate insulin and dopaminergic signaling [144]. Due to its competence, mutations in this gene may affect PD specifically by impairing dopaminergic homeostasis and T2DM by altering insulin signaling [145].

Common environmental risk factors

Some environmental factors have also been associated with both diseases, including heavy metals and pesticides. Heavy metals exposure has been studied in the context of PD, in which blood of PD patients often presented high concentrations of iron, manganese, and copper [146]. Commonly, in T2DM it is known that exposure to heavy metal implicates islet dysfunction and disease progression [147]. Pesticides, such as rotenone increases an individual's risk to develop PD [56], while in T2DM, it correlates with insulin resistance and β-cell dysfunction [148]. Altogether, some environmental factors are a risk for both PD and T2DM development.

Shared mechanistic pathways

Aging is the major risk factor for both metabolic disorders and neurodegenerative diseases, in particular, T2DM and PD respectively [4]. Aging is accepted to dysregulate proteins and organelles [149]. Moreover, both diseases present protein degradation impairment and consequently protein accumulation. In PD, aSyn accumulates and aggregates in neurons [109], while in T2DM IAPP accumulates and aggregates in β-cells [137]. Furthermore, ER and mitochondrial dysfunction may impact lipid and glucose metabolism or protein folding capacity, triggering T2DM [150] and neurodegeneration pathways [151]. The oxidative energy metabolism is also impaired in both conditions, inducing alterations in lactate concentrations and glucose hypometabolism [152]. Altogether, glucose metabolism is commonly dysregulated in PD [65] and T2DM [153], although the precise underlying mechanism is not completely defined.

Dysregulation of sugar metabolism/ Sugar impairment

Brain requires 20% of the total energy of the body, using glucose as main source of energy [135]. This energy ensures the normal function of the brain, including cellular homeostasis, generation of action potential, maintenance of ion gradients and biosynthesis of neurotransmitters [135]. The CNS communicates with the peripheral organs, regulating body glucose levels through neuronal signaling cascades [135].

Brain metabolic signaling can be mediated by insulin receptors (IR) and glucose transporters (GluTs), having respectively insulin-dependent and independent mediators [135]. Although, peripheral glucose uptake is mediated by insulin and glucagon-like peptide-1, the uptake of glucose from the blood into the brain appear

to be insulin-independent [135]. This is ensured by glucose transporters, mainly GLUT1 and GLUT3, which are highly expressed in the BBB and in neurons. GLUT1 mediates glucose uptake from the endothelial membranes into the extracellular fluid, and also mediates the uptake from extracellular fluid into astrocytes, oligodendrocytes and microglia [135]. However, neuronal uptake is mainly maintained by GLUT3, which has a higher transport rate to respond to glucose neuronal needs [135]. Moreover, since it presents high affinity to glucose, it allows glucose uptake also in periods of hypoglycemia [135]. Once inside the cell, glucose is irreversibly converted to glucose-6-phosphate (G6P) by hexokinase (HK). G6P can then be further metabolized by the glycolytic pathway, diverted to the pentose phosphate pathway (PPP) or can be used for glycogen synthesis, that in the brain only occurs in astrocytes. In glycolysis, G6P is metabolized to pyruvate, which is actively transported into the mitochondria where it is converted to acetyl coenzyme A (acetyl-CoA). Acetyl-CoA undergoes TCA cycle reactions producing NADH and $FADH_2$ that are subsequently re-oxidized in the electron transport chain (ETC). ETC transport protons across the inner mitochondrial membrane into the intermembrane space and the resulting flux of protons back to mitochondrial matrix is mediated by ATP synthase which regulates oxidative metabolism by generating ATP from ADP [154]. PPP is a metabolic pathway parallel to glycolysis, which is the major source of NADPH required for anabolic processes. Glucose-6-phosphate dehydrogenase (G6PDH) is the rate controlling enzyme of this pathway and results in the formation of ribose-5-phosphate (R5P) and NADPH, which regenerates oxidized antioxidants, such as glutathione (GSH) [154].

Dysregulation of sugar metabolism could be one of the first symptoms of PD [155]. Patients at early stages present elevated levels of several sugar forms in the cerebrospinal fluid [156]. Being a slowly and progressive disorder, advanced stages of PD progression may result in decreased glucose tolerance and hyperglycemia. These findings are supported by post-mortem analysis which showed that the levels of key enzymes involved in the PPP are decreased in PD patients, an important mechanism involved in glucose metabolism [155]. Altogether, these mechanisms may explain why 60% of PD patients present impairment in insulin signaling and glucose intolerance [157].

Glycation, a consequence of hyperglycemia

A causative link between PD and diabetes/ hyperglycemia is glycation [158]. Being aSyn a long-lived protein is likely to be glycated over time [127]. Glycation is a non-enzymatic reaction between reducing carbohydrates/reactive dicarbonyls and amino compounds/groups, that gives rise to different reaction products, known as, advanced glycation end products [47]. Glycation of proteins depends on the amount of precursors and the availability of potential target sites for the modification that controls the degree to which individual protein undergoes glycation [159]. Reducing sugars, such as methylglyoxal (MGO) are unavoidable

products of glucose metabolism [46]. In diabetes, hyperglycemia leads to the accumulation of glycating agents that potentiates glycation of proteins, which associates the higher glycation levels with diabetic complications [159]. In PD, aSyn is found to be glycated [119], and Lewy bodies in the *SNpc* of patients' brain are positive for glycated proteins [160]. In fact, glycation has been found to participate on aSyn pathology: glycation exacerbates aSyn oligomerization, aggregation and toxicity, and impairs both proteasome and autophagy clearance systems [127,161]. The observed phenotypes were rescued using the MGO scavengers aminoguanidine and tenilsetam.

Alterations of insulin metabolism in brain

Although glycation is a consequence of hyperglycemia, glucose alterations may be escorted by insulin impairment. Commonly, T2DM and PD present reduced insulin mediated glucose uptake [152,162]. This can be due to the inhibition of early insulin secretion and long-term hyperinsulinemia and hyperglycemia after glucose loading [65]. The major role of insulin in the brain is to regulate its associated signaling pathways, rather than a direct neuronal regulation of glucose uptake [163].

Through downstream effectors, insulin can stimulate cell survival or death [164]. Insulin binding IR results in pleiotropic effects. The signal transduction cascade seems to be one of the most relevant roles of insulin and it is mediated through two main pathways, including PI3K/Akt and mitogen-activated protein kinase (MAPK) pathways. PI3K is mostly believed to be involved in the stimulation of neuronal survival, and the MAPK pathway is mostly involved in cell death. In particular, PI3K pathway initiation, activates insulin degrading enzyme (IDE) which plays a role in the modulation of amyloid peptides degradation [144]. In collaboration with IDE, mTOR is a negative feedback regulator of PI3K signaling, which inhibition may promote protein clearance pathways, enhancing autophagy [144]. Activation of these pathways modulate several downstream effectors, such as, glycogen synthase kinase–3B (GSK-3B), mammalian target of rapamycin (mTOR), caspase-9, transcription factor FoxO and others. Insulin signaling may regulate cell survival by its involvement in mitochondrial mechanisms, inflammation, oxidative stress and particularly protein clearance pathways [144].

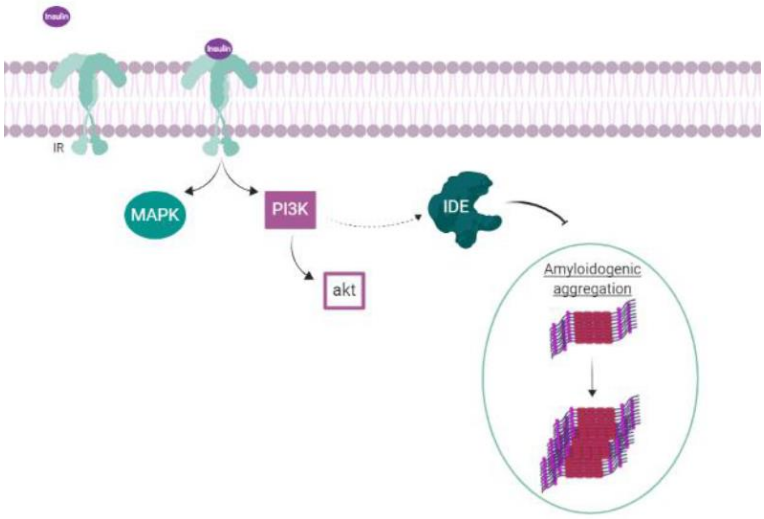


Figure 3. Insulin signaling stimulate IDE activity on amyloidogenic substrates.

Insulin binding to insulin receptors (IR) results in pleiotropic effects. The signaling cascade is mediated through two main pathways, including PI3K/Akt and mitogen-activated protein kinase (MAPK) pathways. PI3K pathway initiation, activates IDE which modulates amyloidogenic aggregation process.

Previous studies demonstrated that brain insulin controls not only cell survival but also the dopaminergic system [145]. Insulin impacts dopaminergic neurons, influencing DAT [145] and the clearance of dopamine from the synapse [20]. DAT is a key regulator of neurotransmission and important for cognitive ability [18]. Dopamine association with insulin metabolism is directly supported by the existence of insulin-like growth factor (IGF-1) within *SNpc* [165] and more specifically, by the densely presence of IR found in these dopaminergic neurons [152]. Considering, that these players may induce insulin signaling and stimulate cell survival, they may have a role in rescuing dopaminergic neurons from apoptosis [20].

Insulin dysregulation may lead to insulin resistance and this pathological feature has been associated with neurodegenerative disorders [154]. In particular, recent studies found that insulin resistance in the brain changes dopamine turnover and may result in movement disorders [166]. Furthermore, brains from PD patients present reduced levels of IR, and of TH, the rate limiting enzyme for dopamine synthesis [167]. To compensate this pathological mechanism, restoring the levels of insulin have been shown to protect dopaminergic neurons in the *substantia nigra* in a rat model of PD [168]. Additionally, recent *in vitro* and *in vivo* studies have demonstrated that approved drugs to treat insulin resistance, such as exenatide, present protective effects in PD [140].

1.5 Insulin degrading enzyme

Although, glucose uptake in the brain is mostly insulin-independent, insulin regulates several brain functions [163]. Insulin resistance have been associated with defective insulin clearance [169]. IDE also known as insulysin, is a highly conserved Zn metallopeptidase encoded by a gene located in chromosome 10 in humans [170]. As the name suggests this enzyme was first identified as an insulin degradative enzyme. Insulin signaling cascades stimulate the production and activity of IDE [144]. Although, the best defined role of IDE is the biochemical processing of insulin hydrolysis [171], it can also degrade a variety of other small peptides including IAPP, Aβ, glucagon, amylin, bradykinin, somatostatin and others [171,172]. This function is very important in the context of AD, since by degrading Aβ, it may prevent the pathological formation of toxic Aβ aggregates [173]. It is also very important, in the context of diabetes, as IAPP is also able to oligomerize and induce toxicity in pancreatic β-cells [174]. In pathological conditions such as insulin resistance, the reduced activation of IR may reduce IDE levels. These alterations may result in mitochondrial dysfunction and oxidative stress, triggers of neurodegeneration [144]. IDE can play different roles depending on its cellular location, that depend on each cell type [175]. In the brain, IDE can exist intracellularly (cytosolic IDE), at the cell surface (membrane-associated IDE) or can be secreted by microglia. However, cytosolic IDE is the most common form of the protein (95%) in the body, whether endosomal, peroxisomal, mitochondrial, extracellular and cell surface IDE accounts for 5% [176,177]. In fact, in neurons IDE seem to be mainly membrane-associated [177].

