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# Investigating the interplay between α-Synuclein and fragile X mental retardation protein in Parkinson's disease

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## Abstract

Parkinson's disease (PD), the second most common neurodegenerative disorder, is associated with the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). PD pathology is characterized by the deposition of α-Synuclein (α-Syn) in these neurons, which is the main component of Lewy bodies (LB). The reasons for its accumulation are manifold and not yet fully understood, however, it has been suggested that α-Syn-induced changes in neuronal excitability precede neuronal cell death. The fragile X mental retardation protein (FMRP) regulates protein synthesis and synaptic plasticity through a variety of mechanisms. Therefore, we studied the interplay of α-Syn and FMRP in vitro and in vivo PD models. Most importantly, we found the expression of FMRP to be significantly decreased in response to α-Syn overexpression in cultured human dopaminergic neurons. Similar to the pathology of fragile X syndrome (FXS), protein networks involved in translational mechanisms were upregulated in these neurons, including increased phosphorylation of ribosomal protein S6, eukaryotic translation initiation factor 4E (eIF4E) and extracellular signal-regulated kinase (ERK), and increased expression of matrix metalloproteinase 9 (MPP-9). We developed a protocol to isolate and quantify synaptic ion channels, and showed that the abundance of N-type calcium channels is increased in synaptosomes of α-Syn-injected mouse brain tissue. Reminiscent of the neuroprotective effect of metformin in FXS, we tested if metformin also rescues dysregulated protein translation in α-Syn overexpressing cultured human dopaminergic neurons as we observed a dose-dependent trend towards normalization of hyperphosphorylated eIF4E and ERK and overexpression MMP-9. In summary, our results highlight the important role of FMRP in the pathogenesis of α-Syn-mediated pathologies by establishing a link between molecular patterns found in PD models and FXS.

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## List of tables

## List of symbols and abbreviations

| | |
|---|---|
| **% (w/v)** | Percent weight/volume |
| **α-Syn** | α-Synuclein |
| **AAV** | Adeno-associated virus |
| **AD** | Alzheimer's disease |
| **Ad5** | Adenovirus serotype 5 |
| **ALS** | Amyotrophic lateral sclerosis |
| **AMPAR** | α-amino-3-hydroxyl- 4-isoxazole propionic acid receptors |
| **AMPK** | 5'-adenosine mono-phosphate-activated protein kinase |
| **ANOVA** | Analysis of variance |
| **ATP** | Adenosine triphosphate |
| **BCA** | Bicinchoninic acid |
| **BDNF** | Brain-derived neurotrophic factor |
| **bFGF** | Basic fibroblast growth factor |
| **BSA** | Bovine serum albumin |
| **cAMP** | Cyclic adenosine monophosphate |
| **CREB** | cAMP-responsive element-binding protein |
| **CYFIP1** | Cytoplasmic FMRP-interacting protein |
| **CYT** | Cytosol |
| **DA** | Dopamine |
| **DALY** | Disability-adjusted life-years |
| **DAPI** | 4',6-diamidino-2-phenylindol |
| **DBS** | Deep brain stimulation |
| **DLB** | Dementia with Lewy bodies |
| **DMEM/F12** | Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **DPBS** | Dulbecco's phosphate-buffered saline |
| **DPT** | Days post transduction |
| ***EGFP*** | Enhanced green fluorescent protein |
| **eIF4E** | Eukaryotic translation initiation factor 4E |
| **4E-BP1** | Eukaryotic translation initiation factor 4E-binding protein 1 |
| **4E-BPs** | Eukaryotic translation initiation factor 4E-binding proteins |
| **ERK** | Extracellular signal-regulated kinase |
| **FBS** | Fetal bovine serum |
| ***Fmr1*** | Fragile X mental retardation 1 |
| **FMRP** | Fragile X mental retardation protein |

| | |
|---|---|
| **FN** | Fibronectin |
| **FXS** | Fragile X syndrome |
| **FXTAS** | Fragile X-associated tremor/ataxia syndrome |
| **GABA** | γ-aminobutyric acid |
| **GDNF** | Glial cell line-derived neurotrophic factor |
| **GFP** | Green fluorescent protein |
| **GluR1** | Glutamate receptor 1 |
| **GWAS** | Genome-wide association studies |
| **GPi** | Globus pallidus internus |
| **HCN1** | Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1 |
| **ICC** | Immunocytochemistry |
| **kDa** | Kilodalton |
| **Lb** | Luria Bertani |
| **LB** | Lewy bodies |
| **LBP** | Lewy body pathology |
| **LM** | Light plasma membranes |
| **LRRK2** | Leucine-rich repeat kinase 2 |
| **LTD** | Long-term depression |
| **LTP** | Long-term potentiation |
| **LUC** | Luciferase |
| **LUHMES** | Lund human mesencephalic |
| **mA** | Milliampere |
| **mGluR** | Metabotropic glutamate receptor |
| **MNKs** | MAP kinase-interacting serine/threonine-protein kinases |
| **MOI** | Multiplicity of infection |
| **MPTP** | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| **mRNA** | Messenger RNA |
| **MSA** | Multiple system atrophy |
| **mTOR** | Mammalian target of rapamycin |
| **mTORC1** | Mammalian target of rapamycin complex 1 |
| **NEAA** | Non-essential amino acids |
| **NES** | Nuclear export signal |
| **NL** | Nuclear lysate |
| **NLS** | Nuclear localization signal |
| **NM** | Nuclear membrane |
| **NP** | Nuclear pellet |

| | |
|---|---|
| **PCR** | Polymerase chain reaction |
| **PD** | Parkinson's disease |
| **PKC** | Protein kinase C |
| **PI3K** | Phosphatidylinositol 3-kinase |
| **PIKE** | PI3K enhancer |
| **PLL** | Poly-L-lysine |
| **PP2A** | Protein phosphatase 2A |
| **RNA** | Ribonucleic acid |
| **ROT** | Rotenone |
| **RT** | Room temperature |
| **S6K1** | S6 kinase 1 |
| **SDS** | Sodium dodecylsulfate |
| **SEM** | Standard error of the mean |
| **siRNAs** | Small interfering RNAs |
| ***SNCA*** | *Synuclein Alpha* |
| **SNpc** | Substantia nigra pars compacta |
| **SNPs** | Single nucleotide polymorphisms |
| **SSRIs** | Selective serotonin reuptake inhibitors |
| **STN** | Subthalamic nucleus |
| **TEA** | Tetraethyl ammonium |
| **TH** | Tyrosine hydroxylase |
| **TTX** | Tetrodotoxin |
| **VGCC** | Voltage-gated calcium channels |
| **WB** | Western blot |

# 1 Introduction

## 1.1 An overview of Parkinson's disease

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease following Alzheimer's disease (AD) (Adams, Kouzani, Tye, Bennet, & Berk, 2018; Bergman & Deuschl, 2002). As life expectancy and population growth continuously increase the global burden of PD including prevalence, disability-adjusted life-year (DALY), and mortality rates have considerably risen from 1990 to 2015 (Feigin et al., 2017).

PD is a chronic, heterogeneous disease with both motor- and non-motor features (DeMaagd & Philip, 2015; Ghika, Kyrozis, Potagas, & Louis, 2015). Movement-related symptoms typically include rest tremor, limb rigidity, bradykinesia, postural and gait impairment (L. V. Kalia & Lang, 2015). Other "non-motor" symptoms involve cognitive impairment, autonomic dysfunction like constipation or hypotension, sleep behavior disorders, and psychiatric disorders like depression, anxiety, and hallucinations (Paleacu, Schechtman, & Inzelberg, 2005; Tibar et al., 2018). Thereby, non-motor symptoms can occur long before the onset of motor symptoms and the clinical diagnosis of PD (Poewe, 2008). Due to differences in clinical manifestation and prognosis, the identification of PD subtypes has been made a priority of clinical PD research to target high-risk populations (Sieber et al., 2014). So far, however, there is no consensus on the distinction of subtypes as there is disagreement about the existence of individual entities or the spectrum of different stages of progression of a single disease (Fereshtehnejad & Postuma, 2017).

The onset of most cases of PD is most probably the result of a complex interaction between environmental and genetic risk factors (Cannon & Greenamyre, 2013; Cooper & Van Raamsdonk, 2018; Goldman, 2014). The incidence of PD is higher in people exposed to certain environmental factors such as pesticides, traumatic brain injuries, rural living, consumption of dairy products, and well water drinking. The incidence is lower in smokers, alcohol drinkers, and caffeine users (Ascherio & Schwarzschild, 2016; Noyce et al., 2012). It is well established that familial forms of PD exist, which indicate an inherited susceptibility to the disease (Spellman, 1962; Tune, Folstein, Rabins, Jayaram, & McHugh, 1982). To date, several genes have been associated with inherited, early-onset PD, including *SNCA, LRRK2, GBA, PRKN, VPS35, PINK1,* and *DJ-1* (Bandres-Ciga, Diez-Fairen, Kim, & Singleton, 2020). For sporadic cases of PD, the largest genome-wide association studies (GWAS) have identified > 90 genetic loci to be associated with the disease (Nalls et al., 2019). Thereby,

genes related to familial PD also play a role in sporadic PD (Nalls et al., 2014). For instance, the *SNCA* gene was found to be affected by deoxyribonucleic acid (DNA) multiplications (duplications, triplications) and missense mutations that cause familial PD (Chartier-Harlin et al., 2004; Kiely et al., 2013; Zarranz et al., 2004). Single nucleotide polymorphisms (SNPs) at the *SNCA* locus were on the other hand linked to sporadic PD (Edwards et al., 2010; Nalls et al., 2014).

The neuropathology of PD is characterized by two principal features: (1) the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) which is associated with the cardinal motor symptoms, and (2) the deposition of α-Synuclein (α-Syn) in neurons (Poewe et al., 2017). Neuronal loss shows regional selectivity, starting in ventrolateral and mediolateral areas of SNpc in early stages and spreading further as the disease progresses (Dijkstra et al., 2014; Fearnley & Lees, 1991). Other brain regions, including the amygdala, basal nucleus of Meynert, hypothalamus, locus ceruleus, and medullary tegmentum were also identified to be consistently affected by neuronal loss (Dickson, 2012).

The second neuropathological hallmark of PD is the Lewy body pathology (LBP), which represents the aggregation of misfolded fibrillary α-Syn formations to Lewy bodies (LB) and Lewy neurites (Goedert, Spillantini, Del Tredici, & Braak, 2013). However, LBP is not specific for PD as the pathology also occurs in other synucleinopathies, including dementia with LB, and multiple system atrophy (MSA) (Gomperts, 2016; Jellinger, 2007). The progression of LBP in PD has been characterized by six stages in the clinical course of the disease, which start from the nuclei of the vagus and glossopharyngeal nerve and the olfactory bulb, “spread” in a stereotyped pattern to other regions of the brain, and eventually manifest themselves in the neocortex (Braak et al., 2003).

In addition to the two hallmarks described above, there are other features relevant to PD pathology, including neuroinflammation and gliosis (Tansey & Goldberg, 2010), different forms of α-Syn aggregation (Cremades & Dobson, 2018; Saito et al., 2003; Schulz-Schaeffer, 2010), and other types of protein aggregation such as β-amyloid plaques and neurofibrillary tangles caused by the hyperphosphorylation of tau protein (Braak & Braak, 1990; Lei et al., 2010). Further pathological mechanisms identified to mediate neuronal cell death are related to mitochondrial function (Bose & Beal, 2016; Schapira, 2007), oxidative stress (Dias, Junn, & Mouradian, 2013), calcium homeostasis (Surmeier, Guzman, Sanchez-Padilla, &

Schumacker, 2011; Surmeier et al., 2017), and axonal transport (Braak et al., 2006; Sulzer, 2007).

To date, there is no known cure for PD, and treatments are mainly focused on reducing symptoms and improving patients' quality of life. To compensate for motor symptoms, disease-modifying drugs that enhance dopamine (DA) levels or stimulate the dopamine receptor, such as DA agonists, levodopa, and inhibitors of the DA degradation, are used (Connolly & Lang, 2014). Furthermore, non-dopaminergic treatments are used to manage non-motor symptoms (Chaudhuri, Healy, & Schapira, 2006) and to reduce complications of long-term dopaminergic treatment such as psychosis, symptoms fluctuation, and dyskinesia (Kaakkola, 2000; Metman et al., 1998). Apart from pharmacological options, patients also benefit from deep brain stimulation (DBS), which is the surgical implantation of an electrode that targets the subthalamic nucleus (STN) or globus pallidus internus (GPi) (S. K. Kalia, Sankar, & Lozano, 2013).

## 1.2 The role of α-Synuclein in PD

The family of human synuclein consists of α-, β- and ɣ-Synuclein (Lavedan, 1998). α-Syn forms signature lesions which are characteristic of synucleinopathies (Giasson et al., 2000). The peptide NAC35 derived from α-Syn was also found in β-amyloid plaques of patients with AD (Uéda et al., 1993).

The 14-kDA α-Syn with 140 amino acids consist of seven KTKEGV repeats, variable acidic C-terminal regions, and a hydrophobic domain found to be relevant for oligomerization and aggregation (Cookson, 2005; Giasson, Murray, Trojanowski, & Lee, 2001). α-Syn is located in the presynaptic terminals, the nucleus, and probably mitochondria and is believed to play a role in various cellular mechanisms (Burré, 2015; Burré, Sharma, & Südhof, 2017; Li et al., 2007; Maroteaux, Campanelli, & Scheller, 1988). These involve mitochondrial function (Nakamura et al., 2008), intracellular transport, synaptic processes including vesicle dynamics, membrane remodeling, modulation of transporters (Wales, Pinho, Lázaro, & Outeiro, 2013), and as a chaperone (Souza, Giasson, Lee, & Ischiropoulos, 2000).

α-Syn was first linked to PD when (1) the A35T mutation of α-Syn was associated with familial PD (Goedert, 1997), and (2) α-Syn was identified as a main component of LBP (Spillantini et al., 1997). Accumulated α-Syn mediates dopaminergic neurotoxicity in vivo (Periquet, Fulga, Myllykangas, Schlossmacher, & Feany, 2007). However, the exact

mechanisms by which α-Syn is involved in the pathogenesis of PD remain unclear. It has been hypothesized that in the physiological state endogenous α-Syn occurs in an unfolded, α-helical, tetrameric structure which breaks down into monomers before the protein misfolds (Bartels, Choi, & Selkoe, 2011). At the same time, it is believed that α-Syn may also exist in a state of equilibrium between monomeric and tetrameric forms (Alderson & Bax, 2016). It was found that during pathogenesis, the soluble α-helical α-Syn turns into a β-sheet structure, and via an off-pathway folding process, forms oligomers and finally insoluble amyloid fibrils, which are the main component of LB and Lewy neurites (Zhang et al., 2018). Regarding neurotoxic properties, there are conflicting views on the role of oligomers and amyloid fibrils, as both have proven to be cytotoxic (Cremades et al., 2012; Forloni, Artuso, La Vitola, & Balducci, 2016; Peelaerts et al., 2015).

