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iPSC-derived Neurons and Astrocytes: a novel patients-specific model to study the pre-degenerative molecular alterations in Parkinson's Disease

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Abstract

Dopamine (DA) plays a fundamental role in the regulation of different physiological functions including motor activity, cognition, learning, reward mechanisms and working memory. The functional role of DA is such that the progressive degeneration of DA neurons underlies the development of Parkinson's disease (PD). The effects of DA are mediated by five receptors (D1-D5) that differ for structure, pharmacology and transduction signalling (Missale et al., 1998). One of the characteristics of DA receptors is their propensity to directly interact with other receptor subtypes to form heteromers with peculiar transductional, functional, and pharmacological properties (Bono et al., 2020). We recently reported that in mouse dopaminergic (DA) neurons, the dopamine D3 receptor (D3R) and the nicotinic ion channels (nAChR) interact to form a heteromeric complex (D3R-nAChR heteromer) that exerts neurotrophic effects when activated by nicotine, leading to neurons with enlarged cell bodies and increased dendrites arborization. We also found that the D3R-nAChR heteromer is present in human DA neurons derived from induced pluripotent stem cells (iPSC) and that nicotine, by activating this complex, protects DA neurons against neuronal injury. The direct interaction between the D3R and the beta2 subunit of nAChR was defined by using bioluminescence resonance energy transfer assays in transfected cells and proximity ligation assay in both mouse and human DA neurons (Bontempi et al., 2017; Bono et al,2019). However, the biochemical properties of the D3R-nAChR heteromer have been never investigated yet. In this study, mouse DA neurons and human iPSC-derived DA neurons were used for investigating the pharmacological and signalling properties of the D3R-nAChR complex. In particular, mouse and human DA neurons have been treated with both nicotine and the D3R agonist quinpirole for different times and analyzed for morphological effects and biochemical analyses; the activation of the ERK/MAPK cascade involved in neuronal long-term modifications, has been mainly analyzed. Morphological and biochemical experiments have been also performed by incubating cells with nicotine or quinpirole in the presence of specific interfering peptides able to disrupt the D3R-nAChR heteromer. Together, the results obtained in this study further elucidate the molecular and biochemical mechanisms underlying the neuroprotective effects of nicotine and quinpirole induced by the activation of the D3R-nAChR heteromer. In particular, our results indicate that nicotine and quinpirole-induced morphological effects in mesencephalic dopaminergic neurons involve nAChR along with dopamine D3R-mediated recruitment of ERK / PI3K signalling.

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the loss of DA neurons in the pars compacta of the substantia nigra. Although the majority of cases are sporadic, a

proportion of PD are linked to genetic mutations. In particular, the G2019S mutation in the LRRK2 gene, that encode for a large multidomain protein involved in multiple cellular processes (Goldwurm et al., 2005), is the most frequently observed. One of the various functions associated with LRRK2 activity is the regulation of vesicle trafficking (Sanna et al., 2012; Cirnaru et al., 2014; Rassu et al., 2017).

We have provided evidence that the heteromeric complex D3R-nAChR is crucially involved in morphological plasticity and neurons homeostasis, thus indicating that abnormal D3R-nAChR function may be associated with the vulnerability of DA neurons. Along this line, through the use of iPSC technology we studied the impact of the properties of the heteromeric complex D3R-nAChR in PD patient-derived DA neurons with LRRK2 G2019S mutation.

The results show that DA neurons derived from PD patients with LRRK2 mutation are resistant to the neurotrophic effects of nicotine and D3R agonist, show a reduced expression of both the dopamine D3 receptors (D3R) and the nicotinic acetylcholine receptors (nAChR) and the formation of the D3R-nAChR heteromer. Interestingly, D3R and nAChR as well as the corresponding heteromer membrane localization were rescued by inhibiting the abnormally increased kinase activity.

Moreover, analysis of synaptic function demonstrated a remarkable dysregulation of receptor mechanisms controlling dopamine release, suggesting that G2019S mutation significantly affects the expression and trafficking and function of key receptors controlling DA function.

Since its crucial role in preserving DA neurons homeostasis, the D3R-nAChR heteromer may represent a novel target for drugs designed for supporting DA neurons plasticity and survival against toxic damages in various pathologies, such as Parkinson's disease.

To study the mechanisms of neurodegeneration in PD, researchers have focused their attention primarily on the affected DA neurons. However, astrocytes play a significant role in the vulnerability of these neurons (Lee et al., 2019). However, due to the difficulty in obtaining human astrocytes, their role in these pathologies is still poorly characterized. On this line, we have developed a protocol to differentiate iPSC derived from healthy control, patients with LRRK2 G2019S mutation (LRRK2-PD) and their respective corrected isogenic lines (LRRK2-ISO) in astrocytes to investigate their possible involvement in PD. Through immunofluorescence and functional assays, we have shown that our protocol consistently produces an enriched and functional population of mature astrocytes. A full set of neurotransmitter receptors are expressed in astrocytes, including dopamine D2 receptors (D2R), shown to act as key negative regulator of neuroinflammation. We found that astrocytes derived from iPSC of PD patients carrying the LRRK2 G2019S mutation are characterized by altered D2R localization

at the plasma membrane suggesting that in these patients, astrocyte D2R defects may represent a relevant molecular event leading PD progression. Targeting astrocytes thus represent an attractive alternative approach to develop new therapies to treat neurological disorder.