Relationship between structure and function

Structurally, IDE is a monomer with 110kDa that is composed by two equal sized domains (55kDa), IDE-N and IDE-C. These domains are connected by a 28-residues extended loop and consequently, IDE adopts a clamshell-like structure enclosing a large central chamber [178]. The formation of this crypt prevents the entry or escape of the substrates [178]. IDE presents a complex molecular machinery and is rewarded by the ability to change its conformational state in tertiary (intra-subunit) and quaternary (inter-subunit) structures to interact with surrounding molecules [179]. Importantly, ATP is the allosteric regulator of these processes [180]. IDE exists in an equilibrium of dimers and tetramers, although its activation by ATP promote the transition from tetrameric to dimeric forms [180], which are predicted to be the most active forms of the enzyme. Moreover, ATP binding can also facilitate the transition from the closed to the open state [180].

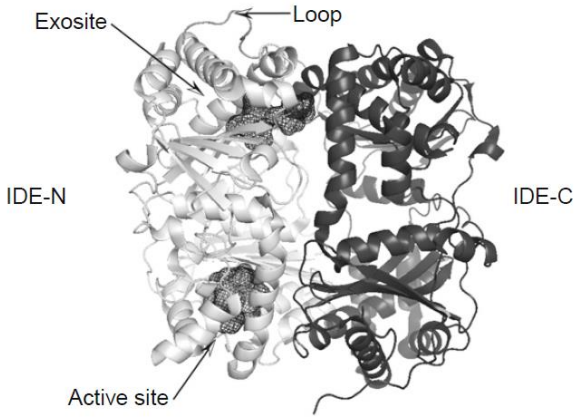


Figure 4. Schematic of IDE structure.

Structurally, IDE is composed by two equal sized domains, IDE-N and IDE-C. These domains are connected by a loop which allows the formation of the central chamber. IDE-N terminal includes its active site and also the exosite, which are the main substrate binding sites of IDE. Adapted from [178].

IDE structure reveals important information about its catalytic function and ability to interact with different substrates [178]. It presents multiple related binding sites which allow for more than one place of interaction with peptides [181]. IDE-N-terminal domain includes its degradative active site which is located inside the central chamber and requires conformational changes for substrate binding [182]. This site is located inside the crypt and due to its enriched charge, polarity and hydrophobicity, it is responsible for the interactions with both IDE-C terminal and substrates [182]. IDE substrate binding to the central chamber may imply an open conformational change to enable electrostatic interactions inside the chamber [178]. IDE-N domain also contain a distal and positively charged binding site, named exosite which recognizes larger peptides [182]. This site may play a role in the positioning of the substrates for cleavage [180,182], being a key regulator of IDE protease activity [178]. In addition, recent studies have reported that the binding of small molecules to the exosite have the ability to activate the catalytic site of the enzyme [183,184], improving IDE selectivity in substrate binding and recognition. IDE-N and IDE-C terminal interactions may also control the catalytic rate of IDE [185]. Disturbance of these interactions was found to increase the catalytic rate of IDE, due to the destabilization of the closed conformation state [178].

There are several factors known to control IDE activity, in particular the conformational state, the presence of ATP, the oligomeric state, the specific substrate binding and specially, the presence of the structural crypt which engulfs the peptides. Likewise, structural mutations in IDE may affect its functionality. In particular, Glutamic acid 111 is involved in the activation of a catalytic water molecule that mediates peptide hydrolysis. Therefore, the replacement of glutamic acid at position 111 by a glutamine (IDE-E111Q) results in a catalytic inactive form of IDE for insulin [177,186].

IDE is a multifunctional protein

IDE is considered a multifunctional protein due to its vast mechanisms of action [179]. As an alternative to its proteolytic activity, there is evidence that IDE can have chaperone-like activity on amyloidogenic peptides [187]. Moreover, recent studies revealed that IDE can present this chaperone-like activity on aSyn [188]. More specifically, IDE can act as a "dead-end" chaperone, in which it performs a proteolysis-independent dead-end kinetic trap [189]. Chaperone like role of IDE was confirmed by, recent evidences which showed that IDE could inhibit the amyloidogenic fibrillation of aSyn, through a non-proteolytic mechanism [188]. Instead, IDE binding to aSyn would occur during the first phases of oligomerization when aSyn monomer uncover the C-terminal domain, rendering it able to interact with IDE exosite while the rest of the protein would remain excluded from the catalytic site [190]. Interestingly, aSyn binding is proposed to stabilize the open-conformation of IDE, which appears then a structural pre-requisite for executing the "dead-end" chaperone activity.

Another considered role of IDE is its possible function as a canonical Heat Shock Protein (HSPs) [191]. Moreover, IDE expression can be modulated by stressful conditions such as heat, oxidative stress and nutrient starvation, in a similar way to that of the HSPs family [191].

Altogether, these findings suggest that IDE is a moonlight enzyme, which means that it is involved in a variety of cellular processes by performing different and often unrelated functions [192], either associated with its catalytic activity or not. IDE as a moonlighting enzyme is expected to be a relevant player in the linkage of different and independent cellular pathways [179,192]. Concluding, IDE multi-functional role present high relevance in multiple mechanisms, due to its involvement in intracellular binding, regulatory and degradative protein processes [179].

1.6 Interplay between IDE and aSyn

IDE have been mostly studied in a diabetic context due to its important role in insulin metabolism. However, IDE has also been studied in a context of neurodegenerative disorders in particular in AD [173]. In fact, IDE activation may prevent from Aβ accumulation and aggregation, displaying preventive effects in AD [193,194].

Surprisingly, a recent study has described an inverse correlation between increased aSyn levels and impaired insulin secretion in the pancreatic β-cells [195], which supports the idea that IDE dysregulation may explain the association between T2DM and PD. Moreover, the inverse correlation was confirmed in pancreatic β-cells of IDE knockout mice and in T2DM patients [88,195]. aSyn is not an IDE proteolytic substrate [188], most likely due to its size of 140 residues in comparison to the 51 presented by insulin, which is the largest molecule that IDE chamber can accommodate [185]. In fact, aSyn cannot fit within the limited size of IDE catalytic core. However, recent studies showed that IDE can interact with aSyn, via an alternative site, the exosite [188]. *In vitro* studies

have shown that, IDE may recognize and bind the exposed C-terminus of monomeric or oligomeric aSyn and suppress further aggregation into amyloids [190]. The molecular mechanisms underlying the interaction of these proteins can be supported by the electrostatic attraction of the acidic and negatively-charged residues present in aSyn C-terminal, to the basic and positively charged exosite region in IDE-N domain [190]. aSyn-exosite interaction not only blocks oligomer assembling thus preventing aSyn amyloid formation, but also enhances IDE proteolytic activity on small substrates [190]. This activation of the proteolytic activity is supported by the idea that filling the exosite blocks the nonproductive substrate binding and substrates can more easily reach the catalytic chamber [190].

1.7 Models of type-2 Diabetes and Parkinson's disease

Mouse models of Type 2 Diabetes

Animal models of T2DM mainly recapitulate insulin resistance and/or β-cell failure [196]. However, the most used mouse models are the obese models. The most widely used monogenic models of obesity are defective in leptin signaling. Leptin induces satiety, and thus, a lack of functional leptin in these animals causes hyperphagia and subsequent obesity. These models can result from deficiencies in leptin (Lepob/ob) or in the leptin receptor (Leprdb/db) [196]. There are also a wide variety of polygenic mouse models of T2DM with obesity and/or glucose intolerance, allowing a variety of genotypes and susceptibilities to be studied. Polygenic models of obesity may provide a more accurate model of the human condition. The most found genetic models of obesity are KK, New Zealand Obese and the TallyHo mouse [196].

Since obesity in humans is mainly induced by environmental rather than genetic influences, diet-induced obesity is often used to model T2DM [196]. The high fat diet (HFD) model [197] is based on feeding mice with a specific diet that may lead to obesity, hyperinsulinemia and altered glucose homeostasis [198]. Normal chow diet presents a caloric basis around 26% protein, 63% carbohydrate and 11% fat. In HFD, the number of calories is increased substantially, around 16.4% of protein, 25.6 % of carbohydrate and 58% of fat. The weight gain from the high-fat-fed mice is detectable within a week of feeding [198], although typically mice are fed for several weeks to induce a more pronounced weight gain. The weight gain is associated with insulin resistance, and lack of β-cell compensation that leads to impaired glucose tolerance [196].

High fat feeding for 12 weeks is a defined protocol that lead to obesity and pre-diabetes in mice [198]. Consequently, the presence of elevated intracellular long-chain fatty acids may inhibit the catalytic activity of IDE, decreasing insulin clearance which leads to hyperinsulinemia and insulin resistance [153,178]. Alterations in insulin regulation may induce early stages of hyperglycemia when β-cells become exhausted by compensating insulin resistance increasing insulin production. Recent studies have shown that this mouse

model present memory, synaptic plasticity and adult neurogenesis impairment [199], and also, that insulin resistance, caused by a HFD, can exacerbate mice dopaminergic degeneration [200].

These common alterations in both insulin metabolism and neurodegenerative processes found in HFD mouse model, render it as a good approach to unveil specific players involved in the shared mechanisms of both T2DM and PD.

PD mouse model

Currently, there are no models that fully recapitulate PD phenotype [201]. The most commonly used models to study PD are either toxin-induced or based on causative genetic mutations of PD [201]. Toxin-induced models are based on neurotoxins that induce dopaminergic degeneration such as 6-OHDA, MPTP, rotenone, paraquat or methamphetamine [201].

The genetic based models allow for the understanding of the signaling cascades/mechanisms associated with the known genetic mutations in PD [201]. The frequently targeted genes include LRRK2, aSyn, Parkin, Pink1 and DJ-1 [201]. Being aSyn the hallmark of PD, one of the most commonly used models is based on the overexpression of the human form of aSyn in the brain under Thy1 promoter (Thy1 mice) [202]. These mice recapitulate the accumulation and aggregation of aSyn, as well as several motor features of the disease [202,203].

Cellular models of PD

The most used cellular models of PD are based on the overexpression of aSyn [204]. In particular, an immortalized human neuroglioma cell line (H4 cells) is widely used since it is easy to transfect with human aSyn, and to express aSyn in a short period of time [205]. Most importantly, it is possible to recapitulate the formation of aSyn toxicity and protein inclusions formation [205] upon expression of SynT, a C-terminally tagged aSyn variant more prone to aggregation [206].

More recently, researchers have been using mid-brain neurons derived from human induced pluripotent stem cells from PD patients [207], either genetic (including duplication or triplication of aSyn gene) or sporadic cases.

2. Objectives

The main objective of this study is to understand if IDE plays a protective role in aSyn pathology.

Specifically, we propose to:

1) Investigate if diet-induced (pre)diabetes decreases IDE brain levels, and if aSyn levels are inversely correlated, in WT mice fed with normal chow vs high-fat diets.

2) Investigate if IDE regulates aSyn pathogenesis in a cellular model of synucleinopathies, by evaluating the impact of IDE or IDE-catalytically inactive variant in the toxicity and aggregation of aSyn in H4 cells.

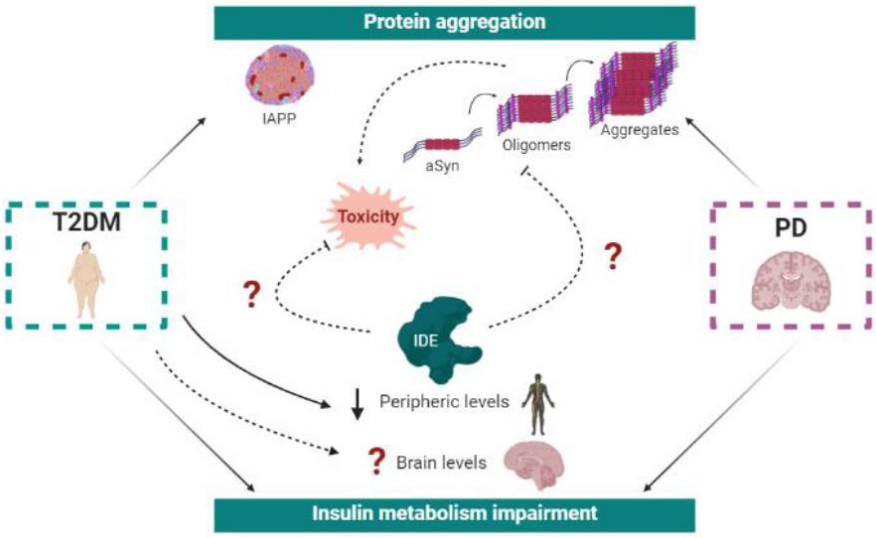


Figure 5. Schematic of the rationale of the thesis hypothesis.