The causes for aggregation are manifold and involve genetic mutations that trigger misfolding, the structural properties of α-Syn (Fink, 2006; Uversky et al., 2002), the concentration of partially folded conformation of α-Syn (Uversky, Lee, Li, Fink, & Lee, 2001), and impairments in proteasomal and autophagic degradation of α-Syn (Cuervo, Stefanis, Fredenburg, Lansbury, & Sulzer, 2004; C.-W. Liu, Corboy, DeMartino, & Thomas, 2003; Xilouri, Brekk, & Stefanis, 2013). It was also hypothesized that the “spreading” of LBP could be attributed to prion-like transmission of α-Syn (Angot, Steiner, Hansen, Li, & Brundin, 2010; Brundin, Melki, & Kopito, 2010; Visanji, Brooks, Hazrati, & Lang, 2013).

## 1.3 Animal and cellular models of PD

In PD research, a major goal is to achieve the most accurate replication of the pathophysiological processes possible, to be able to study molecular, and cell biological mechanisms in detail and to assess disease-modifying interventions. Since the human disease progresses is comparably slow, it is necessary to model neurodegeneration observed in patients in a much shorter time course. Many insights into the pathogenesis of PD result from experimental models.

In toxic animal models, the neurotoxic effect of pharmacological agents such as reserpine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA), paraquat, rotenone (ROT), and lactacystin was used to mimic dopaminergic depletion (Beal, 2001; Dawson, Mandir, & Lee, 2002; McNaught et al., 2002). Genetic models are based on the alterations of genes such as *SNCA, LRRK2, PRKN,* and *PINK1* (Blesa, Phani, Jackson-

Lewis, & Przedborski, 2012). Considering the role of α-Syn in the pathogenesis of PD, transgenic and non-transgenic, viral vector-based α-Syn animal models have been developed (Bazzu et al., 2012; Kahle, Neumann, Ozmen, & Haass, 2000; Kirik et al., 2002; St Martin et al., 2007).

In parallel, various experimental in vitro models are used. Popular cellular models to investigate the pathogenesis of PD include the human neuroblastoma cell line SH-SY5Y (Hasegawa et al., 2004; Xicoy, Wieringa, & Martens, 2017), the pheochromocytoma cell line PC12 (Z. Liu, Yu, Li, Ross, & Smith, 2011; Malagelada & Greene, 2008), the neuronal progenitor cell lines MN9D and CSM14.1 (Haas & Wree, 2002; Hermanson et al., 2003), and primary midbrain cultures (Falkenburger, Saridaki, & Dinter, 2006).

In this present research, the Lund human mesencephalic (LUHMES) cell model was used (Lotharius et al., 2005). This cell line, which was first described in Lund, is a subclone of a human mesencephalic progenitor cell line, termed MESC2.10 cells, which is immortalized by transformation with the v-myc oncogene. In comparison to non-immortalized fetal progenitor cells, these cells offer the advantage to be easily expanded in vitro. Cell growth can be suppressed by adding tetracycline which regulates the expression of v-myc via a tetracycline-controlled transactivator (Lotharius et al., 2002). By inducing differentiation with tetracycline, glial cell line-derived neurotrophic factor (GDNF), and dibutyryl cyclic adenosine monophosphate (db-cAMP) LUHMES cells develop a dopaminergic neuronal phenotype (Lotharius et al., 2002; Schildknecht et al., 2013). For instance, the cells form neurite networks, express tyrosine hydroxylase (TH) and other neuronal markers, and show similar electrophysiological characteristics to human dopaminergic neurons (Höllerhage et al., 2014; Scholz et al., 2011). Further, this model is applicable to investigate the influence of accumulated α-Syn, since α-Syn overexpression by adenoviral transduction quickly develops the pathology by showing small oligomeric forms of α-Syn and gradual cell death (Höllerhage et al., 2014).

## 1.4 An overview of Fragile X syndrome

After trisomy 21, fragile X syndrome (FXS) is the second most common cause of mental retardation (Rousseau, Rouillard, Morel, Khandjian, & Morgan, 1995). Initially, an anomaly of the long (q) arm of the X chromosome was found in the karyotyping of four men with mental retardation from a single family (Lubs, 1969). This abnormality was later located on Xq27-q28

and was called „fragile site“ because it was believed to be susceptible to chromosomal breakage (Giraud, Ayme, Mattei, & Mattei, 1976).

FXS is caused by trinucleotide repeat expansion. In this case, the fragile X mental retardation 1 (*Fmr1*) gene contains excessive repeats of cytosine, guanine, and guanine (CGG) in the 5′ untranslated region (Grigsby, 2016). Individuals carrying a “full mutation” (> 200 CGG repeats) are likely to develop FXS as the transcriptional or translational processes of the fragile X mental retardation protein (FMRP) are disturbed through epigenetic silencing (Nelson, Orr, & Warren, 2013; Tassone et al., 1999). The lack or absence of FMRP as a cause of FXS was validated through *Fmr1*-knockout animal models that developed FXS-related phenotypes (Bakker & Oostra, 2003). While the full mutation leads to FXS in early development, individuals with 55-200 CGG repeats carry a “premutation” which can further destabilize in following generations. These people are likely to develop fragile X-associated tremor/ataxia syndrome (FXTAS), a late-onset movement disorder which is characterized by cerebellar symptoms, PD-like motor symptoms, and cognitive deterioration (Hagerman & Hagerman, 2004; Tassone et al., 2012). The *Fmr1* gene is highly conserved across species, allowing the use of animal models to identify relevant pathogenetic mechanisms connected to FXS. Findings include changes in synaptic plasticity such as an increase of long-term depression (LTD) in the cerebellum and hippocampus (Bear, Huber, & Warren, 2004; Huber, Gallagher, Warren, & Bear, 2002) and decreased long-term potentiation (LTP) in the amygdala, hippocampus and the anterior cingulate cortex (M.-G. Zhao et al., 2005). Further, animal models demonstrated increased messenger RNA (mRNA) disruption of γ-aminobutyric acid (GABA)-related signaling pathways (D’Hulst et al., 2009; Greenblatt & Spradling, 2018).

FXS is associated with a variety of symptoms ranging from motor and psychiatric signs to connective tissue abnormalities. In general, children with FXS are hypotensive and initially present with regurgitation and poor sucking during breastfeeding. Later, anxiety, sensory hyperarousal, and delays in motor and language development are common (Hagerman et al., 2017; Mattei, Mattei, Aumeras, Auger, & Giraud, 1981). As for the physical aspects, children show macroorchidism, asymmetric facies, low-set large ears, a prominent jaw, and large hands (Lubs, Watson, Breg, & Lujan, 1984; Lubs, Travers, Lujan, & Carroll, 1984). Other features are related to connective tissue abnormalities, such as hypermobility of finger joints, mitral valve prolapse, and flat feet (Davids, Hagerman, & Eilert, 1990; Hagerman & Synhorst, 1984; Opitz, Westphal, & Daniel, 1984). There is also a phenotype of FXS similar to Prader-Willi syndrome

in which patients develop hyperphagia and subsequently obesity and present with delayed puberty and small genitalia (Nowicki et al., 2007). It has been shown that the expression level of FMRP can be used as a prognostic indicator in FXS in men (Tassone et al., 1999). In women, the other X chromosome has the capability to transcribe FMRP. Therefore, women typically have less severe manifestations of symptoms than men (Hagerman et al., 2017).

As for PD, to date, there is also no cure for FXS, and patients usually require lifelong, multidisciplinary treatment (Hagerman et al., 2017). In particular, patients benefit from physical and occupational therapy, behavioral and speech interventions (Hagerman et al., 2009). Stimulants, selective serotonin reuptake inhibitors (SSRIs), α-adrenergic agonists, and atypical antipsychotics can be used to treat behavioral abnormalities (Hagerman et al., 2017; Hagerman et al., 2009). Animal models advanced the field of targeted treatment. It has been shown that mGluR5 antagonists such as AFQ056 and GABA receptor agonists have the potential to reverse some of the FXS manifestations (Braat & Kooy, 2015; Levenga et al., 2011). Other targeted treatments include lovastatin, metformin, and trofinetide/NNZ-2566 (Deacon et al., 2015; Gantois et al., 2017; Pellerin et al., 2016).

## 1.5 The role of fragile X mental retardation protein in FXS

FMRP, the gene product of *Fmr1*, is ubiquitous but is predominantly expressed in the brain and reproductive organs (Hinds et al., 1993). In neurons, FMRP is localized in the perikarya, particularly in ribosome-rich regions such as the endoplasmic reticulum, the neuronal nucleoplasm, within nuclear pores, and in dendritic spines (Feng et al., 1997).

The protein structure of FMRP contains three ribonucleic acid (RNA) binding motifs (K homology domains 1 and 2, and the arginine-glycine-glycine (RGG) box) that demonstrate its role as an RNA-binding protein for regulating protein translation (Jin et al., 2004; Siomi, Siomi, Nussbaum, & Dreyfuss, 1993). Furthermore, FMRP contains nuclear localization signal (NLS) and nuclear export signal (NES) motifs (Eberhart, Malter, Feng, & Warren, 1996). The tandem Tudor (Agenet) domain enables FMRP to bind to trimethylated lysine residues of histones, indicating a function in the DNA damage response through chromatin binding (Alpatov et al., 2014).

As a multifunctional protein FMRP is involved in a variety of molecular processes. These include pre-mRNA alternative splicing (Zhou et al., 2017), protein translation (Darnell & Klann,

2013), neuronal transport (Kao, Aldridge, Weiler, & Greenough, 2010), and ion channel interaction (M. R. Brown et al., 2010; Ferron, Nieto-Rostro, Cassidy, & Dolphin, 2014).

The NLS and NES allow FMRP to shuttle back and forth between the cytoplasm and nucleus. FMRP probably exits the nucleus through interaction with the bulk mRNA exporter Tap/NXF1 protein while bound to mRNAs (Eberhart et al., 1996; Kim, Bellini, & Ceman, 2009). mRNAs are then transported to dendrites and axons in a stimulus-induced manner where FMRP which is associated with polyribosomes suppresses their translation (Antar, Afroz, Dictenberg, Carroll, & Bassell, 2004; Penagarikano, Mulle, & Warren, 2007).

In the context of protein translation, FMRP interacts with the eukaryotic initiation factor 4E (eIF4E) and cytoplasmic FMRP-interacting protein (CYFIP1) and thus blocks the translation initiation machinery (Napoli et al., 2008). In FXS neurons, overall protein synthesis is increased by ~15–20% (Qin et al., 2013). This is explained by the metabotropic glutamate receptor (mGluR) hypothesis, which states that the absence of the inhibitory effect of FMRP leads to increased protein synthesis mediated by mGluR pathways (Bear et al., 2004). In particular, the extracellular signal-regulated kinase (ERK) and the mammalian target of rapamycin complex 1 (mTORC1) signaling pathways (**Figure 1**) are affected (Richter, Bassell, & Klann, 2015). In particular, the phosphatidylinositol 3-kinase (PI3K) and the upstream activator PI3K enhancer (PIKE), are disinhibited. As a consequence, phosphorylation of mTOR itself and its targets ribosomal protein S6, 4E-binding proteins (4E-BP), and eIF4FE is enhanced (Gkogkas et al., 2014; Sharma et al., 2010). ERK-dependent translation is based on the phosphorylation of MAP kinase-interacting serine/threonine-protein kinases (MNKs) and eIF4E (Waskiewicz et al., 1999).

In FXS, the resulting burst of protein translation is responsible for the internalization of α-amino-3-hydroxyl-4-isoxazole propionic acid receptors (AMPAR), resulting in a mGluR-depended LTD. Besides, the increased expression of matrix metalloproteinase 9 (MMP-9) leads to abnormalities in spinal morphology (Janusz et al., 2013). These processes occur even without mGluR activation and at normal levels of ERK signaling, indicating a hypersensitivity protein translation mechanisms in FXS (Osterweil, Krueger, Reinhold, & Bear, 2010; Santoro, Bray, & Warren, 2012). In support of these findings, rescue experiments have shown that genetic reduction of $mGluR_5$, MNKs, phosphorylated eIF4E, and MMP-9 ameliorates FXS-related phenotypes in *Fmr1*-knockout mice (Dölen et al., 2007; Gkogkas et al., 2014; Sidhu, Dansie, Hickmott, Ethell, & Ethell, 2014).

Besides the presence or absence of FMRP, protein translation is also affected by the phosphorylation status of FMRP, which is regulated by S6 kinase 1 (S6K1) and protein phosphatase 2A (PP2A) (Narayanan et al., 2007, 2008). Upon activation of mGluR, FMRP is dephosphorylated, which allows protein translation to proceed (Ceman et al., 2003; Narayanan et al., 2007).

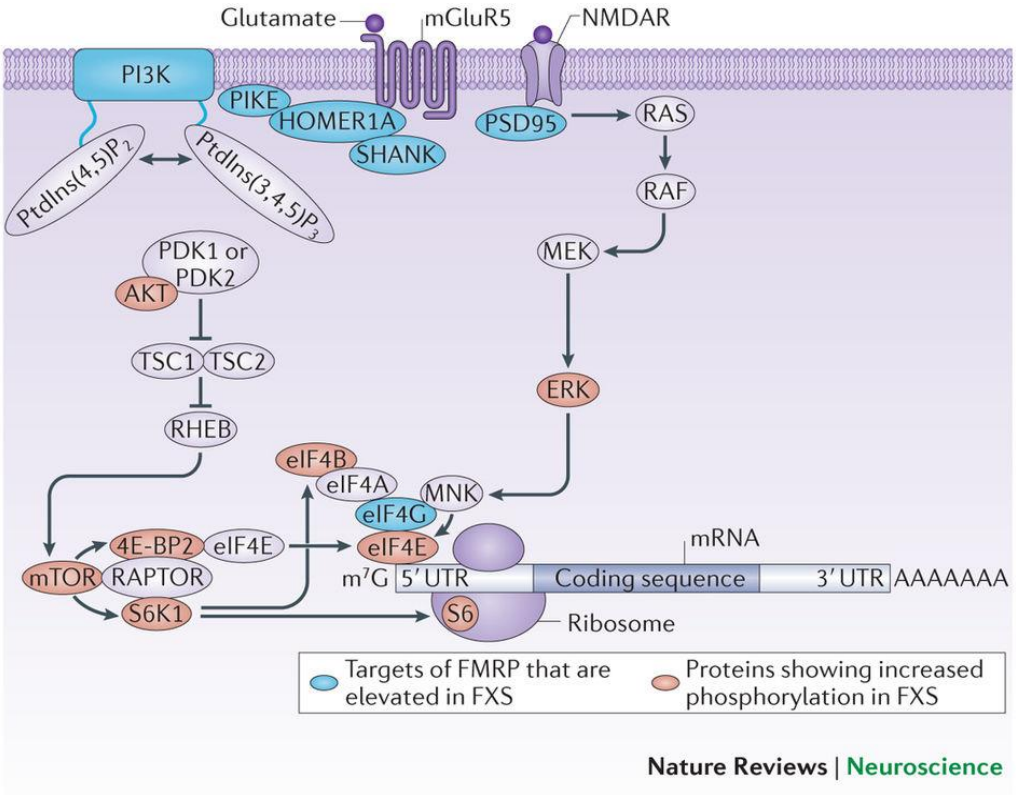


**Figure 1: ERK and mTORC1 signaling pathways in FXS.** This figure was used from Richter et al. 2015 with permission.

## 1.6 Preliminary findings

The context of this dissertation is provided by previous research of Dr. Yi Tan and Dr. Thomas Köglsperger on the role of voltage-gated calcium channels (VGCCs) in PD. VGCCs are divided into three groups according to their electrophysiological and pharmacological characteristics: $Ca_V1$, $Ca_V2$, and $Ca_V3$ (C.-Y. Wang, Lai, Phan, Sun, & Lin, 2015). $Ca_V2$ comprises P/Q-type ($Ca_V2.1$), N-type ($Ca_V2.2$), and T-type ($Ca_V2.3$) calcium channels (Dai, Hall, & Hell, 2009). The role of calcium channels in the pathogenesis of PD became more evident when it was found that perturbed calcium homeostasis of SNc DA can trigger neurodegeneration (Duda, Pötschke, & Liss, 2016).

Electrophysiological experiments show that LUHMES cells reproduce features that are similar to dopaminergic neurons and are therefore suitable as a model for further investigations. As such, α-Syn-transduced LUHMES cells displayed an activated inward calcium current (**Figure 2**) when $Na^+$ and $K^+$ channels were blocked with tetrodotoxin (TTX) and tetraethyl ammonium (TEA). Subsequently, we identified neuronal N-type calcium ($Ca_V2.2$) channels to be the relevant subtype involved in altered calcium homeostasis (Tan et al., 2019). Regarding the molecular mechanisms connected to an increased synaptic calcium inward current, it has been found that FMRP regulates the expression and density of $Ca_V2.2$ channels and modulates vesicle exocytosis. In particular, FMRP knockdown was found to increase calcium currents and the density of $Ca_V2.2$ channels in presynaptic terminals and perikarya of dorsal root ganglion neurons (Ferron et al., 2014). Based on these findings, we aimed to further investigate the regulation of $Ca_V2.2$ channels through FMRP and, its role in PD models and its interplay with α-Syn.

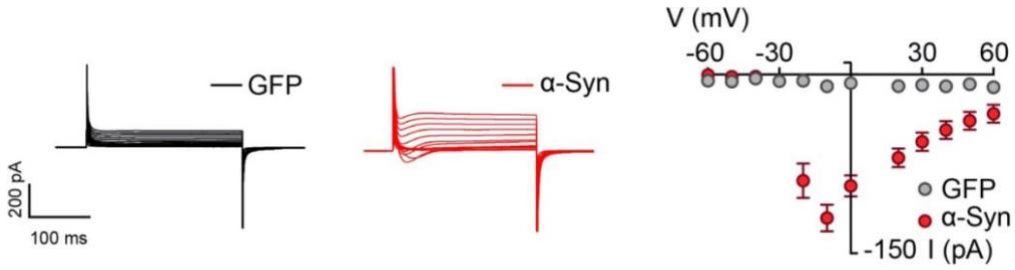


**Figure 2: α-Syn transduced LUHMES cells show activated inward currents.** The use of this figure was authorized by Acta Neuropathologica and it can be found in (Tan, 2020; Tan et al., 2019).

## 1.7 Research question and aims of the study

In general, the experiments and results of this study are closely related to earlier scientific work, which was mainly carried out by Dr. Yi Tan, Dr. Thomas Köglsperger, and Prof. Günter U. Höglinger. Since my work is linked to the PhD project of Dr. Yi Tan, parts of the methods and results described in this paper overlap with respective sections of the doctoral dissertation of Dr. Yi Tan (Tan, 2020). Some of the results and findings of this present research were part of a publication in *Acta Neuropathologica* (Tan et al., 2019).

Because α-Syn has been shown to increase calcium influx through $Ca_V2.2$ channels and FMRP knockdown was linked to increased calcium currents and expression of $Ca_V2.2$ channels (introduction section 1.6), we hypothesized an interplay between FXS and PD associated pathologies. In particular, we suggested that α-Syn regulates $Ca_V2.2$ channels through FMRP. To study the molecular overlap between the pathologies and the interplay between α-Syn and FMRP three aims were defined:

1. Our first aim was to characterize the role of FMRP in PD-models. This study mainly focused on the LUHMES cell model, using Western blot (WB), immunocytochemistry (ICC), and producing different lentiviral vectors to investigate the distribution and function of FMRP.
2. Secondly, we aimed to further address the role of FMRP and α-Syn in the regulation of $Ca_V2.2$ in vitro and in vivo. ICC was used to investigate the expression of calcium channels in LUHMES cells. Besides, we developed a protocol to enrich neuronal membranes from mouse brain tissue to quantify the expression of $Ca_V2.2$ channels by WB.
3. Based on previous research that suggests a rescue effect of metformin on FXS-related phenotypes of *Fmr1*-knockout mice (Gantois et al., 2017) our third aim was to investigate the possibility of a rescue effect of metformin on α-Syn-transduced LUHMES cells. Thereby, we examined the expression of FXS-associated signaling pathway proteins in response to different treatments with metformin by WB.

# 2 Materials and methods

## 2.1 Materials

### 2.1.1 Antibodies

| Antibody | Cat. No. | Dilution (WB) | Dilution (ICC) | Supplier |
|---|---|---|---|---|
| Alpha-synuclein 15G7 | ab195561 | | 1:50 | Abcam |
| Alpha-synuclein | 2642S | 1:1000 | | Cell Signaling |
| Alpha-synuclein C20 | sc-7011-R | 1:1000 | | Santa Cruz |
| CACNA1B | PA5-21440 | 1:1000 | 1:100 | Thermo Fisher |
| CACNA1B | ACC-002 | 1:1000 | 1:100 | Alomone Labs |
| CACNA1B | Sc-377489 | | 1:200 | Santa Cruz |
| EEA1 | 610456 | 1:1000 | | BD Transduction Laboratories |
| Phospho-eIF4E Ser209 | 9741S | 1:1000 | | Cell Signaling |
| eIF4E | 9742S | 1:1000 | | Cell Signaling |
| FMRP | 4317S | 1:1000 | 1:50 | Cell Signaling |
| Glutamate Receptor 1 | AB1504 | 1:1000 | | Merck Millipore |
| GAPDH(14C10) | 3683S | 1:1000 | | Cell Signaling |
| HDAC2(3F3) | 5113 | 1:1000 | | Cell Signaling |
| MMP-9 | PA5-16851 | 1:1000 | | Thermo Fisher |
| MMP-9 D6O3H | 13667S | 1:1000 | | Cell Signaling |
| $Na^+$-$K^+$-ATPase | 3010S | 1:1000 | | Cell Signaling |
| S6 Ribosomal Protein 5G10 | 2217S | 1:1000 | | Cell Signaling |
| Phospho-S6 Ribosomal Protein Ser240/244 | 2215S | 1:1000 | | Cell Signaling |
| Goat anti-Mouse IgG H&L Alexa Fluor® 488 | ab150117 | | 1:500 | Abcam |
| Peroxidase Labeled Goat anti-Rabbit IgG H&L | PI-1000 | 1:10000 | | Vector Labs |
| Peroxidase Labeled Goat anti-Mouse IgG H+L | PI-2000 | 1:10000 | | Vector Labs |
| Donkey anti-Rabbit IgG H+L Alexa Fluor® 594 | A21207 | | 1:500 | Life Technologies |
| Goat anti-Rabbit IgG H&L Alexa Fluor® 488 | A11008 | | 1:500 | Invitrogen |

| | | | | |
|---|---|---|---|---|
| Donkey anti-Rabbit IgG H+L Alexa Fluor® 680 | A10043 | | 1:500 | Life Technologies |
| Goat anti-Rat IgG H&L Alexa Fluor® 568 | A11077 | | 1:500 | Invitrogen |
| Horse anti-Mouse Biotinylated IgG | BA-2000 | | 1:2000 | Vectastain |
| Goat anti-Mouse Biotin-SP-conjugated IgG H+L | 115-065-068 | | 1:2000 | Jackson ImmunoResearch |

**Table 1:** Antibodies used for WB and ICC

### 2.1.2 PCR Primers

| Gene | Primer Name | Sequence 5' to 3' |
|---|---|---|
| Fmr1 | Forward Primer | ATTCCCGGGCAGATGGAGGAGCTGGTGGTG |
| | Reverse Primer | ACTGCAGAATTCTTAGGGTACTCCATT |
| EGFP | Forward Primer | ATTCCCGGGCCACCATGGTGAGCAAGGG |
| | Reverse Primer | ATCGAATTCGGACTTGTACAGCTCGTCCATGC |

**Table 2:** Primer sequences for Fmr1 and EGFP plasmid cloning

### 2.1.3 Touchdown PCR protocol

| Step | Phase | Temperature (°C) | Time (sec) |
|---|---|---|---|
| 1 | | 98 | 30 |
| 2 | Denaturation | 98 | 10 |
| 3 | Primer Annealing | 70 | 20 |
| 4 | Elongation | 72 | 160 |
| 5 | 20 repeats of steps 2-4 decreasing annealing temperature by 0.5°C every cycle | | |
| 6 | Denaturation | 98 | 10 |
| 7 | Primer Annealing | 59 | 20 |
| 8 | Elongation | 72 | 160 |
| 9 | 12 repeats of steps 6-8 | | |
| 10 | | 10 | ∞ |

**Table 3:** Touchdown PCR protocol

## 2.2 Methods

Some of the methods presented in this section have been published in *Acta Neuropathologica* (Tan et al., 2019) and were also described in the doctoral dissertation of Dr. Yi Tan (Tan, 2020).

### 2.2.1 Cell biology

The cell culture techniques for LUHMES cells complied with the general protocols and specifications used by the research group for translational neurodegeneration of Prof. Dr. Günter U. Höglinger (Bruch et al., 2017; Fussi et al., 2018; Höllerhage et al., 2014; Tan, 2020; Tan et al., 2019).

LUHMES cells were cultivated on Nunc™ Delta Surface tissue culture flasks (Thermo Fisher Scientific, Waltham, MA, USA) and Delta Surface multi-well tissue culture vessels. For proliferation, the flasks were coated with 0.1 mg/ml poly-L-lysine (PLL, Sigma-Aldrich, St. Louis, MO, USA). Thereby, the flasks were incubated overnight with PLL at 4°C and afterwards washed three times with 1x Dulbecco's phosphate-buffered saline (DPBS, Life Technologies, Carlsbad, CA, USA). For differentiation, the multi-well vessels were additionally coated with 5 µg/ml bovine fibronectin (FN, Sigma-Aldrich). Thereby, FN was added after three times washing with DPBS for at least 6 h at 37°C. Before plating-out, the vessels were washed again with DPBS.

Both proliferating and differentiating LUHMES cells were cultivated in HERACell 150i $CO_2$ incubators at 37°C, 5% $CO_2$, and water-saturated air. For the proliferation and differentiation medium, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12, Sigma-Aldrich) and 1% N2 supplement (100x) (Life Technologies) was used. In addition, the proliferation medium consisted of 0.04 µg/ml basic fibroblast growth factor (bFGF, PeproTech, London, UK), the differentiation medium consisted of 1 µg/ml tetracycline (Sigma-Aldrich), 2 ng/ml GDNF (R&D Systems, Minneapolis, MN, USA) and 0.49 µg/ml db-cAMP (Sigma-Aldrich).

For the passaging of LUHMES cells, the culture medium was removed and cell adhesion was reduced by incubation with 500 mg/l trypsin and 200 mg/l ethylenediaminetetraacetate (EDTA, all Sigma-Aldrich) at 37°C and 5% $CO_2$. To antagonize trypsinization, 10% fetal bovine serum (FBS, Sigma-Aldrich) in culture medium was added to the flask. Detached LUHMES cells were resuspended in the medium and afterwards centrifuged for 7 min at 1200 rpm with

a Heraerus Megafuge 16 (Thermo Fisher Scientific). The supernatant was discarded and the pellet dissolved in fresh culture medium. The concentration of cells in the suspension was determined by diluting the sample 1:2 to 1:10 in a 0.4% trypan blue solution (Sigma-Aldrich) and using a Neubauer's chamber for counting individual neurons. LUHMES cells were then plated for further proliferation or differentiation.

For propagation, 2-3 x $10^6$ were seeded in PLL-coated tissue culture flasks with proliferation medium. Cell growth was examined under the light microscope and the proliferation medium renewed every three days. When a density of > 80% was reached, the cells were prepared for passaging. For differentiation, cells were plated out at a density of 125.000 cells/$cm^2$ in PLL/FN-coated multi-well tissue culture vessels and regularly examined under the light microscope.

24 h after seeding for differentiation, LUHMES cells were transduced with adenovirus serotype 5 (Ad5) vectors (Ad5-α-syn, Ad5-GFP (both from BioFocus DPI Ltd, Saffron Walden, England)) at a multiplicity of infection (MOI) of 2.15 to overexpress humane wild-type α-Syn and green fluorescent protein (GFP) (Höllerhage et al., 2014, 2017). In particular, the vector solution was diluted in differentiation medium and added to the vessels by partially exchanging the cell culture medium. After virus transduction, the cells were incubated again for 24 h and washed three times with DPBS to remove the adenovirus and afterwards provided with differentiation medium. After another 4 days, 6 days post-transduction (DPT), the cells were used for further readout and experimentation (**Figure 3**).

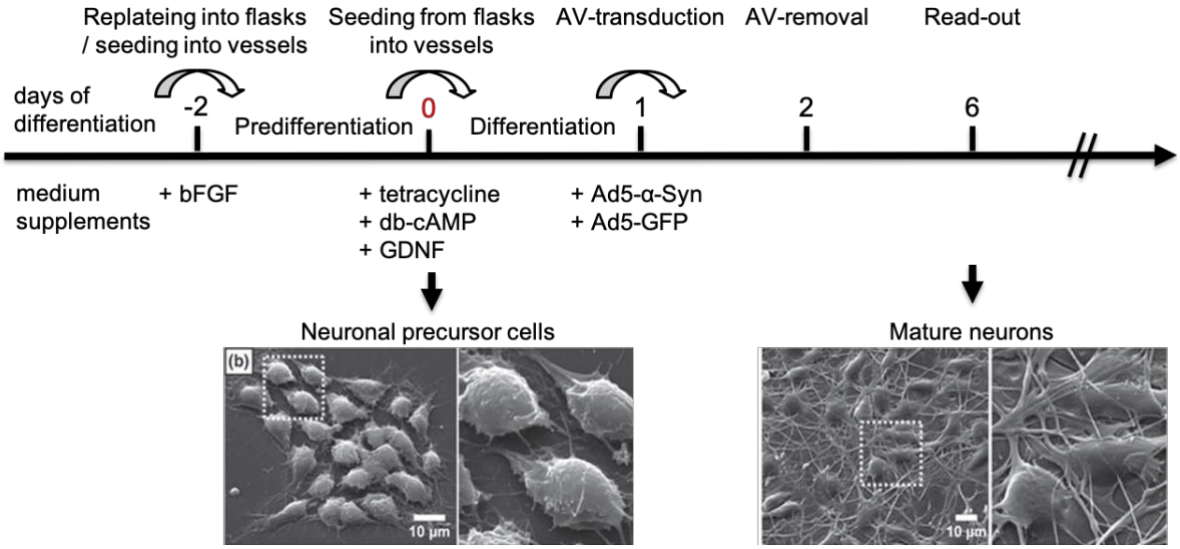


**Figure 3: Time course of seeding LUHMES cells, adenoviral transduction, removal and readout.** This figure was adapted from (Scholz et al., 2011) with permission.