We hypothesize that diabetes may induce IDE impairment in the brain, triggering aSyn pathogenesis in PD.

3. Materials and methods

3.1 Animal approaches

Brain samples

Males C57BL/6J mice from the laboratory of Professor Paula Macedo were separated at 6-weeks age into two groups. The first was fed with a high fat diet (HFD), and the second with continuous normal chow diet (NCD) for 12 weeks. HFD consists in 58% fat from lard, 25.6% carbohydrate, and 16.4% protein, accounting for a caloric basis of 23.4 kJ/g [198]. NCD contained 11.4% fat, 62.8% carbohydrate, and 25.8% protein making a total of 12.6 kJ/g. Animals were weight, glucose tolerance test performed, and glycemic levels determined prior to sacrifice at 18 weeks of age. Brain samples were collected and dissected into different brain regions, including cortex, *cerebellum, hippocampus*, midbrain, *striatum,* brainstem, olfactory bulb and *hypothalamus*.

Glucose Tolerance Test

For glucose tolerance test, overnight fasting mice were intraperitoneal injected with 20% glucose dissolved in a saline solution and glucose measurements from blood have been done at 0, 15, 30, 60, 90 and 120 min after injection.

Glucose measurements

Glucose levels were evaluated from overnight fasting mice prior to sacrifice. Blood samples were collected from the tail and glycemia was measured with a glucometer (Bayer, Contour next, Basel, Switzerland).

Protein extraction

For protein extraction, tissue from each brain region was macerated and sonicated in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Tergitol-type NP-40, 0.1% SDS, 0.25% DOC) at a proportion of 1 µg/1 µl, 3 times for 30 seconds in pulses of 1 second at amplitude of 15 % (Branson Digital Sonifier SFX 150, Emerson, US) with 1 minute incubation on ice between each sonication steps. Samples were centrifuged at 1000 g for 15 minutes at 4ºC, and both supernatant and pellets were collected and stored at -20ºC for future analysis. Protein extracts concentrations was determined relative to BSA protein standards using Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific; Waltham, MA, USA).

SDS page and Western blotting

Brain samples (10 µg) were resolved by SDS-PAGE using a Tetra Cell (Bio-Rad, California, United States of America) in a 12% SDS-polyacrilamide gel (Bio-Rad, California, United States of America) using standard

procedures. Proteins were transferred to nitrocellulose membranes (Bio-Rad, California, United States of America) using the Mini Trans-Blot System (Bio-Rad, California, United States of America). Prestained standard proteins were also loaded on the gel. For immunoblotting analysis, membranes were incubated for 1hour with constant shaking at room temperature with blocking solution, consisting of 5% bovine serum albumin (BSA)(Albumine Bovine Fraction V, NzyTech, Lisbon, PT) or 5% low-fat milk in Tris-HCl buffer saline (1x TBS) (150 mM NaCl, 50 mM Tris-HCl, pH 7.4), according to the primary antibody used. The membranes were incubated overnight at 4ºC with different primary antibodies: rabbit-IDE (Ab32216, Abcam; 1:1000 dilution in TBS + 1% Milk); purified mouse anti-α-synuclein antibody (610787, BD Biosciences; San Jose, CA, USA; 1:1000 dilution in TBS + 5% BSA); mouse monoclonal anti-β-actin antibody (Am4302, Ambion, Thermo Fisher Scientific; Waltham, MA, USA; 1:5000 dilution in TBS + 5% BSA). Membranes were washed 3 times with TBS supplemented with 10% tween-20 solution (TBS-T) and incubated for 1h20 min with anti-mouse-IgGkBP-HRP (sc-516102) or mouse anti-rabbit-IgG-HRP (sc-516102) - horseradish peroxidase-conjugated secondary antibodies (santa cruz biotechnology, Heidelberg, Germany), using a dilution of 1:15000 in the corresponding blocking solution. After 4 washes with TBS-T, detection procedures were performed according to the ECL system (GE Healthcare, Life Sciences; Little Chalfont, UK), using appropriate exposure time in a ChemiDoc™ Touch Imaging System (Bio-Rad, CA, USA). Densitometry of the chemiluminescent signal was performed using ImageJ - Image Processing and Analysis in Java [202].

The levels of the selected proteins were normalized to a loading control protein (β-actin). The ratio to control conditions (NCD group) was calculated. The ratio between the β-actin normalized aSyn and IDE levels within each condition has been also calculated.

3.2 Cellular procedures

DNA extraction

pcDNA3.1 plasmids containing IDE-WT or IDE-E111Q gene were kindly provided by the laboratory of Professor Denis Selkoe (USA). DNA was extracted from whatman paper by eluting with MilliQ water.

Bacteria transformation

Competent DH5α bacteria (50 ul) were transformed with around 50 ng of DNA. DNA insertion was performed by heat shock (20-30 minutes on ice, 90 seconds at 42ºC, 5 minutes on ice). Bacteria was incubated at 37ºC for 1 hour in SOC medium. Transformed bacteria were selected by overnight growth at 37ºC in LB agar plates supplemented with Ampicillin. For DNA isolation, a single colony was inoculated in liquid LB supplemented with Ampicillin for 8 hours at 37ºC.

DNA isolation

Maxi-prep (PureLink® HiPure Plasmid Filter Purification Kits, Thermo Fisher Scientific; Waltham, MA, USA) protocol was followed according to the supplier instructions, and DNA amount measured by NanoDrop™ 2000 (Thermo Fisher, Waltham, MA, USA).

Cell culture

H4 is a human neuroglioma cell line (ATCC® HTB-148™; Manassas, VA, USA). Cells were maintained in Opti-MEM® I Reduced Serum Medium (1x) (Gibco, Thermo Fisher Scientific; Waltham, MA, USA) supplemented with 10% of fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific; Waltham, MA, USA) incubated at 37ºC in a 5% CO2 environment and 1 Million cells were seeded every 2 days in 10 cm dishes (VWR, part of Avantor, Radnor Township, PA, USA). For cytotoxicity assays, cells were seeded in 12-well plates (VWR, part of Avantor, Radnor Township, PA, USA), while for Triton solubility assays, cells were seeded in 10 cm dishes, for protein levels assays, in 6-well plates (VWR, part of Avantor, Radnor Township, PA, USA) and for microscopy studies in 35 mm imaging dishes (µ-Dish, Ibidi, Martinsried, Germany).

Transfection

Cells were transfected with pcDNA3.1, aSyn or SynT, which consists of a plasmid encoding for aSyn fused with a truncated form of green fluorescent protein which forms *in vitro* inclusions [206], and wild type IDE or mutant IDE-E111Q (catalytic inactive form) inserted in pcDNA3.1 backbone (Invitrogen, California, United States of America). Transfection was performed with FuGENE® 6 Transfection Reagent (Promega; Madison, WI, USA), according to the manufacturer's instructions, using 1:3 proportion of plasmid (µg DNA: µl Fugene) to Fugene 6 for single transfections, or 1:5 for double transfections. For 10 cm plates, 10 µg of plasmid was used, for 6-well plates or 35 mm imaging dishes, 2 µg and, for 12-well plates 0,8 µg of DNA. Cells confluence was around 50-80% when transfected. 24 hours post-transfection, the medium was renewed, and cells collected 48 hours post-transfection.

Cytotoxicity assays

Cytotoxicity was analyzed using the LDH kit (Takara, Bio Europe, Saint-Germain-en-Laye, France). This is based on the release of lactate dehydrogenase (LDH) into cell culture media upon loss of plasma membrane integrity. This method is based on the enzymatic reactions in which the formation of formazan is directly proportional to the amount of LDH released by the cells. Human H4 cells, 90.000 cells/well were seeded in 12-well plates (3.8 cm^2). 48 hours post-transfection procedures, conditioned media was collected, and

centrifuged at 750 rpm for 5 minutes (to discard cellular debris). Fresh media containing 1% Triton®X 100 (Panreac; Barcelona, Spain) was added to cells, in order to completely lyse them. This conditioned media was collected and used as 100% cell death control. 5 µl of each culture condition was incubated with the reaction mixture in a 96-well microplate (VWR, part of Avantor, Radnor Township, PA, USA) for 20-30 minutes at 37ºC to determine LDH activity and quantify cell death. The amount of formazan was determined by the difference between the absorption at 490 nm (absorption wavelength of formazan) and absorption at 620 nm (as a reference wavelength). The measured cytotoxicity is given in percentage.

Protein extraction

Cells were suspended in NP40 lysis buffer (150 mM NaCl ,50 mM Tris-HCl, pH 8.0, 1% NP-40) supplemented with cOmplete™, Mini, EDTA-free Protease Inhibitor (Roche; Basel, Switzerland). Cell lysis and protein extraction was performed by 3 cycles of freezing in liquid nitrogen. Protein extracts were centrifuged at 10,000 g, at 4 ºC, for 10 minutes and supernatant collected and stored at -20ºC until further use. Protein extracts concentrations was determined relative to BSA protein standards using Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific; Waltham, MA, USA).

SDS page and Western blotting

Samples (15 µg) were resolved by SDS-PAGE, as previously described. The levels of the selected proteins were normalized to a loading control protein (β-actin). The ratio to control conditions (aSyn + EV) was calculated. The ratio between the β -actin normalized aSyn and IDE levels within each condition has been also calculated.

Clearance

Cells seeded at 190.000 cells/well in 6-well plates were transfected as previously described. 24 hours post-transfection, the medium was renewed, and 43 hours post-transfection, cells were treated with cycloheximide (100 µM) for a period up to 12 hours. This treatment arrests *de novo* protein synthesis, which allows to monitor the clearance of aSyn. 55 hours post-transfection, protein extracts were prepared and SynT levels determined as previously described. The levels of SynT were normalized to the levels present in cells without cycloheximide treatment.

Triton-X 100 solubility assay

The solubility of the proteins can be assessed by a Triton X solubility assay. This technique consists on the separation of proteins according to their detergent solubility at a given percentage. Cells were lysed using phosphate-buffered saline (PBS) (Gibco, Thermo Fisher Scientific; Waltham, MA, USA) supplemented with protease inhibitors (COmplete™, Mini, EDTA-free Protease Inhibitor, Roche, Basel, Switzerland), sonicated 3 times for 30 seconds in pulses of 1 second at amplitude of 15 % (Branson Digital Sonifier SFX 150, Emerson, US) with 1 minute incubation on ice between each sonication step. The resultant supernatant, that represents the "total protein" was quantified using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific; Waltham, MA, USA). Triton®X 100 was added at a final concentration of 1% to 200 µg of protein extract, followed by an incubation, at 4ºC, for 30 minutes. Protein fractions were separate by centrifugation at 16,000 g, at 4ºC for 1 hour. Soluble protein fraction was collected, and the insoluble protein fraction pellet was resuspended in 40 µl of PBS supplemented with SDS at a final concentration of 2% and COmpleteTM, Mini, EDTA-free Protease Inhibitor (Roche, Basel, Switzerland). Total protein (15 µg), soluble fraction (10 µl) and the insoluble fractions (15 µl) were resolved by SDS-PAGE (12% SDS-page gel) and immunoblotted as previously described. SynT and IDE insolubility is the ratio between insoluble fraction and the sum of insoluble and soluble fractions. The ratio of insoluble fraction of the proteins was calculated to control conditions (SynT+EV).

Immunocytochemistry

Cells were collected and softly washed 2 times with PBS. Immunocytochemistry protocol was started by fixation and permeabilization with 100% methanol (Merckmillipore, Darmstadt, Germany) at -20ºC for 10 minutes. Cells were incubated for 1 hour with blocking solution (1.5% Normal Goat serum (NGS) diluted in filtered PBS) at room temperature. Hybridization with primary antibodies rabbit anti-aSyn (Cell Signalling Technology; Danvers; MA; USA; dilution 1:50) and mouse anti-IDE (F-9) (santa cruz biotechnology, Heidelberg, Germany; dilution 1:50) was performed overnight at 4ªC. Cells were washed with PBS and incubated with Alexa Fluor® 488 goat anti-rabbit conjugated secondary antibody (Invitrogen; Carlsbad, CA, USA) for aSyn detection (dilution 1:1000 in blocking solution) and with Rhodamine Red-x-1 donkey anti-mouse conjugated antibody (Jackson ImmunoResearch, Europe) for IDE detection (dilution 1:500 in blocking solution) for 4 hours at room temperature. Cells were washed with blocking solution and kept protected from light at 4ºC in PBS. Microscopy images were acquired in a Widefield fluorescent microscope Zeiss Axiovert 40 (Carl Zeiss MicroImaging) and in a point scanning confocal microscope Zeiss LSM 710 (Carl Zeiss MicroImaging).

3.3 Statistical Analysis

Each assay was performed at least three times, unless stated otherwise, and all values are expressed as normalized average ± standard deviation. Statistical analysis was performed using GraphPad Prism version 8. T-test or one-way ANOVA were used to compare differences among conditions, followed by Tukey's multiple comparison test. Values of $p < 0.05$ were considered significant.

4. Results and discussion

4.1 Diabetes modulates the levels of IDE in the brain

As previously described, HFD model is an appropriated model to evaluate if (pre)diabetes affects the brain levels of IDE and aSyn [198]. However, it is worth describing that inbred C57BL/6 strain can present heterogeneity in the response to the high fat feeding [208].

Males C57BL/6J mice with 6-weeks age were fed either with NCD (control group) or an HFD for 12 weeks. The schematic of the experimental procedure is depicted (Fig. 6A). For validation of the protocol, weigh (obesity), glycemia, and glucose tolerance were investigated. The HFD-treated animals were overweight (Fig. 6B), and showed decreased glucose tolerance, as assessed by glucose tolerance test (GTT)(Fig. 6D), in comparison to the control group. Furthermore, by analyzing the area under the curve (AUC) of glucose measurements [209], we observed that HFD mice had a higher index of glucose excursion (AUC=27807) in comparison with NCD animals (AUC=13436) (Fig. 6D). However, HFD treated group did not exhibit hyperglycemia (Fig. 6C).

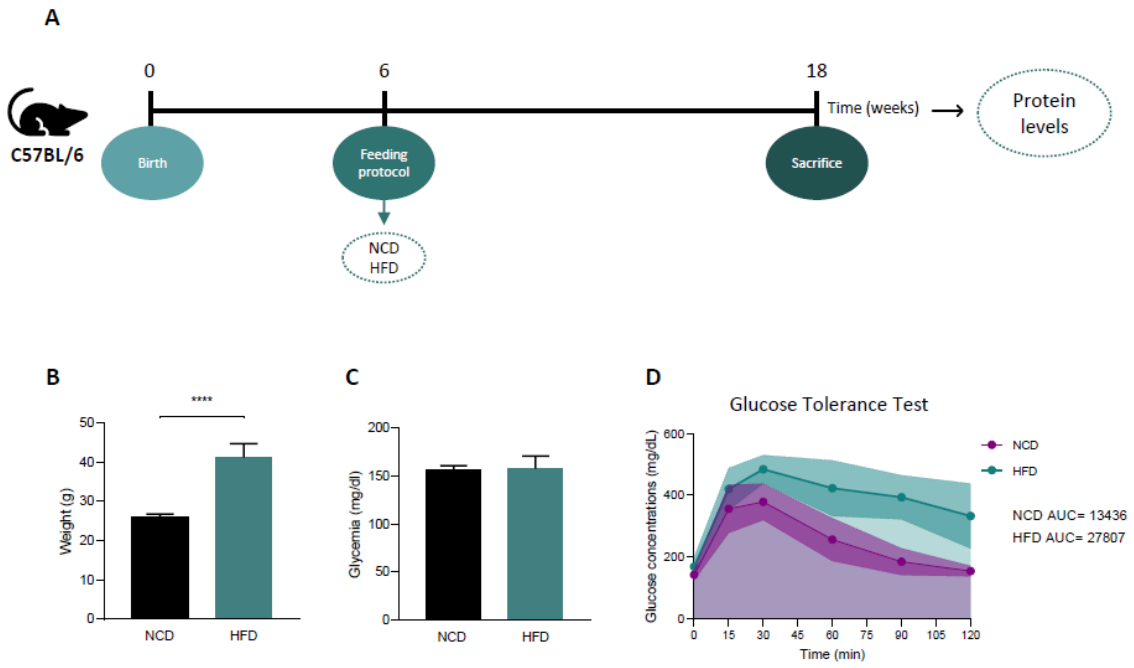


Figure 6. Characterization of the cohort

(A) Schematic of the experimental procedure. At 6-weeks of age, animals were split into two groups and fed with a NCD or a HFD for 12 weeks. Mice were sacrificed at 18 weeks age and brain samples collected and stored at -80ºC. Animals were **(B)** weight and **(C)** blood glucose concentration measured at the day of the sacrifice, in starvation. **(D)** Glucose

tolerance test. Glucose concentrations were measured at different time points after an intraperitoneal injection of 20% glucose. At least n=4. Data in all panels are average ± SD. Unpaired t-test. ****p < 0.0001.

The HFD mice model is an established model of pre-diabetes [198]. It exhibits obesity, impaired glucose metabolism, without hyperglycemia [198] and insulin resistance [210]. Previous studies have demonstrated that cortical IDE expression levels depends on insulin signaling and insulin resistance [211], which are hallmarks of T2DM. IDE levels may therefore decrease with T2DM [212] and slower insulin turnover [213]. In agreement, mice fed an HFD show decreased levels of IDE in the pancreas [214].

Therefore, we hypothesized that the HFD paradigm may also decrease the levels of IDE in the brain. To evaluate our hypothesis, brain tissue from 18-weeks mice fed a HFD or NCD for 12-weeks were collected and dissected in different brain regions including cortex, *cerebellum, hippocampus*, midbrain, *striatum,* brainstem, olfactory bulb and *hypothalamus*. The levels of IDE were determined by western blotting (Fig. 7A). First, we confirmed that IDE was expressed throughout the brain, specifically in the midbrain, cortex, *hippocampus and cerebellum* of WT mice, being more expressed in the *cerebellum* (Fig. 7B). Given the hypothesis that the levels of IDE and aSyn inversely correlate we also ensured the detection of aSyn in different brain regions. aSyn is expressed in all analyzed brain regions, being more expressed in the *hippocampus*, followed by the cortex (Fig. 7C).

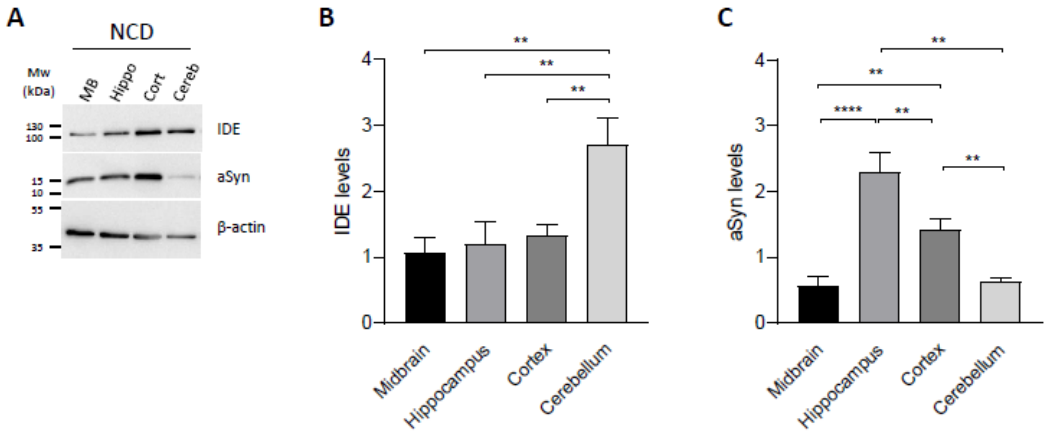
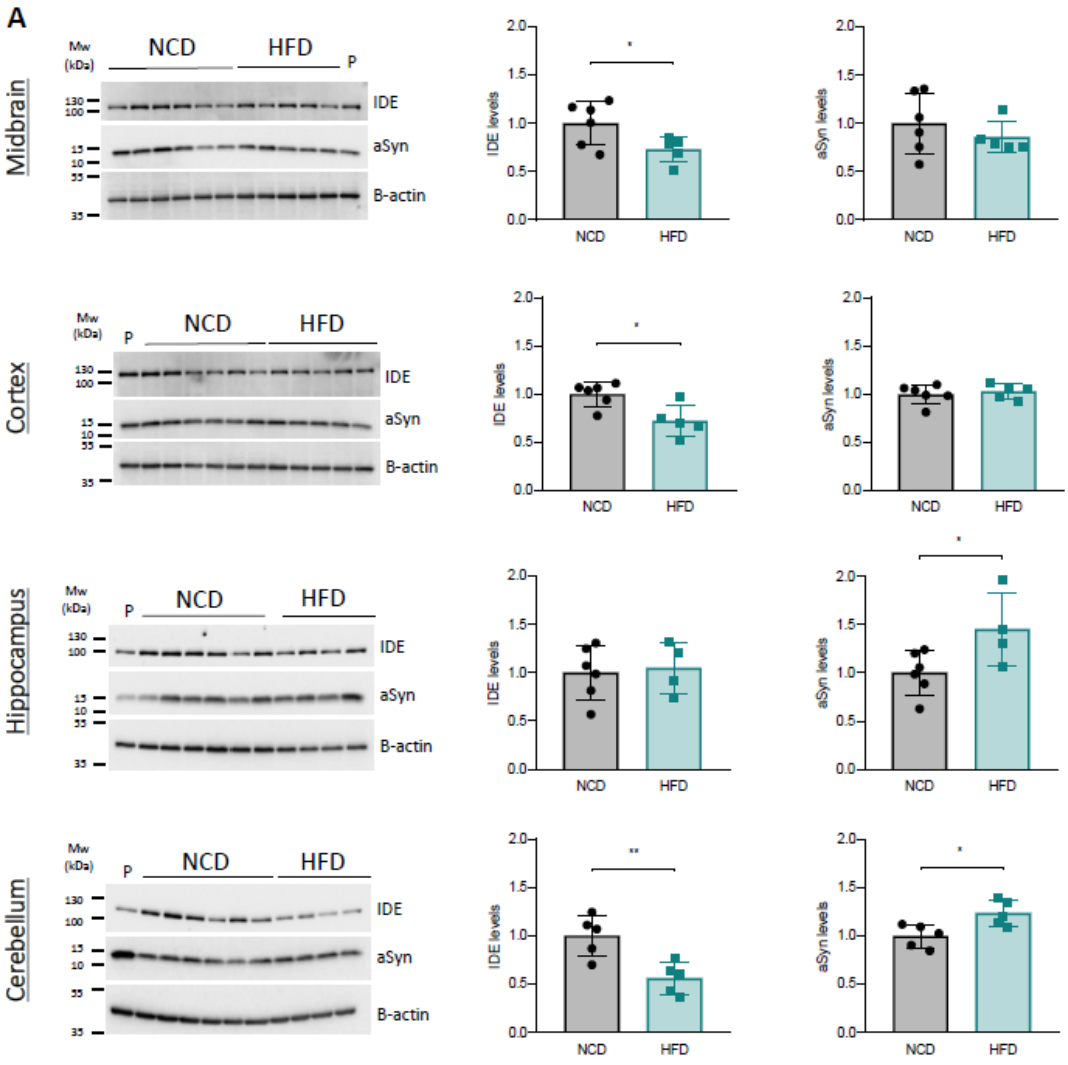


Figure 7. IDE and aSyn is expressed throughout the brain

(A) Brain samples from different brain areas, including midbrain, *hippocampus*, cortex and *cerebellum* were collected from WT mice. Total protein extracts were probed for IDE, aSyn and β-actin for normalization. **(B)** IDE and **(C)** aSyn levels were normalized to β-actin and to a pooled fraction of mid-brain. At least n=4. Data in all panels are average ± SD, One-way ANOVA, followed by Tukey's multiple comparison test, **p < 0.01, ****p < 0.0001.