### 2.2.2 Molecular biology

#### 2.2.2.1 Plasmid cloning

Based on the plasmid pCMV-EGFP-FMR1 (a gift from Dr. Edbauer, German Center for Neurodegenerative Research (DZNE), https://benchling.com/s/seq-Ui78kQNwXayfpWofcbsz) lentiviral and adeno-associated virus FMRP vector plasmids were generated. All primers used for plasmid sequencing and amplification are listed in **Table 2**.

To generate the lentiviral FRMP vector plasmids, first, the mouse *Fmr1* gene was sequenced and amplified using touchdown PCR with the conditions specified in **Table 3**. Subsequently, DNA fragments were digested by a two-hour incubation at 37°C using Xma1 and EcoR1 restriction sites. The DNA fragments were then separated on a 1% (w/v) agarose in Tris-acetate-EDTA (TAE) buffer gel. The DNA was cleaned up and purified using the GeneJET™ PCR purification kit (Thermo Fisher Scientific). The *Fmr1* and *EGFP* genes were then inserted into the lentiviral F2U-ΔZeo backbone by digestion with EcoR1 and Xma1 restriction enzymes and ligation with T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) overnight at room temperature (RT). The resulting clones were then transfected into electrocompetent Escherichia coli (New England Biolabs) and incubated overnight in kanamycin-containing Luria Bertani (Lb) medium at 37 °C. Cells were then grown on ampicillin-containing Lb agar plates (Sigma-Aldrich). The following day, bacterial colonies were picked from the agar plates and incubated in ampicillin-containing LB medium at 37 °C overnight. The plasmid DNA was extracted by using PureLink™ HiPure Plasmid Midiprep kit (Thermo Fisher Scientific) and sequenced for confirmation. In the end, three plasmids were produced:

1. F2U-ΔZeo-EGFP (https://benchling.com/s/seq-RtREwOO2eJtk4j4zDecH),
2. F2U-ΔZeo-EGFP-FMR1 (https://benchling.com/s/seq-bdu6Dm0WtRNRrl8K41PJ),
3. F2U-ΔZeo-FMR1 (https://benchling.com/s/seq-7I2zbxGnzyqX1AggLSPC).

The adeno-associated virus (AAV) 5-FMRP vector plasmid was produced through cloning the *Fmr1* gene from the plasmid pCMV-EGFP-FMR1 into the open reading frame of the AAV expression plasmid pAAV-CAG-GFP (Addgene #37825, https://benchling.com/s/seq-76hc2INyjc60mZtI9lMr) using the restriction enzymes BamH1 and EcoR1. At the viral vector core facility of the Technical University of Munich, the plasmid was then packed into an AAV5 (https://benchling.com/s/seq-pduhmdscce1BxCtJuoxc).

#### 2.2.2.2 Lentivirus production

The plasmids produced were transfected into HEK293T cells as described previously (Bruch et al., 2017; Kuhn et al., 2010). For this purpose, 5 million cells were seeded in 8 ml DMEM/F12 and 10% FBS (both Sigma-Aldrich) HEK medium on Nunc™ Delta Surface tissue culture petri dishes (Thermo Fisher Scientific). 18 h after seeding, HEK medium was changed to pure OptiMEM® (Life Technologies) and the cells transfected with the F2U-ΔZeo vectors and the plasmids psPAX2 and pCNA3(-)-VSV-G using Lipofectamine 2000 and OptiMEM® (both Life Technologies). After another 6 h the medium was changed to high BSA packaging medium containing DMEM, Pyruvate, GlutaMAX™ (Life Technologies), 100x non-essential amino acids (NEAA, Life Technologies), 10% FBS (Sigma-Aldrich) and 20% (w/v) bovine serum albumin (BSA, Cell Signaling Technology, Danvers, MA, USA). 24 h after transfection, the medium was retrieved and replaced with fresh packaging medium. The collected medium was centrifuged at 3000 rpm for 10 min, passed through 0.45 µm, and stored overnight on ice. 48 h after transfection the second sample of packaging medium was collected, centrifuged, and filtered as described above. The packaging medium was then ultra-centrifuged at 22.000 rpm for 2 h at 4°C and the supernatant was discarded. The pellet was diluted in 175 µl TBS5 solution containing 50 mM Tris-HCl, 130 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 10% (w/v) BSA and incubated on ice for at least 4 h or overnight. Later, the virus was aliquoted in microcentrifuge tubes and stored at -80 °C.

#### 2.2.2.3 Lentivirus transduction

6 h after seeding for differentiation, LUHMES cells were treated with different concentrations of lentivirus particles in differentiation medium ranging from 1:1500 to 1:500. The final concentration of the virus 1:1000 was determined by using MTT assay to assess cell viability and ICC to obtain a clear signal in confocal microscopy.

#### 2.2.2.4 MTT assay

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) was added to LUHMES cells in differentiation medium up to a final concentration of 0.5 mg/µl. The cells were incubated for at least 30 min at 37°C. When intracellular purple formazan crystals became visible under the microscope, the medium was removed and the cells stored at -80°C for 1 h. The cells were thawed and incubated with 300 µl dimethyl sulfoxide (DMSO,

AppliChem, Darmstadt, Germany) per well at RT for 2 h. As the cells lysed and purple crystals dissolved, the absorbance at 570 nm was measured with the CLARIOstar® Plus microplate reader (BMG Labtech). The data was then analyzed using MARS data analysis software (BMG Labtech). The absorbance reading of the blank ($abs_{blank}$) was subtracted from all samples and the absorbance readings of the samples ($abs_{sample}$) were corrected based on the readings of the control samples ($abs_{control}$). The cell viability was thus calculated according to the formula below:

$$\% \text{ viable cells} = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} \times 100$$

### 2.2.3 Protein biochemistry

#### 2.2.3.1 Western blot

LUHMES cells were harvested by removing the differentiation medium, washing the vessels one time with DPBS, removing the DPBS, adding RIPA-buffer (Sigma-Aldrich) containing EDTA-free protease and phosphatase inhibitors (Complete™ protease inhibitor cocktail, PhosStop™ phosphatase inhibitor cocktail, both Roche, Rotkreuz, Switzerland) and by carefully scratching the cells from the vessels surface. The lysates were then incubated on ice for 30 min being vortexed every 10 min. Afterwards the lysates were centrifuged at 5.000 g for 15 min at 4°C with a Heraeus™ Fresco™ 17 microcentrifuge (Thermo Fisher Scientific) and the supernatant was retrieved for further experimentation.

The protein concentration of the samples was determined by using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific), a spectrophotometer (NanoDrop™, Thermo Fisher Scientific) and a microplate reader (CLARIOstar® Plus) with MARS data analysis software (both from BMG Labtech Ortenberg BMG Labtech).

After the protein concentration of the samples was adjusted, they were boiled in a heating block (Eppendorf Thermomixer Comfort, Eppendorf, Germany) at 75°C for 15 min or 95°C for 5 min with 4x Laemmli sample buffer (Bio-Rad Laboratories) containing 10% 2-mercaptoethanol (Sigma-Aldrich). In general, 20-30 µg of protein were loaded to Cirterion™ TGX™ precast gels or mini-protean™ TGX™ precast protein gels (both Bio-Rad Laboratories). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Tris-glycine-based running buffer and a PowerPac™ basic power supply (Bio-Rad Laboratories, Hercules, CA, USA). They were then transferred to a polyvinylidene

fluoride (PVDF) membrane (Bio-Rad Laboratories) at 150 mA for 60-120 min on ice. Nonspecific antibody binding was blocked by incubating the membranes with 5% (w/v) dry milk (Sigma-Aldrich) in TBST (Tris-buffered saline containing 0.05 % Tween) washing buffer for at least 1 h at RT. They were then incubated overnight at 4°C with the primary antibody while being carefully shaken in 5% (w/v) BSA (Cell Signaling Technology) in TBST. The next day, the membranes were washed three times with TBST washing buffer and incubated with corresponding secondary antibodies (Vector Labs, Burlingame, CA, USA) in 5% (w/v) dry milk (Sigma-Aldrich) in TBST for 1-2 h at RT. Subsequently, they were washed three more times with TBST to reduce the background signal. All antibodies used for WB experiments are listed in **Table 1**.

For imaging, the membranes were incubated with Clarity™ Western blot ECL substrate (Bio-Rad Laboratories) for 10 min or ECL Prime™ (GE Healthcare, Chicago, IL, USA) for 2-5 min at RT. Chemiluminescence of the protein bands was detected by using the Odyssey Fc (LI-COR Biosciences, Lincoln, NE, USA) imaging system. The images were then analyzed with Image Studio software (LI-COR Biosciences).

To quantify the optical densities of the protein bands using Fiji software (http://fiji.sc) was used. The density of the target protein bands was normalized to the density of the respective housekeeping proteins (e.g. GAPDH). The normalization factor was obtained by dividing the density of each loading control with the highest value of the housekeeping protein on the membrane. At least three independent control experiments were performed for each condition.

For further experimentation, the membranes were stripped with 2% (w/v) SDS, 62.5 mM Tris-HCl (both Carl Roth, Karlsruhe, Germany), 2-mercaptoethanol (Sigma-Aldrich) stripping buffer with pH 6.8 for 40 min at RT. They were subsequently washed multiple times with distilled water and three times with TBST washing buffer before being incubated again with primary antibodies.

#### 2.2.3.2 Immunocytochemistry

LUHMES cells were incubated with 4% paraformaldehyde (PFA) preheated to 37°C for 5 min at RT. Afterwards the cells were washed twice with DPBS for 5 min before being incubated with 1% (w/v) BSA (Cell Signaling Technology) and 0.1% Triton X-100 (Sigma-Aldrich) in DPBS (Life Technologies) permeabilization solution. The solution was removed and the cells were incubated with 1% BSA (w/v) and 1% goat serum (Vector Labs) in DPBS blocking solution

for 5 min at RT to block unspecific antibody binding. The cells were incubated with 1% BSA (w/v) in DPBS primary antibody solution overnight at 4°C or for 4 h at RT. To remove all unbound primary antibodies, the cells were washed three times with DPBS. They were then incubated with 1% (w/v) BSA in DPBS Alexa Fluor-conjugated secondary antibody solution for 1 h at RT. From this time on, the cells were placed in a low-light environment to prevent photobleaching. To remove unbound secondary antibodies, the cells were washed again three times with DPBS before their DNA was stained with a 1:1000 DAPI (Thermo Fisher Scientific) in DPBS solution for 5 min at RT. After another two washes in DPBS, the cells were air-dried, embedded in fluorescence mounting medium (Dako, Jena, Germany), and sealed with nail polish under the cover glass on a glass microscope slide. The slides were examined with a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) and a ZEISS LSM 880 AiryScan confocal microscope (Carl Zeiss Microscopy, Jena, Germany). Multiple pictures per coverslip were taken and visualized with Fiji software. All antibodies used for ICC are listed in **Table 1**.

#### 2.2.3.3 Subcellular fractionation

The protocol for subcellular fractionation by differential centrifugation (**Figure 4**) was developed based on a cell fractionation procedure already described (Taha et al., 2014). LUHMES cells were separated into five different fractions, including cytoplasm, light membranes (containing smooth endoplasmic reticulum, polysomes, and Golgi apparatus), heavy membranes (containing plasma membrane and rough endoplasmic reticulum), nuclear membranes (rough endoplasmic reticulum) and nuclear lysate (Taha et al., 2014).

On day 6 after transduction, LUHMES cells were trypsinized for 5 min and centrifuged at 1.200 x g for 7 min at 4°C. The pellet was homogenized with a pre-chilled Dounce homogenizer in a detergent-free lysis buffer containing 10 mM Tris-HCl (pH 7.4) 10 mM NaCl, 0.5 mM $MgCl_2$ (all Carl Roth), and EDTA-free Complete™ protease inhibitor cocktail (Roche). All differential centrifugation steps were carried out at 4°C. The homogenates were centrifuged at 1.200 x g for 5 min. The supernatants ($S_1$) were then again centrifuged at 1.200 x g for 5 min to obtain the crude cytosolic fraction ($S_2$) while the pellet ($P_2$) was discarded. The crude cytosolic fraction was further separated into the heavy membranes fraction ($S_3$) and the post-nuclear supernatants ($P_3$) by centrifugation at 16.000 x g for 10 min. By ultracentrifugation at 130.000 x g for 90 min, the post-nuclear supernatants were separated into the light membrane ($P_4$) and

the cytoplasm ($S_4$) fractions. The pellets ($P_1$) resulting from the first centrifugation step were resuspended in a 250 mM sucrose (Carl Roth) solution containing 10 mM $MgCl_2$ before being centrifuged through an 880 mM sucrose cushion containing 0.5 mM $MgCl_2$ at 1.200 x g for 10 min. The resulting crude nuclear fraction ($P_5$) was resuspended in the detergent-free lysis buffer and gently homogenized. The homogenized nuclei were centrifuged for 20 min through an 880 mM sucrose cushion containing 0.5 mM $MgCl_2$ at 2.000 x g to separate the nucleolar pellet ($P_6$) and the post-nucleolar supernatant ($S_6$). Subsequently, the latter was ultracentrifuged at 130.000 x g for 90 min to obtain the nuclear membrane fraction ($P_7$). The protein concentration of all fractions was determined by the BCA protein assay kit (Thermo Fisher Scientific). The protein samples were then boiled at 75°C for 15 min with 4x Laemmli sample buffer (Bio-Rad Laboratories) containing 10% 2-mercaptoethanol (Sigma-Aldrich), separated by an SDS gel electrophoresis and subjected to WB as described above.

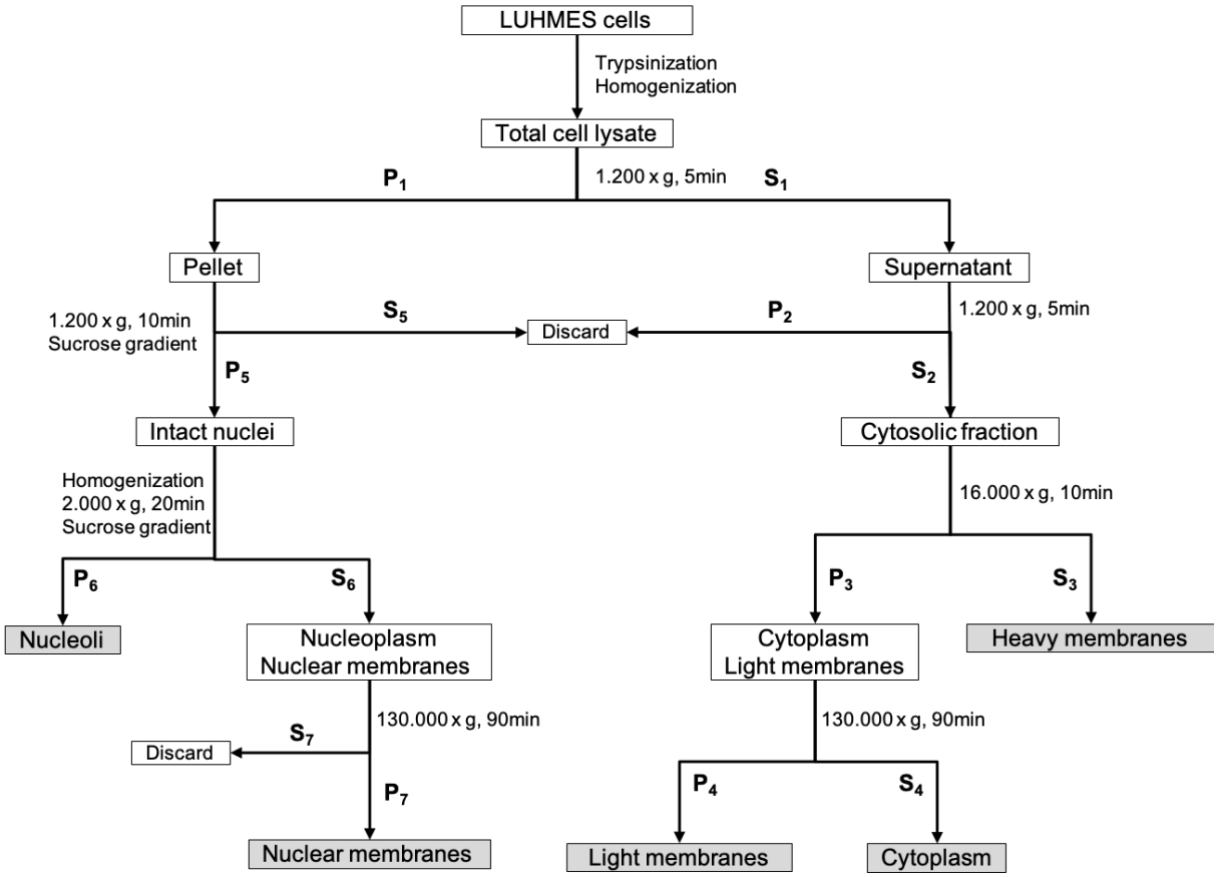


**Figure 4: Experimental subcellular fractionation protocol employing differential centrifugation.** S = supernatant, P = pellet. This figure was adapted from (Tan, 2020) with permission.