To evaluate the effects of (pre)diabetes, the levels of IDE and aSyn were compared in the brains of mice fed with HFD or NCD. Our results demonstrate that HFD generally reduces the levels of IDE in the brain (Fig. 8A).

In contrast, the levels of aSyn are either unaltered or are increased (Fig. 8A). In particular, we observed that both in the midbrain (0.7-fold) and in the cortex (0.7-fold), HFD group showed a significant decrease in the levels of IDE, while the levels of aSyn did not change (Fig. 8A). In contrast, in the *hippocampus* no significant differences were observed in the levels of IDE in the HFD animals, however the levels of aSyn were significantly increased (1.5-fold) (Fig. 8A). The major alterations were found in the *cerebellum*. Interestingly, we observed a significant decrease in the levels of IDE (0.6-fold), followed by an increase in the levels of aSyn (1.2-fold) in HFD mice (Fig. 8A). Since the levels of IDE and aSyn seem to be inversely correlated, we calculated the ratio between aSyn and IDE levels from each animal. Surprisingly, at the exception of midbrain region, the difference is significantly different in cortex (1.5-fold), *hippocampus* (1.3-fold) and even more expressive in the *cerebellum* (2.3-fold) of mice fed an HFD (Fig. 8B). Moreover, there is a significant negatively correlation between IDE and aSyn levels in the *cerebellum* (Fig. 8C).



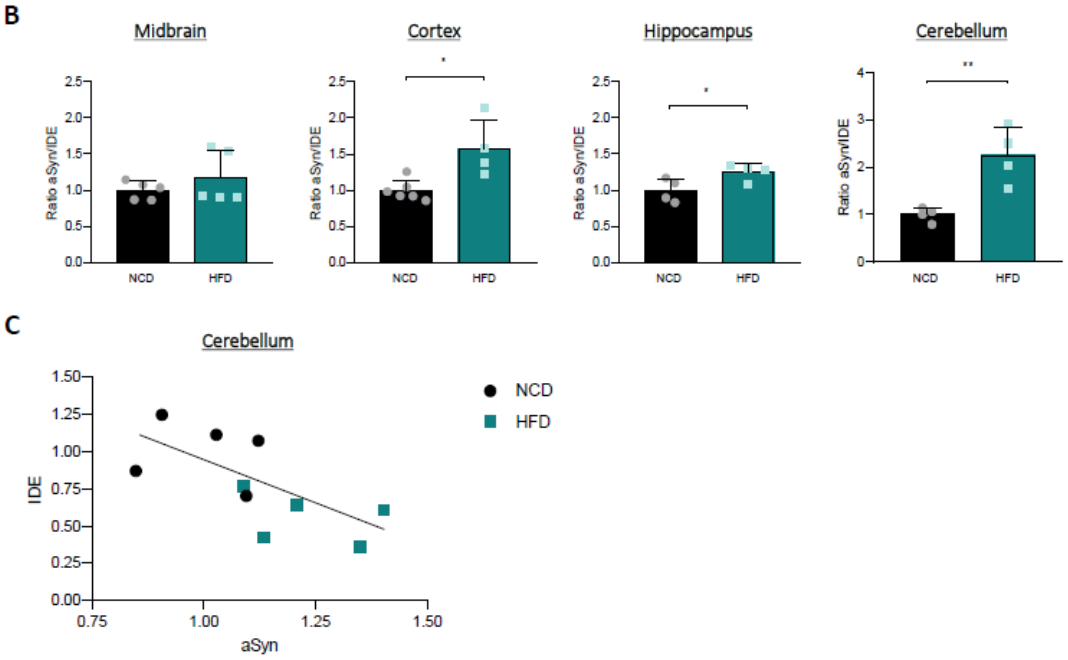


Figure 8. IDE levels decrease while aSyn levels increase in prediabetic mice.

Protein extracts from brain samples of NCD and HFD mice were probed for IDE, aSyn and normalized to β-actin. Protein levels were normalized intra-gel to a Pool and then inter-gel to the average levels of NCD. **(A)** Representative blots of mid-brain, cortex, *hippocampus* and *cerebellum*. The levels of IDE and aSyn are normalized to NCD levels. **(B)** The ratio between aSyn/IDE is presented and normalized to NCD levels. **(C)** Correlation of aSyn and IDE levels in the *cerebellum*. (at least n=4). Data in all panels are average ± SD. Unpaired t-test. *p < 0.05, **p < 0.01.

This data suggests that, diabetes decreases the levels of IDE in the brain. Moreover, it also suggests that IDE failure may induce aSyn accumulation in the brain. These findings are in agreement with the inverse correlation found in the pancreas [88], therefore validating our hypothesis that IDE failure in the brain may contribute for aSyn pathology in PD.

4.2 IDE modulates aSyn pathogenesis

Given the hypothesis that IDE is able to suppress the fibrilization of aSyn, we aimed to evaluate if IDE modulates aSyn pathogenesis. To that purpose, we investigated the interplay between aSyn and IDE *in vitro*. H4 neuroglioma cells were co-transfected with pcDNA3.1 (EV), aSyn WT or SynT (aggregation-prone variant) together with EV, IDE-WT or IDE-E111Q (catalytically inactive) according to the described timetable (Fig. 9A). 48 hours post-transfection, several aSyn pathological readouts were determined, including aSyn toxicity, proteostasis and aggregation (Fig. 9B).

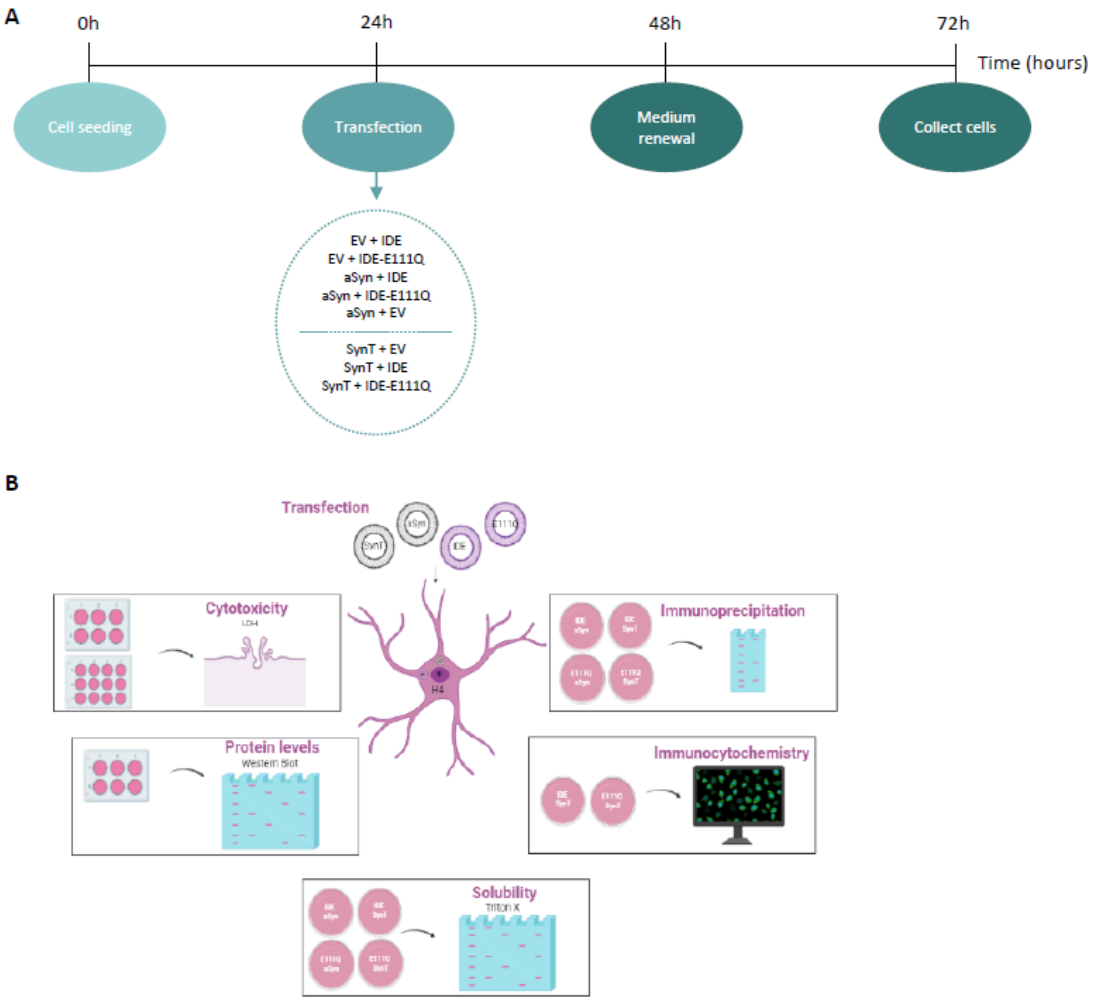


Figure 9. Schematic of the experimental setup of in vitro experiments.

(A) H4 cells were co-transfected 24 hours post-seeding with aSyn or SynT together with IDE-WT or mutant IDE-E111Q. Medium was replaced 24 hours post-transfection and cells harvested for analysis 48 hours post-transfection. **(B)** Cytotoxicity, protein levels, solubility, interaction and aggregation assays were analyzed.

4.2.1 IDE prevents aSyn cytotoxicity

To assess if IDE is able to protect from aSyn cytotoxicity, H4 cells were treated as previously described (Fig. 9A). Cytotoxicity was assessed by LDH release to the cells conditioned media, that occurs only upon loss of cell membrane integrity. Remarkably, IDE-WT or IDE-E111Q expression is able to significantly decrease (10-15%) the cytotoxicity of aSyn (Fig. 10A). However, no differences were observed with SynT (Fig. 10B). These findings suggest that IDE prevention of aSyn toxicity may not be dependent on its catalytic activity, however, it is only possible if aSyn is not altered in its C-terminal domain, since no effects were observed in the toxicity of C-terminally tagged aSyn (SynT).

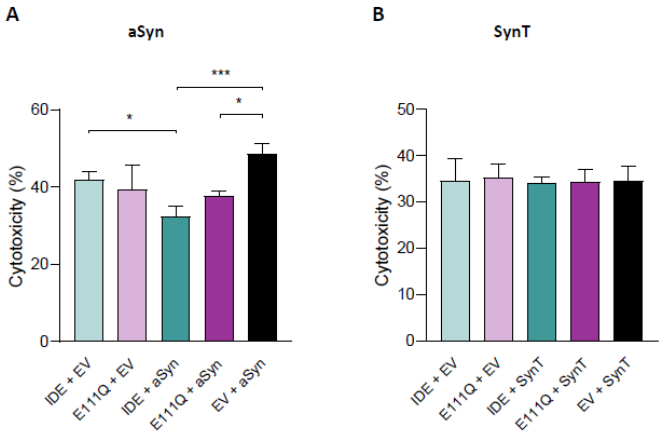


Figure 10. IDE suppresses aSyn toxicity, but not of SynT.

H4 cells were co-transfected with **(A)** aSyn or **(B)** SynT or EV together with EV, IDE-WT or IDE-E111Q. 48 hours post-transfection, the levels of released LDH were determined. Cytotoxicity was determined by normalizing to total cell loss (induced by triton x-100). Data is presented in percentage. At least n=3. Data in all panels are average ± SD, One-way ANOVA, followed by Tukey's multiple comparison test *p < 0.05, ***p < 0.001.