#### 2.2.3.4 Membrane protein extraction

The protocol to extract membrane proteins from mouse hemispheres (**Figure 5a**) was developed based on procedures previously described in animal models and adapted for our purposes (Bi et al., 2017; Di Giovanni, Sun, & Sheng, 2012; Suski et al., 2014; Wong, Li, & Stanley, 2013). The aim was to isolate and purify synaptic terminals, so-called “synaptosomes” from other subcellular structures. All centrifugation steps were carried out at 4°C.

First, the mouse hemisphere was homogenized in 10% (w/v) 320 mM sucrose, 5 mM HEPES (both Carl Roth) low concentrated buffer containing Complete™ protease inhibitor cocktail (Roche) on ice using a Dounce homogenizer mounted on a commercial drill. The homogenizate was centrifuged at 1.000 x g for 10 min and the supernatant ($S_1$) was collected. The pellet ($P_1$) was resuspended again in the buffer solution and subjected to the same centrifugation step to pool the supernatants ($S_1$+ $S_2$). The pooled supernatants were then centrifuged at 4.000 x g for 10 min. The pellet ($P_3$) was discarded while the resulting supernatant ($S_3$) was centrifuged at 40.000 x g for 15 min to obtain the crude synaptosome fraction ($P_4$). The supernatant ($S_4$) was subjected to ultracentrifugation at 100.000 x g for 60 min to obtain the fraction containing microsomes and small vesicles ($P_5$). The crude synaptosome fraction was resuspended in the low concentrated buffer, layered on top of a gradient consisting of 0.8 M sucrose, 5 mM HEPES buffer, and 1.6 M sucrose, 5 mM HEPES buffer, and ultracentrifuged at 54.000 x g for 90 min (**Figure 5b**). The resulting interface containing the synaptosomes was collected with a plastic pipette, resuspended in the low concentrated buffer, and pelleted ($P_7$) by ultracentrifugation at 100.000 x g for 60 min. Both pellets ($P_5$ and $P_7$) were resuspended in RIPA-buffer (Sigma-Aldrich) including protease inhibitor cocktail (Roche) and incubated on ice for 30 min while being vortexed every 10 min. The lysates were then pelleted by centrifugation at 12.000 x g for 7 min. As described above, the protein concentration was determined by the BCA protein assay kit (Thermo Fisher Scientific). The protein samples were boiled with 4x Laemmli sample buffer (Bio-Rad Laboratories) containing 10% 2-mercaptoethanol (Sigma-Aldrich), separated by SDS-PAGE, and subjected to WB.

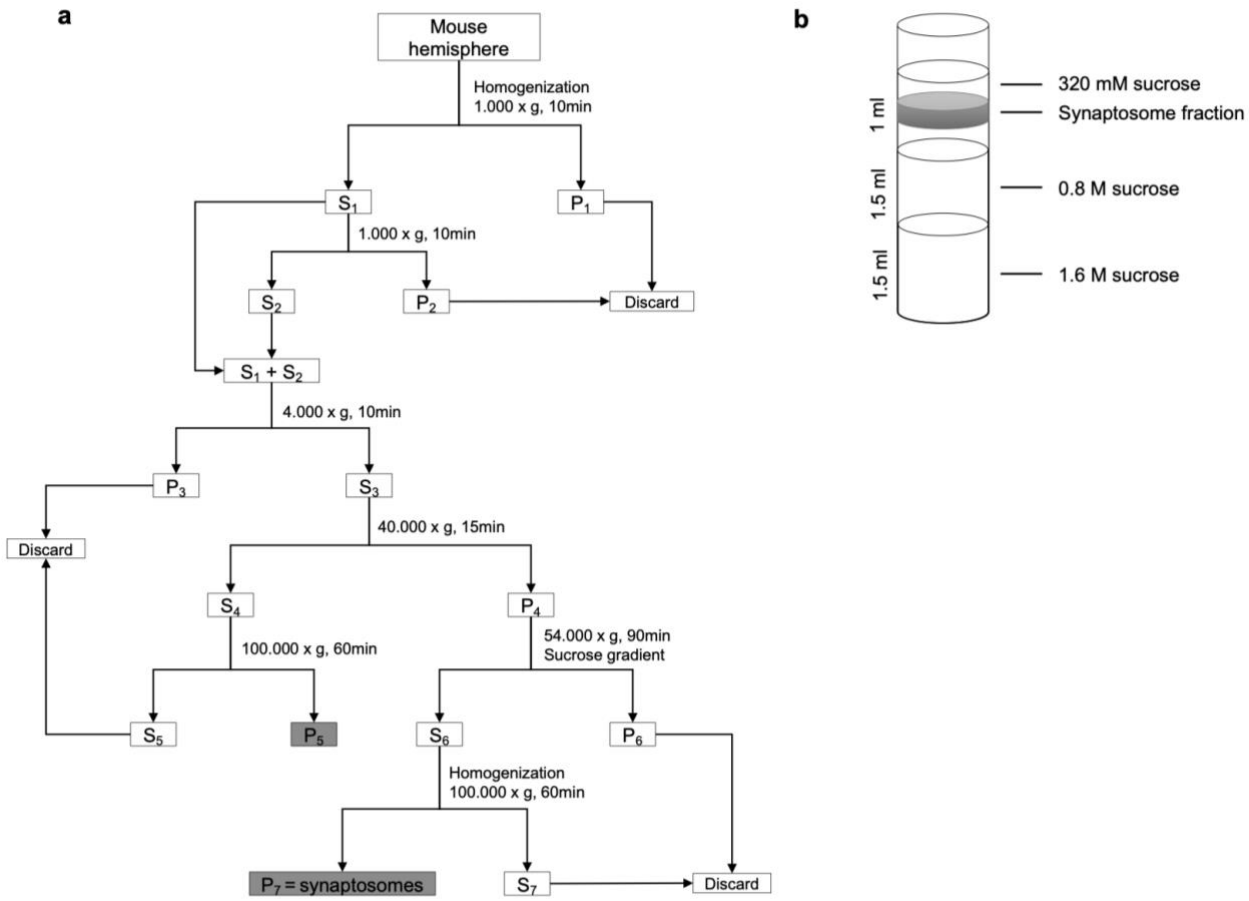


**Figure 5: Illustration of the membrane protein extraction protocol.** (a) Experimental protocol for membrane protein extraction employing differential and sucrose gradient centrifugation. S = supernatant, P = pellet. (b) Resulting interface containing the synaptosome fraction in the sucrose gradient after centrifugation.

## 2.3 Statistical analysis

The statistical analysis and design of graphs were performed using GraphPad Prism 8.4.3 (GraphPad Software, La Jolla, CA, USA). The data shown in the figures are presented as mean value ± standard error of the mean (SEM). Two data sets were analyzed using unpaired t-test. Multiple datasets with only one variable were compared by one-way ANOVA and Tukey's or Dunnett's multiple comparisons test. Results with p-values $< 0.05$ were considered statistically significant.

# 3 Results

Some of the results presented in this section have been published in *Acta Neuropathologica* (Tan et al., 2019) and can also be found in the doctoral dissertation of Dr. Yi Tan (Tan, 2020). The letter of authorization to use the published figures is attached below. I have worked on the following results individually or together with my collaborators Dr. Yi Tan and Diana Mahlstedt under the supervision of Dr. Thomas Köglsperger and Prof. Dr. Günter U. Höglinger. The results of Figure 6 and Figure 16 were not generated directly by me, but they are included in the results section for completeness. An overview of the figures in the publication and their corresponding figures in this thesis is given in **Table 4.**

| **Figure in Tan et al., 2019** | **Equivalent figure in this dissertation** | **Related figure in this dissertation** |
|---|---|---|
| Suppl. Figure S2 | Figure 4 | |
| Figure 1a | Figure 6 | |
| Figure 1b<br>Suppl. Figure S 1a, b | | Figure 7 |
| Figure 1d | Figure 8 | |
| Figure 1e, f | Figure 9 | |
| Figure 1c | | Figure 10 |
| Figure 4 a, b<br>Suppl. Figure 5 a, b | | Figure 14 |
| Figure 5 c - g | Figure 16 | Figure 15 |

**Table 4:** List of figures found in the *Acta Neuropathologica* publication (Tan et al., 2019) to which this scientific work has directly or indirectly contributed.

## 3.1 Aim 1: to characterize FMRP in cultured human dopaminergic neurons

### 3.1.1 α-Syn-transduced LUHMES cells show a significant decrease of FMRP

Investigating the relation of α-Syn and FMRP, in a prior experiment, my collaborators quantified the expression of FMRP in α-Syn transduced LUHMES cells 6 days after seeding out for differentiation by WB. Thereby, a significant reduction of FMRP was found in α-Syn overexpressing neurons compared to GFP-transduced neurons (**Figure 6**). The western blot results were supported by our results of immunofluorescent staining, in which the intensity of the FMRP signal was reduced in α-Syn-transduced neurons compared to GFP-transduced neurons. Among the α-Syn-transduced neurons, a negative correlation between FMRP and α-Syn was demonstrated by cells with a relatively strong α-Syn signal (grey arrow) showing weaker FMRP intensity (white arrow) compared to neurons with a weak α-Syn signal (**Figure 7**).

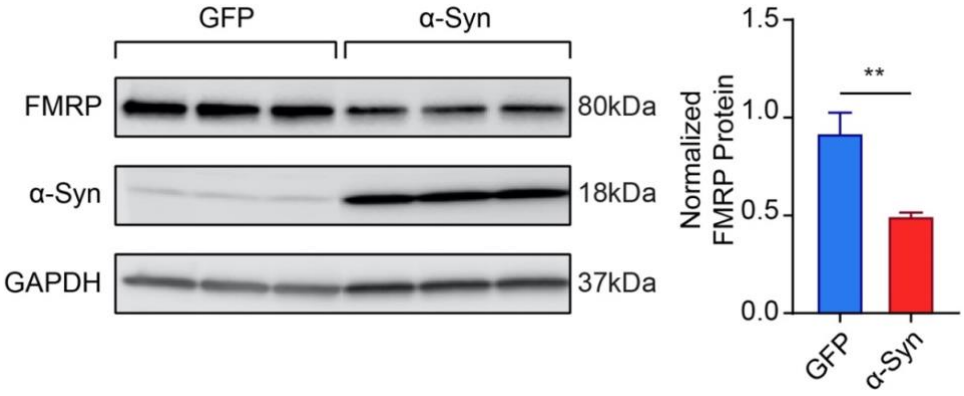


**Figure 6: FMRP expression is reduced in α-Syn-transduced LUHMES cells.** WB and quantification demonstrating a significant reduction of FMRP in α-Syn-transduced neurons compared to GFP-transduced neurons. For comparison of the means, a two-tailed unpaired t-test was used. Data are shown as means ± SEM. **P < 0.01. The use of this figure was authorized by Acta Neuropathologica and it can be found in (Tan, 2020; Tan et al., 2019).

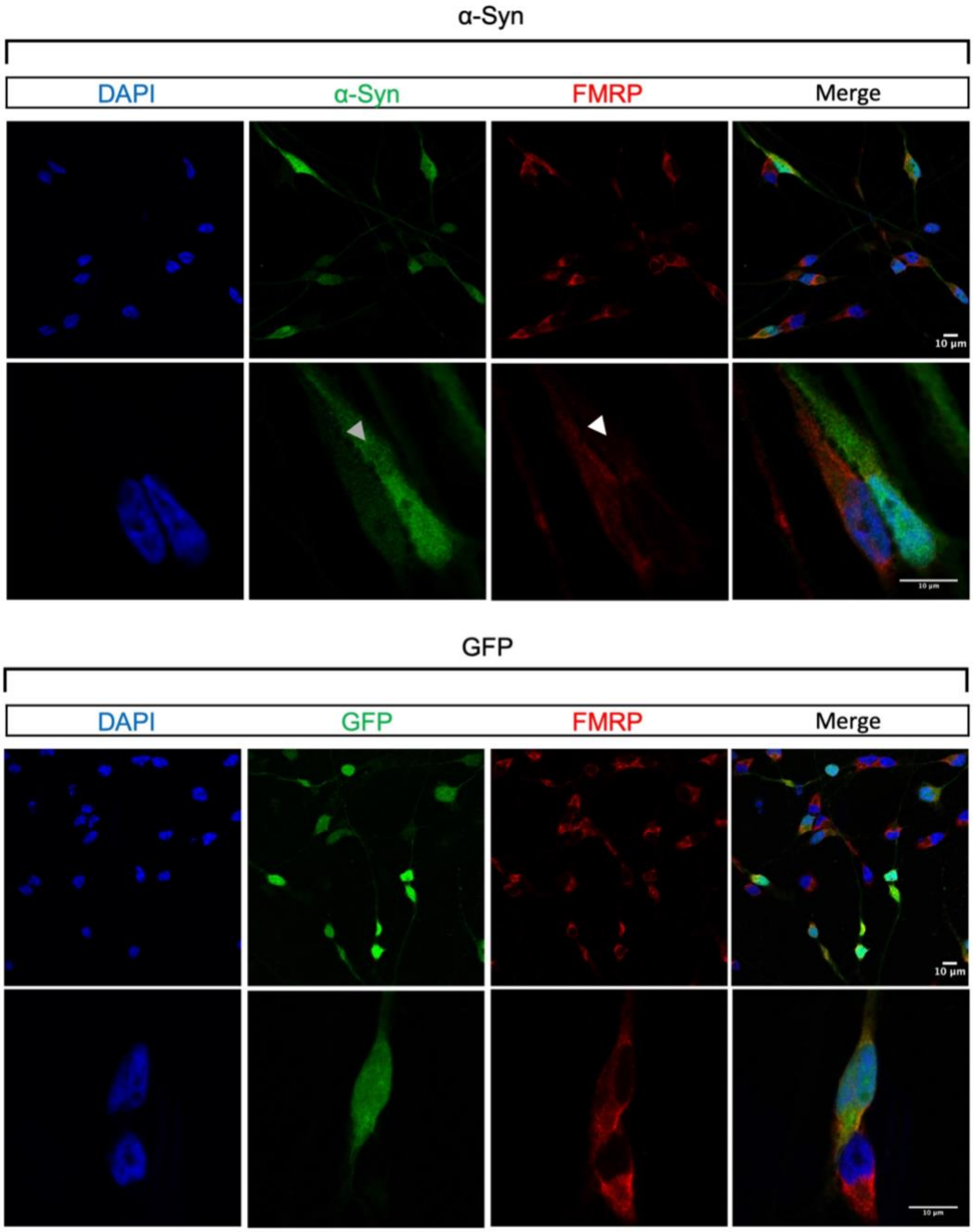


**Figure 7: FMRP signal is decreased in α-Syn-transduced LUHMES cells.** Results of ICC indicating a reduction of the intensity of the FMRP signal in α-Syn-transduced neurons in comparison to GFP transduced neurons. Among α-Syn-transduced neurons, neurons with a strong α-Syn signal (gray arrow) exhibited a weaker FMRP signal (white arrow) as compared to neurons with a relatively weak α-Syn signal.