4.2.2 IDE modulates aSyn levels and vice versa

The protective effect of IDE over aSyn toxicity raises the question about whether IDE modulates the levels of aSyn or SynT. To that purpose, IDE and aSyn levels were determined by western-blotting of H4 cells following our previously described paradigm (Fig. 9A).

We observed that in cells overexpressing aSyn, the overexpression of both IDE-WT and IDE-E111Q increases the levels of aSyn (comparing to EV co-transfected cells). Likewise, in cells overexpressing IDE, the overexpression of aSyn increases the levels of both IDE-WT and IDE-E111Q (comparing to EV co-transfected cells) (Fig. 11A). However, the same phenotype was not observed if cells were overexpressing SynT (Fig. 11B). We still did not perform the experiment with the co-transfection of IDE-WT or IDE-E111Q with EV, therefore the impact of SynT expression in the levels of IDE cannot be evaluated. When calculating the ration between aSyn or SynT and IDE, no changes are observed (Fig. 11A, B).

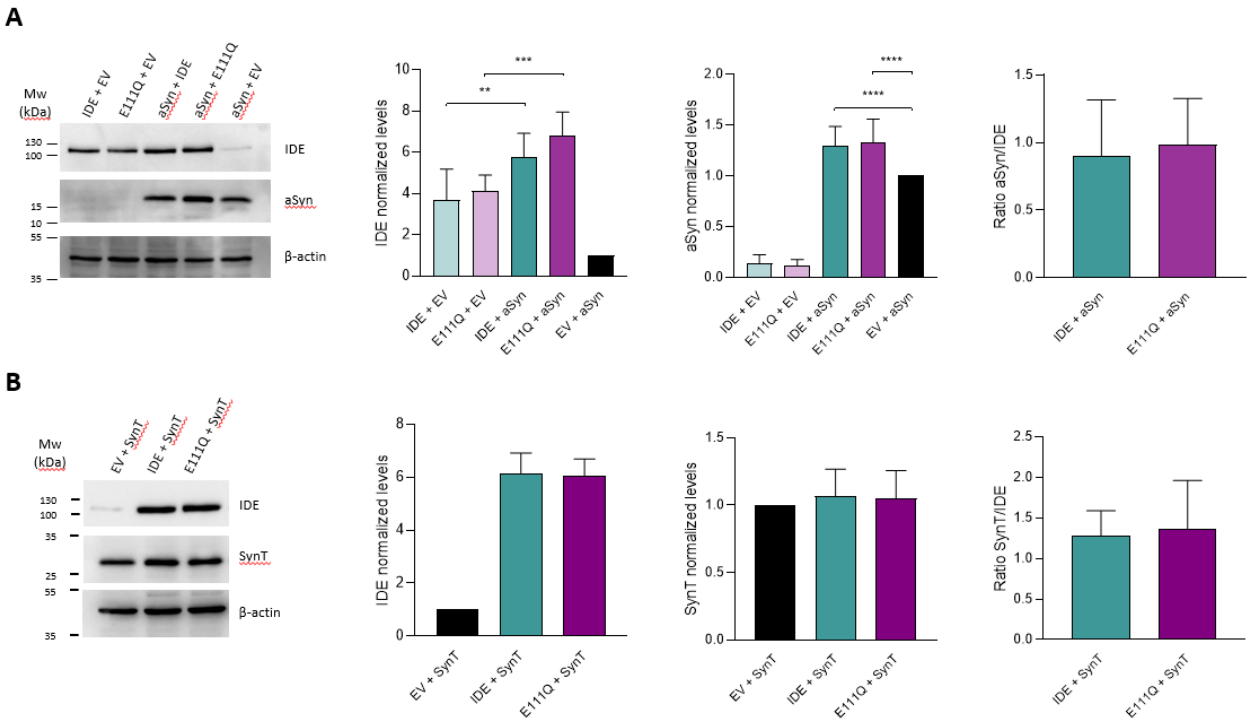


Figure 11. IDE increases the levels of aSyn but not of SynT. aSyn but not SynT also increase IDE levels.

(A) Protein extracts from cells co-expressing aSyn or EV together with EV, IDE-WT or IDE-E111Q were probed for aSyn, IDE and β-actin, for normalization. Levels of IDE or aSyn were normalized to aSyn + EV. Ratio between the levels of aSyn and IDE-WT or IDE-E111Q are presented. **(B)** Protein extracts from cells co-expressing SynT together with EV, IDE-WT or IDE-E111Q were probed for aSyn, IDE and β actin, for normalization. Levels of IDE and SynT were normalized to SynT+EV. Ratio between the levels of SynT and IDE-WT or IDE-E111Q are presented. At least n=3. Data in all panels are average ± SD, One-way ANOVA, followed by Tukey's multiple comparison test, **p < 0.01, ***p < 0.001, ****p < 0.0001.

These findings suggest that IDE can modulate the levels of aSyn and vice-versa. However, the same findings are not recapitulated upon SynT overexpression. In fact, we also proposed to study this variant to address if changes in the C-terminal of aSyn could alter the interplay between aSyn and IDE. In agreement, as previously described, IDE was not able to suppress the toxicity of SynT (Fig. 10B), therefore supporting the requirement of a functional aSyn C-terminal to allow interaction with IDE [190]. To address this hypothesis, we are currently measuring aSyn and/or SynT interaction with IDE via co-immunoprecipitation experiments.

4.2.3 IDE-E111Q plays a role in aSyn clearance

To better understand how overexpression of IDE (WT or E111Q) could modulate aSyn stability, we evaluated protein clearance. In fact, IDE was described to modulate the activity of both the UPS (playing a role in Ubiquitin-activation, and interacting with the uncapped 20S proteasome and single-capped 26S proteasome)

and the chaperone-mediated autophagy (CMA), which represent important pathways involved in the clearance of damaged proteins [179]. However, the mechanism by which IDE modulates protein clearance is not completely elucidated. Therefore, according to IDE multifunctionality and its specific effects in the main pathways of protein clearance [179], it would be expected that IDE could play a role in the clearance of aSyn.

To investigate if the clearance of aSyn and SynT were affected by IDE, H4 cells were treated as previously described (Fig. 9A). Briefly, 43-, 47- or 51-hours post-transfection, cells were treated with cycloheximide to arrest *de novo* protein synthesis for 12 hours. 55 hours post-transfection, cells were collected and aSyn levels were monitored by western-blotting. For unknown reasons, we gathered irreproducible data in the setup of aSyn overexpression. However, the analysis was possible in cells expressing SynT. In these conditions, the overexpression of IDE-WT did not alter the kinetics of SynT clearance (Fig. 12). In contrast, the expression of IDE-E111Q impaired the clearance of SynT (Fig. 12). More specifically, the levels of SynT 12 hours post-cycloheximide treatment were significantly higher upon expression of IDE-E111Q (1.8-fold).

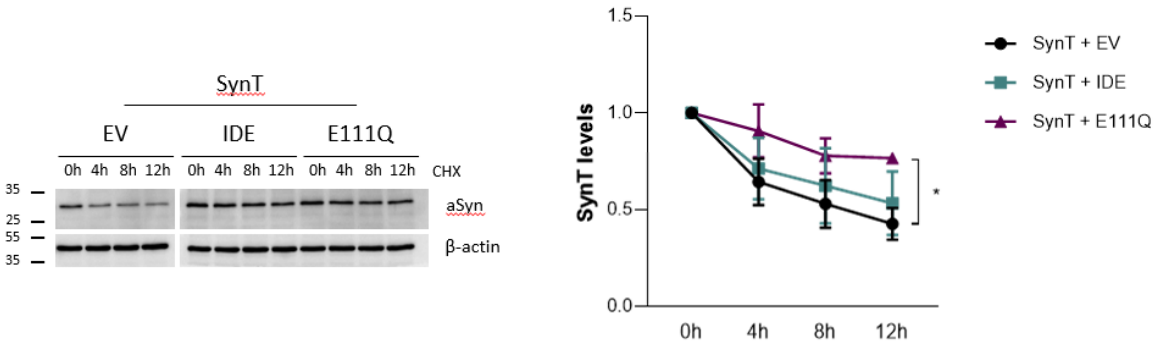


Figure 12. Catalytic inactive IDE (IDE-E111Q) impairs SynT clearance.

H4 cells co-expressing SynT together with EV, IDE-WT or IDE-E111Q were collected 56 hours post-transfection after treatment with cycloheximide (CHX) for the last 0, 4, 8 and 12 hours. Protein extracts were probed for aSyn and β-actin, for normalization. SynT protein levels were normalized to 0 hours treatment. At least n=3. Data in all panels are average ± SD, One-way ANOVA, followed by Tukey's multiple comparison test. *$p < 0.05$.

Since we previously observed that IDE-E111Q did not alter the levels of SynT, but it impacts SynT clearance, we hypothesize that IDE-E111Q expression may be influencing SynT expression at a transcriptional level. We thus propose to analyze SynT mRNA levels by qPCR technique.

4.2.4 IDE impacts aSyn insolubility and aggregation

One major hallmark of aSyn pathology is its aggregation [215]. Given that IDE was described to interact with aSyn in its oligomeric state and thus preventing its further aggregation [190], we aimed to understand if IDE

(WT or E111Q) is able to modulate aSyn solubility. H4 cells were treated as previously described (Fig. 9A). To determine the insolubility of aSyn and SynT, Triton X-100 solubility assay was performed. Briefly, native (non-denatured) protein extracts were solubilized in 1% Triton X-100 solution, and the soluble and insoluble fractions separated by high-speed centrifugation. Soluble proteins remain in the supernatant, while insoluble protein is retained in the pellet. aSyn levels in both soluble and insoluble fractions were determined by western-blotting and insolubility calculated as the ratio between aSyn levels in insoluble fractions and in total fraction (combination of soluble and insoluble aSyn levels). We observed that the levels of insoluble aSyn were not modulated in the presence of IDE-WT neither IDE-E111Q (Fig. 13A). Moreover, no differences in the insolubility of IDE were observed (Fig. 13A). In what regards to SynT, IDE-WT expression did not modulate its insolubility (Fig. 13B). However, SynT is more soluble in IDE-E111Q expressing cells when comparing to IDE-WT expressing cells (reducing around 15% the insolubility) (Fig. 13B). Additionally, IDE-E111Q is more soluble then endogenous IDE in SynT expressing cells (Fig. 13B).

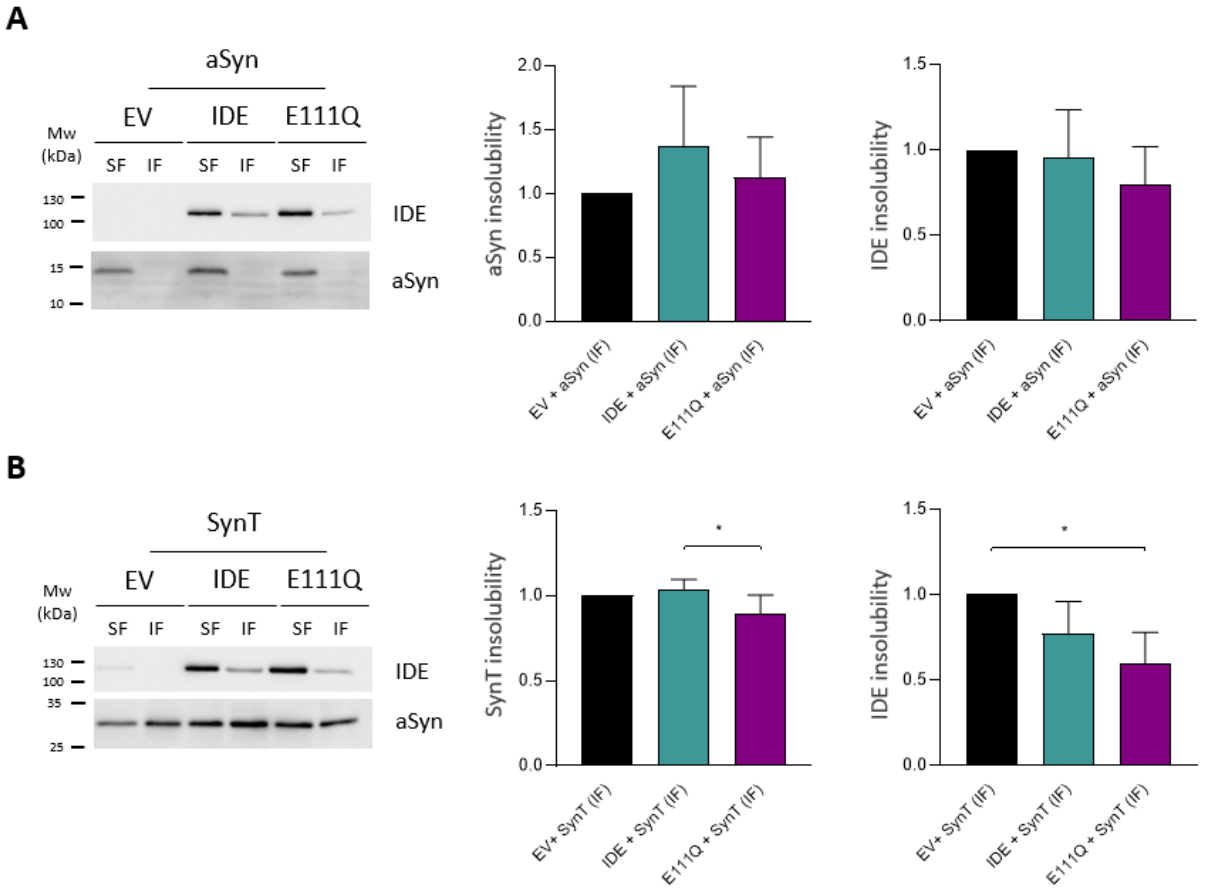


Figure 13. IDE impact in insolubility of aSyn and SynT.