### 3.1.2 FMRP decrease in α-Syn-transduced LUHMES cells is time-dependent

Next, the effect of α-Syn on FMRP over time was assessed by harvesting LUHMES cells on consecutive DPT. The WB result showed a decrease of FMRP on day 2 and sustained downregulation in α-Syn-transduced neurons compared to GFP-transduced neurons. In contrast, the GFP-transduced neurons showed no change in FMRP expression levels (**Figure 8**).

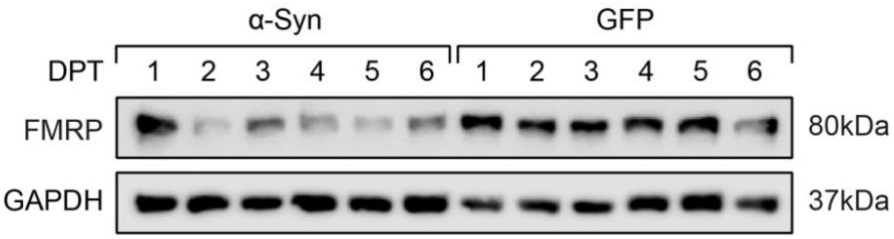


**Figure 8: FMRP expression is persistently decreased in α-Syn-transduced LUHMES cells.** WB result demonstrated decreased FMRP levels in α-Syn-transduced LUHMES 1-6 DPT, starting at 2 DPT. The use of this figure was authorized by Acta Neuropathologica and it can be found in (Tan, 2020; Tan et al., 2019).

### 3.1.3 FMRP expression is not related to cell death

Further, it was assessed whether a reduced FMRP expression is specific for α-Syn. Therefore, we treated LUHMES cells with the neurotoxic agent ROT in increasing concentrations (0-1 µM) for 24 h to induce apoptotic cell death. The WB results did not show significant changes in FMRP expression indicating that FMRP is not related to neuronal cell death, but mediated by α-Syn.

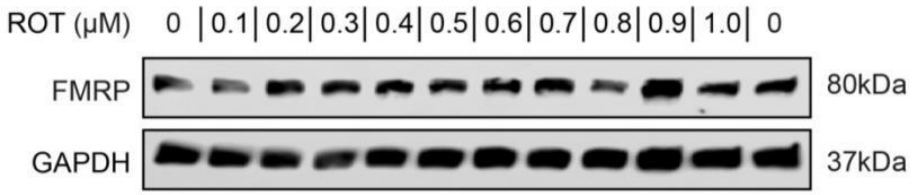


**Figure 9: Expression of FMRP remains stable in rotenone (ROT) treated LUHMES cells.** WB result showing no significant changes in FMRP expression in response to ROT-treated (0-1 µM) LUHMES cells for 24 h. The use of this figure was authorized by Acta Neuropathologica and it and it can be found in (Tan, 2020; Tan et al., 2019).

### 3.1.4 FMRP expression is decreased in solid compartments of α-Syn-transduced LUHMES cells

To study the distribution of FMRP in LUHMES cells, a protocol for subcellular fractionation (methods section 2.2.3.3.) based on previous research (Taha et al., 2014) was developed. We found that similar to Taha et al., FMRP is largely absent in the cytoplasm and mainly located in solid compartments, including heavy membranes (HM), light membranes (LM), and the nuclear lysate (NL) of LUHMES cells (**Figure 10**). Furthermore, the WB results and quantification indicated that FMRP is decreased in these fractions in α-Syn-transduced LUHMES cells, however not significant in comparison to GFP-transduced neurons (**Figure 10**).

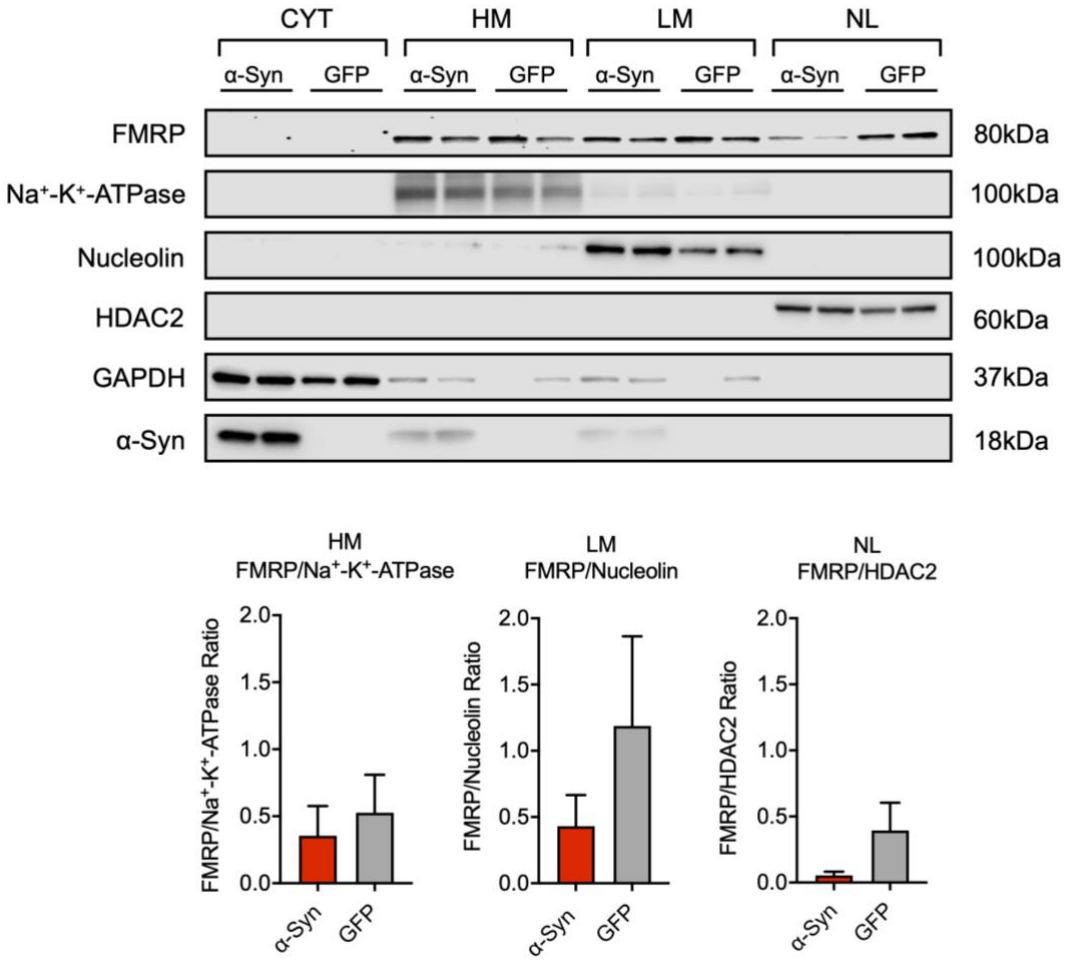


**Figure 10: FMRP is decreased in solid compartments in α-Syn-transduced LUHMES cells.** WB and quantification showing segregation to solid compartments, including heavy membranes (HM), light membranes (LM), and the nuclear lysate (NL) and absence in the cytoplasmatic fraction (CYT) of α-Syn-transduced LUHMES cells (n=4). FMRP expression in solid compartments of α-Syn-transduced LUHMES cells was decreased as compared to GFP-

transduced neurons. Protein concentrations were normalized in all fractions. For comparison of the means, a two-tailed unpaired t-test was used. Data are shown as means ± SEM.

### 3.1.5 FMRP distribution and shuttling can be studied with a lentiviral vector

To further evaluate the subcellular distribution and shuttling of FMRP, we developed a GFP-tagged FMRP lentivirus (methods section 2.2.2). A concentration of 1:1000 F2U-ΔZeo-EGFP-FMR1 lentivirus to transduce LUHMES cells was determined through balancing cell viability by MTT assay and signal intensity by ICC. The results of the MTT assay showed that survival rates of LUHMES cells transduced with different concentrations of virus (1:500 – 1:1500) were ~ 78.8 and 95.0%. There was a higher cytotoxic effect and decreasing viability at an increasing concentration (**Figure 11**). The use of a concentration of 1:1000 F2U-ΔZeo-EGFP-FMR1 lentivirus for further testing was confirmed by ICC, which showed a co-localization of GFP-tagged FMRP and endogenous FMRP and a detectable GFP-FMRP signal in α-Syn-transduced LUHMES cells (**Figure 12**).

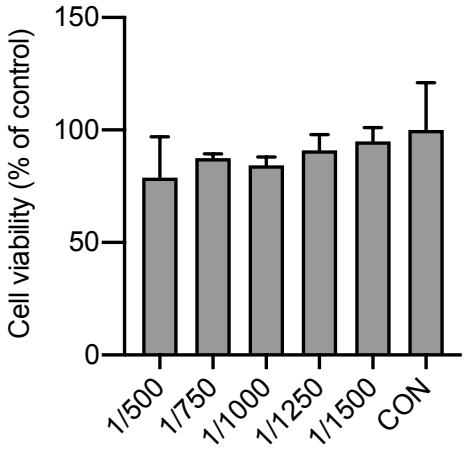


**Figure 11: Viability of LUHMES cells transduced with F2U-ΔZeo-EGFP-FMR1 lentivirus.** 6 h after seeding out for differentiation, LUHMES cells were transduced with F2U-ΔZeo-EGFP-FMR1 lentivirus at varying concentrations (1:500-1:1500). An MTT assay was used to assess cell viability. Data are presented as means ± SEM.

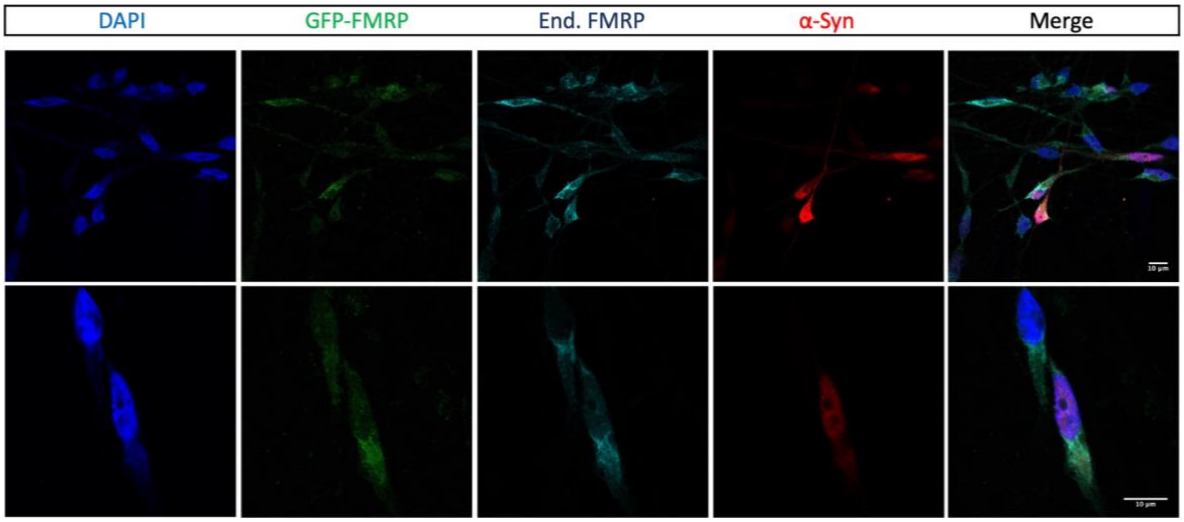


**Figure 12: Co-localization of GFP-FMRP and endogenous FMRP in LUHMES cells.** Results from ICC demonstrating a detectable signal of GFP-FMRP and co-localization with endogenous (end.) FMRP in LUHMES cells transduced with α-Syn and 1:1000 F2U-ΔZeo-EGFP-FMR1 lentivirus.

## 3.2 Aim 2: to investigate the role of α-Syn in regulating N-type calcium channels

After characterizing the role of FMRP in PD-models, we further focused on the role of FMRP and α-Syn in regulating $Ca_V2.2$ channels in vitro and in vivo using ICC in LUHMES cells and a protocol for extracting membrane proteins from mouse hemispheres.

### 3.2.1 α-Syn does not influence the abundance of $Ca_V2.2$ channels as assessed by immunostaining

To assess the expression of $Ca_V2.2$ channels, ICC with different antibodies against the pore-forming calcium voltage-gated channel subunit alpha-1B (CACNA1B) was performed. The results of the two antibodies used did not demonstrate a significant difference in the intensity of the $Ca_V2.2$ signal between α-Syn-transduced and GFP-transduced LUHMES cells.

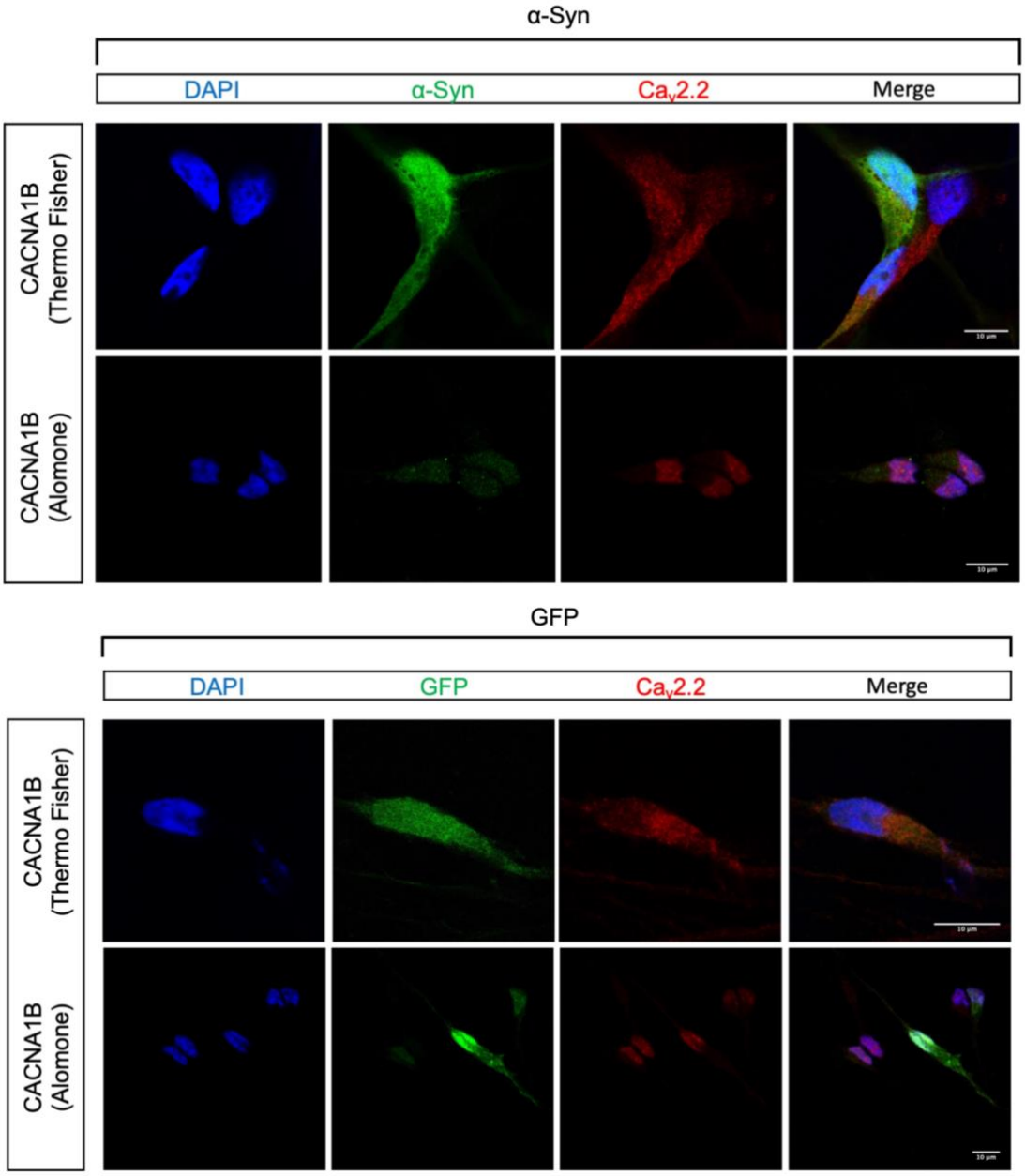


**Figure 13: $Ca_V2.2$ signal is unchanged in α-Syn-transduced LUHMES cells.** Results from ICC showed no difference in the $Ca_V2.2$ signal between α-Syn-transduced neurons in comparison to GFP-transduced neurons using different antibodies against the calcium voltage-gated channel subunit alpha-1B (CACNA1B).

### 3.2.2 α-Syn overexpression increases $Ca_V2.2$ channel expression is increased in synaptosomes of a PD mouse model

Due to the limitations of in-vitro PD-models (Falkenburger & Schulz, 2006) and to validate our previous findings, we also studied the neuropathology of PD in-vivo. Therefore, we investigated the effect of viral-induced α-Syn overexpression on dopaminergic neurons in

mice. Previous research showed that virus-induced models recapitulated pathological hallmarks such as and nigrostriatal and dystrophic neurites, and show slow disease progression similar to human PD (Kirik et al., 2002; Oliveras-Salvá et al., 2013; St Martin et al., 2007). For our study, we acquired the standardized mouse model C57BL/6J from the Jackson Laboratory in Bar Harbor, Maine, USA. All animal studies performed by Dr. Yi Tan comply with the EU Council Directive 2010/63/EU, the Guide for the Care and Use of Laboratory Animals (National Research Council 2011), and the guidelines of the DZNE institutional committee (Tan et al., 2019). Overexpression of α-Syn and luciferase (LUC) was achieved by stereotactic injection of prefabricated adeno-associated viruses (AAVs) to the SNpc of 8-10 weeks old female C57BL/6J mice. AAV5-α-syn and AAV5-LUC were obtained through the Michael J. Fox Foundation from the viral vector core facility at the University of North Carolina, USA (Tan et al., 2019). Four weeks after surgery, the mice were sacrificed and their brains stored at -80°C for the time of further experimentation.

To study the effect of α-syn on the expression of $Ca_V2.2$, we developed a protocol for the extraction of membrane proteins from mouse hemispheres (methods section 2.2.3.4). The aim was to isolate and purify synaptic terminals, so-called “synaptosomes”, from other subcellular structures since their enrichment shows an accumulation of synaptic vesicle proteins and surface membrane marker proteins such as $Ca_V2.2$ and $Na^+$-$K^+$-ATPase (Wong et al., 2013). Performing WB and quantification, we found a significantly increased expression of $Ca_V2.2$ in α-Syn-overexpressing mice compared to LUC-injected controls (**Figure 14**).

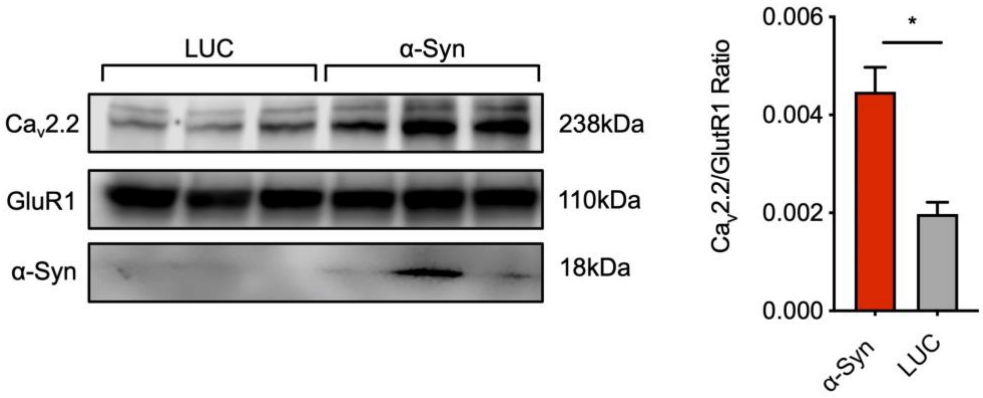


**Figure 14: $Ca_V2.2$ channel expression is increased in synaptosomes of AAV5-α-syn-injected mice.** WB and quantification demonstrating an increase of $Ca_V2.2$ channel expression in α-Syn-overexpressing brain tissue as compared to LUC overexpressing tissue (n=3). Protein concentration was normalized to the density of the glutamate receptor 1 (GluR1) signal. For

comparison of the means, a two-tailed unpaired t-test was used. Data are presented as means ± SEM. *P < 0.05.

## 3.3 Aim 3: to explore a possible rescue effect of metformin

### 3.3.1 α-syn overexpression induces up-regulation of FXS associated proteins in LUHMES cells

In studying the interplay of α-Syn and FMRP, we investigated the response of FXS-associated signaling pathway proteins (introduction section 1.5) to α-Syn overexpression. WB and quantification suggested that the phosphorylation of eIF4E and ERK and the expression of MMP-9 was increased in α-Syn-transduced LUHMES cells compared to GFP-transduced neurons **(Figure 15)**. These results were key to further experiments by my colleagues. They found that the phosphorylation of S6, eIF4E, and ERK and the expression of MMP-9 was significantly increased in α-Syn-transduced LUHMES cells. Furthermore, they transduced α-Syn overexpressing cells with AAV5-FMRP. They discovered that the co-expression of FMRP reversed the upregulatroy effect, suggesting an important role of FMRP in α-syn-mediated phenotypes (**Figure 16**).

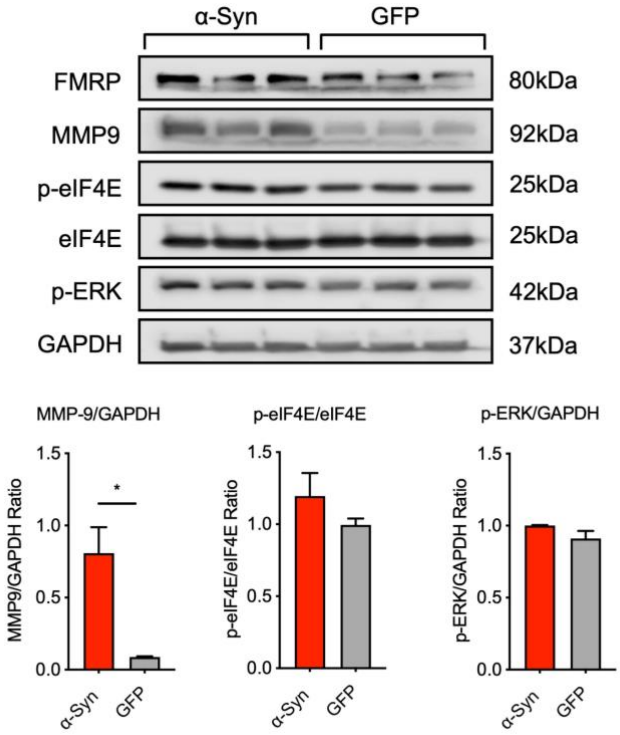


**Figure 15: FXS-associated pathways proteins are upregulated in α-Syn-transduced LUHMES cells.** WB and quantification suggesting increased phosphorylation of eIF4E and ERK, and expression of MMP-9 in α-Syn transduced LUHMES cells. For comparison of the means, one-

way ANOVA with Tukey's multiple comparisons test was used. Data are shown as means ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05.

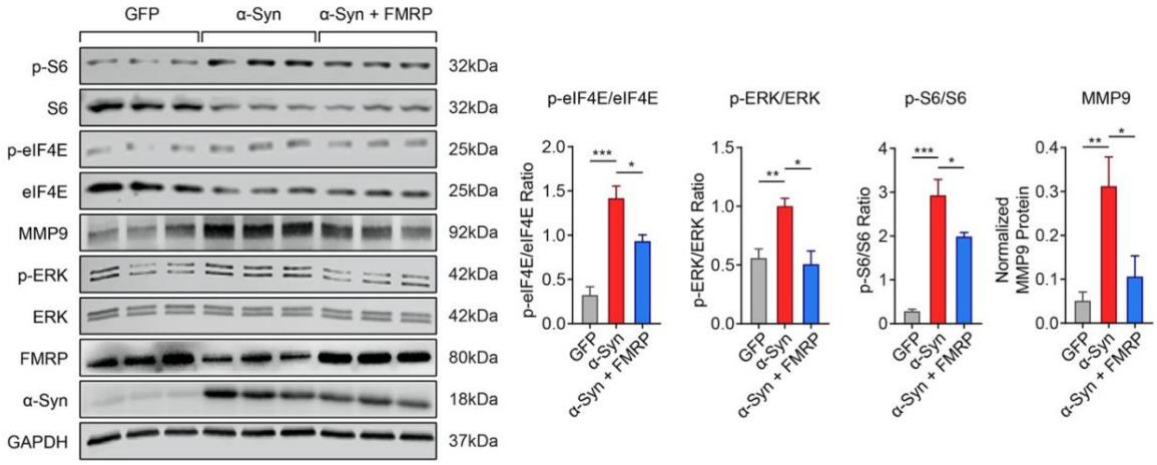


**Figure 16: FXS-associated pathways proteins are upregulated in α-Syn-transduced LUHMES cells, which can be reversed by FMRP co-expression.** WB and quantification demonstrating increased phosphorylation of S6, eIF4E, ERK, and expression of MMP-9 in α-Syn transduced LUHMES cells. The upregulating effect was reversed by the co-expression of FMRP (n=6). For comparison of the means, one-way ANOVA with Tukey's multiple comparisons test was used. Data are shown as means ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05. The use of this figure was authorized by Acta Neuropathologica and it can be found in (Tan, 2020; Tan et al., 2019).

### 3.3.2 Metformin rescues deregulated protein translation in α-Syn-transduced LUHMES cells

Metformin, a commonly used drug for the treatment of 2 diabetes, has emerged as a candidate for possible symptomatic therapy of FXS (Dy et al., 2018; Gantois et al., 2017; Katila et al., 2017). To study the effect of metformin on FXS-associated proteins in the LUHMES cell model, we treated LUHMES cells with a low (100 µm) and high (1 mM) concentration of metformin 1 DPT. We quantified the expression of FMRP downstream targets by WB and found increased levels of phosphorylated eIF4E and ERK, and a significantly increased expression of MMP-9 in α-Syn overexpressing LUHMES cells as compared to GFP-transduced cells as described above. Within α-Syn-transduced neurons treated with different concentrations of metformin, we observed a dose-dependent trend towards reduced phosphorylation of eIF4E and ERK as well as reduced expression of MMP-9, although not statistically significant (**Figure 16**).

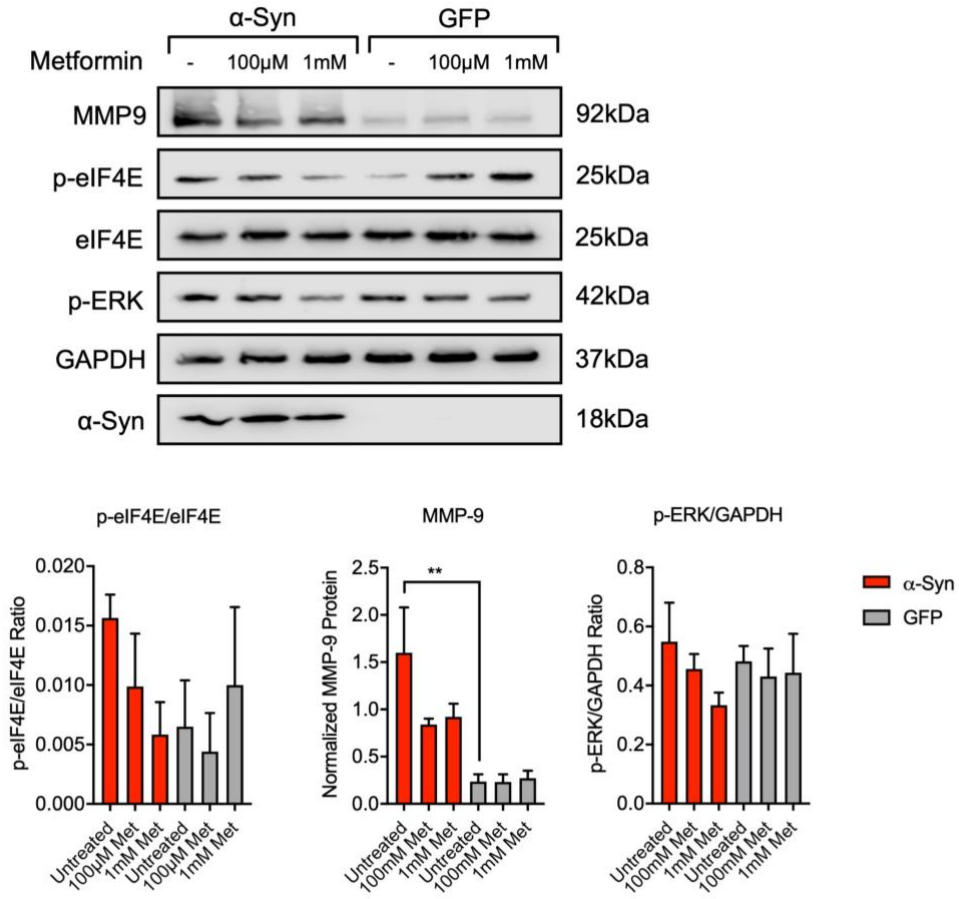


**Figure 17: Potential rescue effect of metformin on deregulated FXS-associated proteins in α -Syn-transduced LUHMES cells.** WB and quantification indicating a trend towards a dose-dependent rescue effect on increased phosphorylation of eIF4E and ERK, and expression of MMP-9 of α-Syn-transduced LUHMES treated with 100 μm and 1 mM metformin at 1 DPT (n=3). For comparison of the means, one-way ANOVA with Tukey's multiple comparisons test was used. Data are shown as means ± SEM. **P < 0.01.