(A) H4 cells were co-transfected with aSyn and EV or IDE-WT or IDE-E111Q. Triton X-100 soluble (SF) and insoluble (IF) fractions were probed for aSyn and IDE. aSyn and IDE insolubility is the ratio between insoluble fraction and the sum of insoluble and soluble fractions. **(B)** H4 cells were co-transfected with SynT and EV or IDE-WT or IDE-E111Q. Triton X-100 SF and IF fractions were probed for aSyn and IDE. SynT and IDE insolubility is the ratio between insoluble fraction and the sum of insoluble and soluble fractions. At least n=3. Data in all panels are average ± SD, One-way ANOVA, multiple comparisons, *p < 0.05.

The impact of IDE on aSyn aggregation was also assessed by immunocytochemistry. In particular, as previously described, cells expressing SynT variant of aSyn recapitulate the formation of protein aggregates [206]. Briefly, cells co-expressing SynT together with EV, IDE-WT or IDE-E111Q were fixed and immunostained for aSyn (Fig. 14A). We counted the percentage of cells that display SynT aggregates. Moreover, we also determined the percentage of cells presenting big aggregates (more than 3 µm) and the cells with more than 10 aggregates. Although the percentage of cells with SynT aggregates and the number of cells with more than 10 aggregates was not modulated by IDE (WT or E111Q) expression, we observed that both IDE forms induced the formation of big aggregates of SynT (25%) (Fig. 14B).

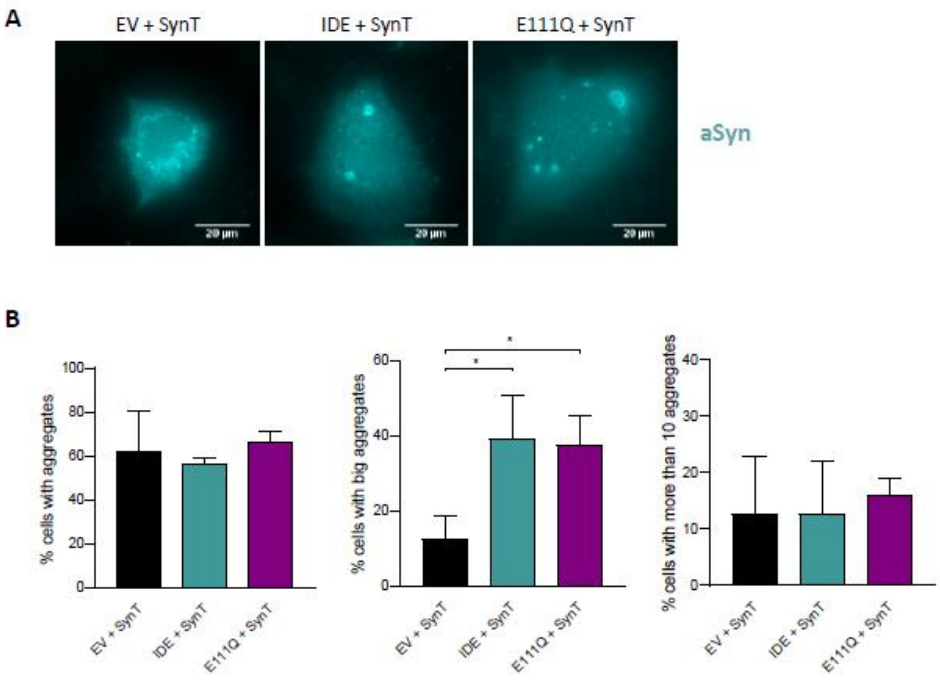


Figure 14. IDE increase the size of SynT aggregates.

(A) H4 cells co-expressing SynT together with EV, IDE-WT or IDE-E111Q were collected 48 hours post-transfection. Cells were fixed with methanol and then probed with aSyn. **(B)** Percentage of cells with aggregates, with aggregates bigger than 3 µm, and with more than 10 aggregates were analyzed. At least n=3. Data in all panels are average ± SD, One-way ANOVA, followed by Tukey's multiple comparison test. *p < 0.05.

These findings seem to be contrary to the observed decrease of insoluble forms of SynT between cells expressing IDE-WT or IDE-E111Q. However, one should note that the insolubility assay does not distinguish between the type of insoluble forms of aSyn. Meaning that a reduction of SynT oligomers into monomers but not of SynT aggregates to SynT monomers would increase SynT solubility, without changing SynT bigger inclusions. Therefore, it is necessary to better understand the type of species that are favored by the interaction with IDE (WT or E111Q). This can be achieved by analyzing native protein extracts by size exclusion chromatography or separating proteins by sucrose gradient approaches, that could better indicate the type of oligomers that are present in both conditions.

Since the formation of bigger inclusions (Fig. 14B) is not followed by increased toxicity of SynT (Fig. 10B), we hypothesize that these structures are not toxic. In fact, it is in agreement with previous studies that describe that trapping aSyn into bigger inclusions could be a neuroprotective mechanism [216] to avoid cells from accumulating more oligomeric toxic species of aSyn [217]. Interestingly, PD patients present large inclusions mainly constituted by aSyn in the surviving neurons [108].

4.2.5 IDE interacts with aSyn and SynT, however they do not colocalize in SynT inclusions

Previous findings from our laboratory, suggest that IDE interacts both with SynT in H4 cells (Fig. 15A) and with aSyn from mouse brain tissue (Fig. 15B). Furthermore, we investigated the intracellular localization of IDE and aSyn in H4 aggregation paradigm. Cells co-expressing SynT and IDE (WT or E111Q) were fixed, immunostained for aSyn and IDE and confocal imaging was performed. Our preliminary results suggest that both IDE (endogenous, WT or E111Q) and aSyn are dispersed throughout the cell, but they do not colocalize with SynT bigger inclusions (Fig. 15C). These findings may suggest that the observed impact of IDE in SynT aggregates could be on the early steps of SynT oligomerization, but not in highly aggregated SynT. Since H4 cells expressing aSyn do not recapitulate the formation of protein inclusions, we cannot ascertain if the same phenomenon would occur in non-tagged aSyn. To disclose this hypothesis, it would be interesting, in the future, to evaluate IDE-aSyn co-localization in aSyn aggregates in the brains of mouse models of PD.

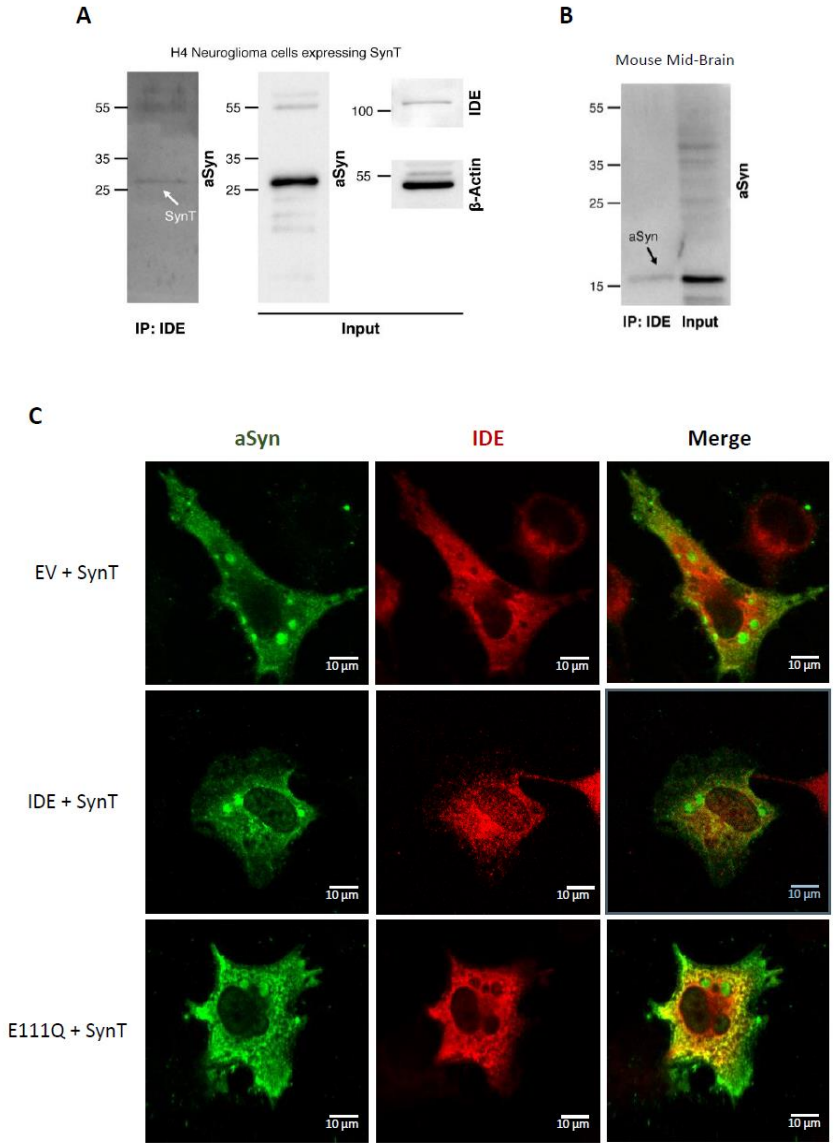


Figure 15. IDE interacts with aSyn or SynT, but not in SynT inclusions.

(A) H4 cells expressing SynT were collected 48 hours post-transfection. Immunoprecipitation of IDE was performed and aSyn co-precipitation evaluated by western-blotting with anti-aSyn. aSyn is co-precipitated. Protein inputs are also presented. **(B)** Brain samples from mouse mid-brain were processed, IDE immunoprecipitated and aSyn co-precipitation were evaluated by western-blotting. aSyn is co-precipitated. Protein input is also presented. **(C)** H4 cells co-expressing SynT together with EV, IDE-WT or IDE-E111Q were collected 48 hours post-transfection. Cells were fixed with methanol and then probed with aSyn and IDE. n=1. (A) and (B) are preliminary data from Vicente Miranda laboratory.