# 4 Discussion

In this present research, we have provided evidence for a molecular overlap between α-Syn-mediated pathologies and FXS by investigating the interplay between α-Syn and FMRP in PD models. We demonstrated that the overexpression of α-Syn leads to a loss of FMRP and subsequently to dysregulation of translational processes similar to FXS, including upregulation of signaling pathway proteins and overexpression of $Ca_V2.2$ channels in vitro and in vivo (**Figure 18**). Furthermore, we showed that metformin, a potential therapeutic for FXS, may have a rescue effect on this pattern of dysregulation in vitro.

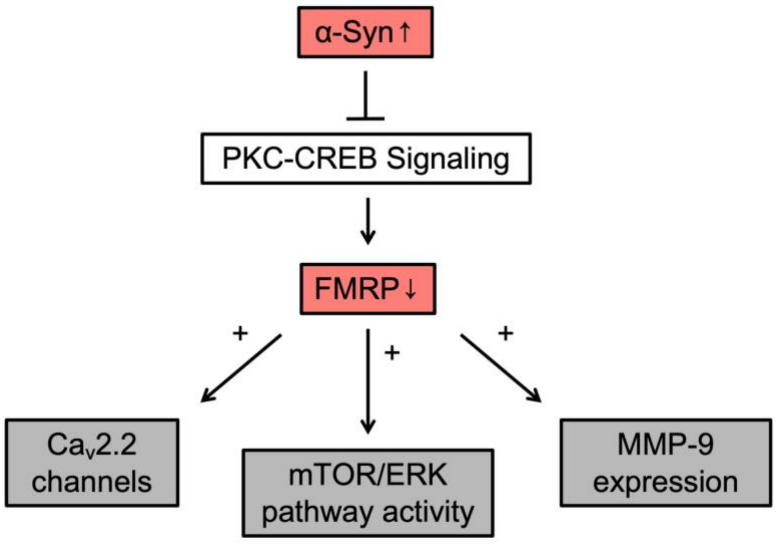


**Figure 18: Schematic summary of the pathological consequences following α-Syn-mediated FMRP loss.** α-Syn inhibits protein kinase C (PKC) and transcription factor cyclic AMP-responsive element-binding protein (CREB) signaling, which leads to a decreased expression of FMRP. As a consequence of FMRP loss, the expression of $Ca_V2.2$ channels, the activity of the mTOR and ERK signaling pathways, and the expression of MMP-9 are increased. This figure was adapted from (Tan, 2020; Tan et al., 2019) with permission.

## 4.1 Overexpression of α-Syn leads to FMRP loss and dysregulation of FXS-associated translation processes

One of our principal findings supporting a molecular overlap between the pathologies of PD and FXS was that the overexpression of α-Syn in cultured human dopaminergic neurons, typical for PD, also resulted in a loss of FMRP (**Figure 6, 7**). This finding was further reproduced in vivo observing a reduction of FMRP in mouse SNpc dopaminergic neurons in response to virus-induced overexpression of α-Syn (Tan et al., 2019). In particular, we found FMRP to be

decreased in solid cellular compartments, mainly in ER-associated and nuclear membranes (**Figure 10**) where its localization was described earlier (Taha et al., 2014). We also found that the decrease of FMRP is not merely a consequence of neuronal cell death, but specific for α-Syn, since treatment with ROT did not show any change in FMRP expression (**Figure 8**).

As mentioned earlier, FMRP-mediated translation activity is regulated through ERK and mTORC1 signaling pathways (Richter et al., 2015). In FXS, the absence of FMRP leads to increased phosphorylation of mTOR and its targets S6K1, 4E-BP, and eIF4FE and subsequently to overall increased protein translation (Gkogkas et al., 2014; Sharma et al., 2010). Accordingly, we found that neurons transduced with α-Syn also showed elevated phosphorylation of S6, eIF4E, and ERK and increased expression of MMP-9. The co-expression of FMRP, in turn, reversed this effect (**Figure 16**). Linking the overexpression of α-Syn with FMRP loss and impaired translation pathways involved in FXS offers a complementary perspective on the pathogenesis of PD.

Deregulated protein synthesis has been associated with several neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), FXTAS, and prion-mediated neurodegeneration (Grigsby, 2016; Lagier-Tourenne et al., 2012; Roffé et al., 2010). In PD, genetic studies have identified susceptibility genes such as *LRRK2, PRKN, PINK1* and *DJ-1* to be involved in protein translation (Gehrke et al., 2015; Martin, Kim, Dawson, & Dawson, 2014; van der Brug et al., 2008). For instance, in *Drosophila*, leucine-rich repeat kinase 2 (LRRK2) was found to interact with the protein translation machinery, particularly with targets of the FXS-associated mTOR pathway such as the eIF4E-binding protein 1 (4E-BP1) stimulating protein translation via eIF4E (Imai et al., 2008). LRRK2 has also been identified to negatively regulate microRNA-mediated translation suppression and to interact with ribosomal proteins (Gehrke, Imai, Sokol, & Lu, 2010; Martin, Kim, Lee, et al., 2014). Due to its role in the pathogenesis of PD, LRRK2 has been suggested as a candidate for targeted therapy using small molecule inhibitors (West, 2017; Y. Zhao & Dzamko, 2019).

FMRP was found to affect the translation of different ion channels, including voltage-gated potassium channels (Gross, Yao, Pong, Jeromin, & Bassell, 2011; Lee et al., 2011) and potassium/sodium hyperpolarization-activated cyclic nucleotide-gated ion channel 1 (HCN1) (Brager, Akhavan, & Johnston, 2012; Richter et al., 2015). Independent of its function as a translational inhibitor, FMRP can also interact directly with ion channels to regulate their activity (M. R. Brown et al., 2010; Deng et al., 2013). Previous research has shown that the loss of

FMRP increases calcium currents and the density of $Ca_V2.2$ channels in dorsal root ganglion neurons by modulating proteasomal degradation (Ferron et al., 2014). A comparable overexpression of $Ca_V2.2$ channels in LUHMES cells could not be confirmed by using ICC, since α-Syn-transduced cells did not demonstrate a significant increase in intensity of the $Ca_V2.2$ signal (**Figure 13**). However, this result might be related to methodological problems, as successful ICC depends on a variety of factors, including the fixation process, antibody concentration, and sensitivity and specificity of antibodies (Glynn & McAllister, 2006). Using a protocol to isolate and purify synaptosomes from mouse hemispheres, we found a significantly increased expression of $Ca_V2.2$ channels in AAV5-α-Syn-injected mice compared to LUC-injected controls (**Figure 14**). This result supports electrophysiological findings of an increased $Ca_V2.2$-mediated calcium influx and increased membrane abundance of $Ca_V2.2$ channels in α-Syn overexpressing cultured human dopaminergic neurons (Tan et al., 2019).

The translation of FMRP was found to be mediated by PKC and CREB (H. Wang et al., 2012). α-Syn on the other hand inhibits PKC (Ottone et al., 2011). In accordance with these results, we found that α-Syn-mediated FMRP loss in LUHMES cells results from suppression of PKC and CREB (Tan et al., 2019). This finding establishes the molecular link between α-Syn overexpression and the pathological consequences of FMRP loss (**Figure 18**).

Collectively, these results show that similar to FXS, the overall protein synthesis is increased in PD and that dysfunctional mRNA translation resulting from FMRP loss plays a central role in the pathogenesis and progression of PD. However, the relationship between protein translation and the onset of PD is still not fully understood. Thus, further investigation of the consequences of FMRP loss and impaired protein translation is required to uncover the molecular processes behind the degeneration of dopaminergic neurons and to explore possible PD treatments.

### 4.2 Neuroprotective effect of metformin

As PD cannot be cured yet, there is a need for disease-modifying drugs in therapeutic strategies. Metformin, in addition to its antidiabetic effect, has shown neuroprotective effects and thus attracted attention as a possible therapeutic. In clinical practice, the use of metformin led to improvements in behavioral deficits of patients such as social avoidance and conversational skills (Dy et al., 2018). This effect, however, has not yet been studied in a controlled trial. In animal models, metformin was identified to ameliorate FXS phenotypes in

*Fmr1*-knockout mice, and to revert the up-regulation of FXS-associated signaling pathways, the hyperphosphorylation of ERK and eIF4E and the increased expression of MMP-9 (Gantois et al., 2017). In an MPTP animal model of PD, metformin showed neuroprotective effects including the recovery of MPTP-induced α-Syn phosphorylation, DA depletion, and behavioral impairment (Katila et al., 2017).

The neuroprotective effect of metformin can be explained in several ways. However, the key molecular mechanisms underlying the neuroprotective effect are not yet fully understood. Metformin is able to inhibit microglia-induced neuroinflammation, to induce autophagy, and to eliminate mitochondrial reactive oxygen species (Lu et al., 2016). Metformin was also found to exert its neuroprotective effect by reducing the phosphorylation of α-Syn through inducing protein phosphatase 2A (PP2A) (Katila et al., 2017). Furthermore, metformin is an activator of 5'-adenosine mono-phosphate-activated protein kinase (AMPK), which preserves cellular energy stores by stimulating mitochondrial adenosine triphosphate (ATP) production. This function of metformin may be relevant for PD since patients show mitochondrial and lysosomal dysfunction (Burbulla et al., 2017; Cantó et al., 2009). For instance, it was found that AMPK activation by metformin protects neurons from α-Syn-induced toxicity in vitro (Dulovic et al., 2014). In vivo, AMPK-mediated neuroprotection was attributed to a reduced formation of dystrophic axons and a limitation of lysosomal material (Bobela, Nazeeruddin, Knott, Aebischer, & Schneider, 2017) as well improvement of dopaminergic and mitochondrial dysfunction due to the protective effect of epigallocatechin gallate (Ng et al., 2012).

Based on the findings by Gantois et al. we explored similar effects of metformin on FXS-associated proteins in LUHMES cells. WB results demonstrated a dose-dependent trend towards reduced phosphorylation of eIF4E and ERK as well as reduced expression of MMP-9 in α-Syn-transduced neurons treated with low (100 µm) and high (1 mM) concentration of metformin (**Figure 17**). These results support the assumption of the neuroprotective effect of metformin on α-Syn-mediated phenotypes in the LUHMES cell model and serve as a basis for further experiments to study the use metformin as a possible therapeutic strategy to treat PD and FXS.

### 4.3 Potential role of FMRP in α-Syn mediated pathologies

FXS is the most common hereditary form of autism spectrum disorder and intellectual disability, in which patients exhibit behavioral abnormalities, learning deficits, and seizures (R.

J. Hagerman et al., 2017). The neuronal mechanisms underlying the clinical manifestations in FXS include alterations in neuronal plasticity, decreased GABA signaling, and deregulated protein translation leading to hyperexcitability of neuronal circuits (Contractor, Klyachko, & Portera-Cailliau, 2015; D'Hulst et al., 2009; Qin, Kang, Burlin, Jiang, & Smith, 2005). In this study, we demonstrated that FMRP is significantly reduced in α-Syn-overexpressing cultured human dopaminergic neurons (**Figure 6, 7**) resulting in increased expression of $Ca_V2.2$ channels (**Figure 14**) and subsequent calcium current, enhanced activity of FXS-associated signaling pathways and protein translation (**Figure 15, 16**). These results suggest a molecular overlap between the FXS and α-Syn mediated pathologies and offer a new perspective on the pathology of PD.

The role and function of FMRP in the loss of dopaminergic neurons remains complex and cannot be derived one-to-one from FXS. In contrast to FXS, in which FMRP is deficient or reduced due to epigenetic silencing of *Fmr1,* we found that in α-Syn-overexpressing LUHMES cells FMRP expression is decreased through the suppression of PKC and CREB-mediated transcription (Tan et al., 2019). Furthermore, different isoforms FMRP and the FMRP paralogues FXR1P and FXR2P exist, which vary in the ratio and distribution of RNA-recognition elements. Thus, FMRP has a more complex role in neurodegeneration, as it can associate with different target mRNAs that share signaling pathways across various cellular contexts (Ascano et al., 2012). Although extensive research on RNA targets of FMRP has been conducted, further research is required to identify more RNA targets and better understand the manifold function of FMRP and options for molecular treatment (V. Brown et al., 2001; De Rubeis, Fernández, Buzzi, Di Marino, & Bagni, 2012; Krueger & Bear, 2011)

In our work, we found a similar number of SNpc TH-positive neurons in *Fmr1*-knockout mice compared to wild-type mice in response to α-Syn-overexpression. Furthermore, *Fmr1*-knockout mice showed no difference in DA release between LUC and α-Syn-injected hemispheres. These results thus suggest that FMRP loss in α-Syn-mediated pathologies may have a protective, stabilizing effect on DA release despite its dysfunctional effect on protein synthesis (Tan et al., 2019). Therefore, further studies are needed to investigate the relation between FMRP and α-syn overexpression and to better understand its role in the pathogenesis and progression of PD.

# 5 Summary

In this work, we studied the molecular overlap between FXS and α-Syn-mediated pathologies by investigating the interplay between α-Syn and FMRP in different PD models using a variety of imaging and biochemical methods. In particular, we found that FMRP is significantly decreased in response to α-Syn overexpression in vitro, mainly in solid compartments of cultured human dopaminergic neurons. Consequently, FXS-associated signaling pathway proteins were upregulated, including increased phosphorylation of S6, eIF4E, and ERK, and increased expression of MPP-9. Developing a protocol to isolate and quantify synaptic ion channels, we showed that the abundance of $Ca_V2.2$ is increased in synaptosomes of α-Syn-injected mouse hemispheres. Finally, we suggested that the promising neuroprotective effect metformin in FXS may be applied to the LUHMES cell model since we observed a dose-dependent trend associated with metformin treatment towards reduced phosphorylation of eIF4E and ERK as well as reduced expression of MMP-9 in α-Syn-transduced neurons. Together, these results underline the important role of FMRP in the pathogenesis of α-Syn-mediated pathologies and PD, also exploring a possible treatment strategy with metformin.

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# 7 Publication

This study is part of the following publication:

Tan, Y., Sgobio, C., Arzberger, T., Machleid, F., Tang, Q., Findeis, E., Tost, J., Chakroun, T., Gao, P., Höllerhage, M.,·Bötzel, K., Herms, J., Höglinger G. U., Köglsperger, T. (2019): Loss of fragile X mental retardation protein precedes Lewy pathology in Parkinson's disease. Acta Neuropathologica, 139(2), 319-345. https://doi.org/10.1007/s00401-019-02099-5

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