5. Conclusions and future perspectives

Recent epidemiological findings concluded that T2DM is a risk factor for PD. Learning from T2DM pathology, IDE has been demonstrated to suppress the cytotoxicity of amyloidogenic peptides, in particular of IAPP, Aβ and more recently aSyn [190]. For that reason, IDE has been studied in a context of neurodegenerative disorders, specifically in AD, given that Aβ is a proteolytic substrate of IDE [173]. Although, aSyn is not a substrate, IDE may present dead-end chaperone-like function suppressing protein aggregation [188]. Moreover, IDE may display preference for interaction with the C-terminal domain of oligomeric aSyn, forming stable non-toxic complexes [190]. Research on diabetes also showed that the levels of IDE are decreased in T2DM patients [218]. Findings that are recapitulated in mouse model of diabetes showing that not only impairment in insulin secretion upon glucose stimulation, but also increased levels of aSyn in pancreatic islets [88,171]. Moreover, a possible correlation between insulin dysfunction and protein accumulation is observed in these mice [88]. Therefore, we hypothesize that if IDE becomes impaired in PD, it may contribute to aSyn pathogenesis by allowing for its accumulation and aggregation.

In this thesis, the major aim was to investigate if diabetes may decrease IDE levels also in the brain, promoting aSyn accumulation. Additionally, the aim was to understand if IDE is able and how it suppresses aSyn pathogenesis in a cellular model of synucleinopathy.

T2DM patients and (pre)diabetic mice models present decreased peripheral levels of IDE [219]. Here, we showed that mice fed an HFD, generally present lower levels of IDE in the brain. Therefore, our findings suggest that there may be a possible association between the peripheral and brain levels of IDE. Interestingly, an inverse correlation with the levels of aSyn was also observed in several brain regions. We thus hypothesize that it should be important to investigate if PD patients also present lower peripheral levels of IDE, therefore reflecting lower levels of IDE in the brain, and consequent higher levels of aSyn. Moreover, if this hypothesis is validated, IDE peripheral levels could have biomarker potential to identify individuals at higher risk to develop PD.

The chosen analyzed brain areas are considered relevant for the amyloidogenic process in several neurodegenerative diseases. We showed that IDE is present in all analyzed areas, in much higher amount in the *cerebellum*. Notably, the highest HFD-induced decrease of IDE was found in *cerebellum*, with a significant accumulation of aSyn. Moreover, also in the *cerebellum*, IDE and aSyn are inversely correlated, as it was previously in the pancreas. Therefore, our findings demonstrate that diabetes may differently affect IDE/aSyn in a brain area specific manner. Interestingly, previous studies found that *cerebellum* display higher levels of amyloid plaques in early stages of dementia [220]. This suggests that *cerebellum* may play a role in early stages of protein accumulation and spread. However, the performed brain dissection procedure may not isolate

pure *cerebellum* fragments, possibly containing brain stem. Therefore, we cannot state that the observed effect in this region is exclusive to the *cerebellum*.

In our analysis, we observed that in the mid-brain, where dopaminergic neurons are present, IDE levels are also decreased in HFD-mice. However, to our surprise, no significant differences in aSyn total levels are present. Nevertheless, our protein extraction strategy does not allow to discriminate between the type of evaluated species. Knowing that oligomeric forms of aSyn are accepted as the most cytotoxic [217], it would be also important to correlate IDE levels and activity with the different species of aSyn present in these regions. Moreover, the analysis of brain *striatum*, to where dopaminergic neurons project, would be important to understand if IDE impairment drives to aSyn accumulation in the dopaminergic system. Additionally, analysis of IDE alterations on the olfactory bulb would also be relevant, since this region contains dopaminergic neurons, and it is known to be affected in PD [221].

In neurons, IDE is mostly membrane-associated, and was described to play an important role in AD pathology [222]. In particular, decreased levels of membrane-associated IDE are observed in the *hippocampus* of AD brains [222]. However, our approach is not suitable to distinguish between cytoplasmic or membrane-bond forms of IDE, but it allowed to detect major changes induced by a HFD. Interestingly, in the *hippocampus* we did not observed general changes of IDE levels upon a HFD, however aSyn was significantly accumulated. Therefore, we hypothesize that besides IDE total levels, its specific intracellular location and respective activity may differently contribute to aSyn pathogenesis, that should be addressed in the future. Moreover, further dissected brain areas such as brain stem, olfactory bulb, *striatum* and *hypothalamus* should be evaluated.

To dissect the possible protective role of IDE in aSyn pathogenesis, we chose H4 neuroglioma cells. This model is easily to manipulate, with a well-established phenotype of aSyn biology and pathogenesis [205]. We thus aimed to evaluate the role of IDE-WT, but also its catalytic inactive variant IDE-E111Q, on aSyn pathology. Our findings confirmed that IDE protects from aSyn toxicity. Moreover, since IDE was not able to protect from a C-terminally tagged aSyn (SynT), our findings further support that the C-terminal of aSyn may be relevant for the action of IDE over aSyn [190]. aSyn C-terminal does play a role in its pathology [223]. SynT consists of a 27 kDa C-terminal fusion protein between aSyn WT and a truncated form of GFP that is primed to form aggregated species [206]. Our approach does not allow to determine if aSyn or SynT interacts with IDE exosite, known to be important to modulate IDE function [184]. We may hypothesize that C-terminal domain of aSyn may preferentially bind to IDE exosite [190], which could explain why IDE is not able to suppress the cytotoxicity of SynT. Our findings also suggest that although IDE does not degrade aSyn [188], catalytic active IDE better prevents the toxicity of aSyn. This finding suggests that favoring the activity of IDE could be important to facilitate the suppression of aSyn toxicity. Importantly, diabetes research demonstrated that IDE nitrosylation

is increased in T2DM, and that nitrosylated-IDE degradative activity is reduced [224]. Therefore, it is also important in the future to evaluate if IDE is specifically nitrosylated in the brain, and if nitrosylated-IDE levels are increased in PD individuals. If this hypothesis is validated, strategies aimed at decreasing IDE nitrosylation could present therapeutic potential for PD.

Although the protein levels of SynT and their toxicity are not altered by IDE, we observed that IDE-E111Q but not IDE-WT decreases SynT insolubility. One might hypothesize that anti-aggregation mechanistic action of IDE-E111Q is different from IDE-WT and could favor the interaction with SynT. Since IDE-E111Q is catalytically inactive for its insulin degradative capacity, it may be more unrestricted to interact with other substrates, including SynT. To test this hypothesis, we propose to compare the interaction capacity of both IDE-WT/E111Q with aSyn or SynT.

IDE can control ubiquitin-proteasome degradation system by acting on ubiquitin homeostasis or directly on the proteasome enzymatic activity. Physiologically, IDE is upregulated upon stress exposure therefore modulating ubiquitin proteasome pathway [191]. Although, the role of IDE in the regulation of protein homeostasis is not well defined, its heat shock like function is suggested to facilitate the detection of damaged proteins [191]. It is still unclear if IDE-E111Q keeps this function and could favor the degradation of proteins. Surprisingly, we observe that IDE-WT does not alter the clearance of SynT, and IDE-E111Q actually impairs the clearance of SynT. We tried to address the effects of IDE in the clearance of aSyn, however at the time we faced technical difficulties and propose to study this in the future. This should be essential to understand if, although the total levels of aSyn are not altered, if IDE could be facilitating the clearance of toxic species of aSyn.

Altogether, our findings still do not clarify how IDE-WT is able to suppress the toxicity induced by aSyn. We observe that IDE-WT does not decrease the amount of aSyn, does not favor its clearance neither its solubility, measured by Triton X-100. Therefore, we hypothesize that it is able to act as a dead-end chaperone, reducing the presence of toxic oligomers of aSyn, which we did not assess. In fact, IDE-E111Q is able to increase the solubility of the aggregation-prone variant of aSyn (SynT). Alternatively, it may be acting in an indirect pathway.

To further explore the role of IDE in the modulation of aSyn pathogenesis, we propose to evaluate the impact of the loss of IDE activity. Our experimental setups were based in the overexpression of IDE (WT or E111Q) over endogenous IDE levels. Therefore, in the future, we aim to knockdown (shRNA) or knockout (CRISPR/Cas9) IDE in the cellular models of synucleinopathies and, evaluate if a decrease of IDE increases aSyn susceptibility to aggregate and become cytotoxic.

In this work, we did not study the proteolytic activity of IDE. As previously mentioned, IDE activity is not only related to its protein levels. IDE can be a target of post-translational modifications (such as nitrosylation), that can lead to structural changes that impair its degradative activity [224]. Therefore, it would be important to assess the activity of IDE in brain samples of mice fed a HFD and to measure its nitrosylation levels. Furthermore, as previously reported [190], it would be interesting to analyze how aSyn or SynT could stimulate IDE catalytic activity. In particular, if oligomeric or aggregated species of aSyn may stop promoting IDE degradative function. This is of particular importance, as IDE impairment in the brains of PD individuals could explain why they are hyperinsulinemic. Moreover, since we observed that the levels of IDE in the *hippocampus* of HFD-mice are not altered, but aSyn accumulates, it suggests that although the levels remain unaltered, its activity may be impaired. The measurement of IDE activity may be performed using an established kit (SensoLyte ® 520 IDE Activity Assay Kit *Fluorimetric*, AnaSpec, CA, USA).

The modulation of IDE activity can also be achieved by pharmacological treatment. In particular, pioglitazone, a medication currently used for the treatment of Diabetes that is reported to lower blood glucose levels, and it is also able to stimulate IDE activity [212]. In contrast, ML345-inhibitor is able to decrease the activity of IDE [225]. It would be interesting to explore in both, in the *in vitro* model or in mice, the impact of increasing or reducing IDE activity in the pathogenesis of aSyn. Alternatively, to study a protective role of IDE over aSyn pathogenesis, we propose to overexpress IDE in the brain of PD mouse models, to investigate if it alleviates from aSyn pathogenesis, and reduces dopaminergic neuronal loss and motor impairment.

In conclusion, we were able to validate the hypothesis that diabetes reduces the levels of IDE in the brain, and that aSyn negatively correlates with IDE levels in mice. Secondly, we validated in a neuroglioma cell model of synucleinopathies that IDE is able to suppress aSyn cytotoxicity in an activity-dependent manner. These findings pave the ground for a novel area of research in PD, by exploring the therapeutic potential of IDE to suppress aSyn pathogenesis, and suggesting IDE as a putative biomarker for PD. It also provides evidence that IDE impairment could be a causative mechanism underlying the association between T2DM and PD.

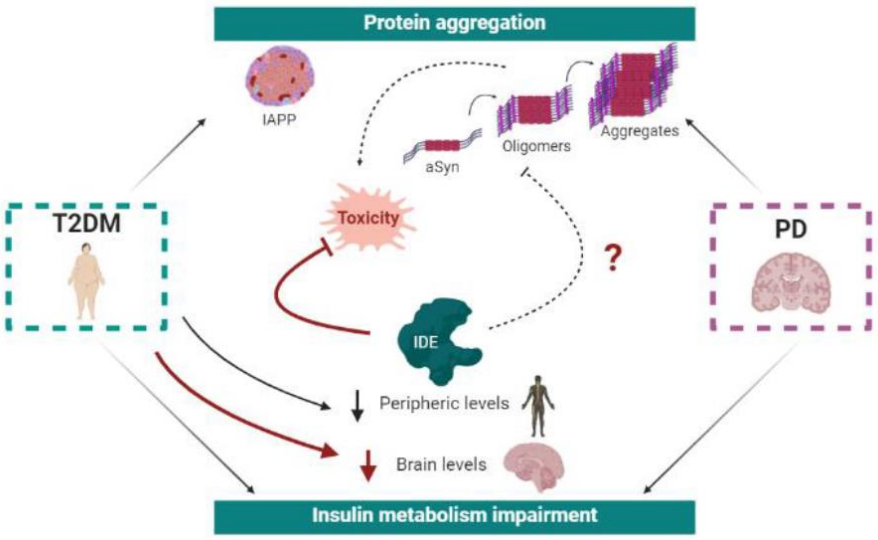


Figure 16. Schematic of the major conclusions.

We conclude that T2DM induce IDE impairment in the brain, decreasing aSyn toxicity. Red lines represent our major findings.

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