RIASSUNTO

La dopamina (DA) svolge un ruolo fondamentale nella regolazione di diverse funzioni fisiologiche tra cui l'attività motoria, l'apprendimento, i meccanismi di ricompensa e la memoria. La progressiva degenerazione dei neuroni dopaminergici (DA) è alla base dello sviluppo del morbo di Parkinson (PD). Gli effetti della DA sono mediati da cinque recettori (D1-D5) che differiscono per struttura, proprietà farmacologiche e di trasduzione del segnale (Missale et al., 1998). Una delle caratteristiche dei recettori DA è la loro propensione a interagire direttamente con altri sottotipi di recettori per formare eteromeri con peculiari proprietà trasduzionali, funzionali e farmacologiche (Bono et al., 2020). Recentemente abbiamo riportato che nei neuroni DA del topo, il recettore della dopamina D3 (D3R) e i canali ionici nicotinici (nAChR) interagiscono tra loro per formare un complesso eteromerico (D3R-nAChR) che quando stimolato dalla nicotina esercita effetti neurotrofici sui neuroni, e questo si traduce in un'aumentata arborizzazione dei dendriti e un ingrandimento dei corpi cellulari. Abbiamo anche scoperto che l'eteromero D3R-nAChR è presente nei neuroni DA umani derivati da cellule staminali pluripotenti indotte (iPSC) e che la nicotina protegge i neuroni DA dalle lesioni neuronali tramite l'attivazione di questo complesso. L'interazione diretta tra la subunità D3R e la subunità beta2 del nAChR è stata definita utilizzando saggi di trasferimento di energia di risonanza di bioluminescenza (BRET) in cellule trasfettate e test di legatura di prossimità (PLA) in neuroni DA sia murini che umani (Bontempi et al., 2017; Bono et al., 2019). Le proprietà biochimiche dell'eteromero D3R-nAChR non sono state ancora studiate. Quindi in questo lavoro di tesi sono stati utilizzati i neuroni DA di topo e i neuroni DA derivati da iPSC umani per indagare le proprietà farmacologiche e biochimiche del complesso D3R-nAChR. In particolare questi neuroni sono stati trattati sia con nicotina che con il quinpirolo un'agonista D3R, per tempi diversi e analizzati per gli effetti morfologici e biochimici; in particolare è stata analizzata l'attivazione della cascata delle ERK/MAPK coinvolta nelle modificazioni neuronali a lungo termine. Sono stati inoltre condotti esperimenti morfologici e biochimici incubando le cellule con nicotina o quinpirolo in presenza di specifici peptidi interferenti in grado di disgregare l'eteromero D3R-nAChR. I risultati da noi ottenuti chiariscono ulteriormente i meccanismi molecolari e biochimici alla base degli effetti neuroprotettivi della nicotina e del quinpirolo indotti dall'attivazione dell'eteromero D3R-nAChR e indicano che gli effetti morfologici indotti dalla nicotina e dal quinpirolo nei neuroni DA mesencefalici coinvolgono il recettore nAChR insieme al reclutamento mediato dal D3R della via di segnalazione ERK / PI3K.

La malattia di Parkinson (PD) è una malattia neurodegenerativa caratterizzata dalla perdita di neuroni DA nella pars compacta della substantia nigra. Sebbene nella maggior parte dei casi sia sporadica, una piccola porzione del PD è legata a mutazioni genetiche. In particolare, la mutazione più frequentemente osservata è la G2019S nel gene LRRK2, che codifica per una proteina multidominio coinvolta in vari processi cellulari (Goldwurm et al., 2005) tra cui la regolazione del trafficking vescicolare (Sanna et al., 2012; Cirnaru et al., 2014; Rassu et al., 2017). Abbiamo fornito prove che indicano che il complesso eteromerico D3R-nAChR è coinvolto in modo cruciale nella plasticità morfologica e nell'omeostasi dei neuroni, indicando così che una funzione anomala del complesso D3R-nAChR può essere associata a una maggior vulnerabilità dei neuroni DA. Su questa linea, attraverso l'uso della tecnologia iPSC abbiamo studiato l'impatto delle proprietà del complesso eteromerico D3R-nAChR nei neuroni DA derivati da pazienti PD con mutazione LRRK2 G2019S. I nostri risultati mostrano che i neuroni DA derivati da pazienti parkinsoniani con mutazione LRRK2 sono resistenti agli effetti neurotrofici della nicotina e dell'agonista D3R, mostrando un'espressione alterata sia dei recettori dopaminergici D3 (D3R) che dei recettori nicotinici dell'acetilcolina (nAChR) e di conseguenza anche della formazione dell'eteromero D3R-nAChR. È interessante notare che inibendo l'aumentata attività chinasica di LRRK2, la localizzazione recettoriale in membrana del D3R e del recettore nAChR, così come del complesso D3R-nAChR, è stata ripristinata. Inoltre, l'analisi della funzione sinaptica ha dimostrato una notevole disregolazione dei meccanismi recettoriali che controllano il rilascio di dopamina, suggerendo che la mutazione G2019S influenza significativamente l'espressione, il traffico e la funzione dei recettori chiave che controllano la funzione dopaminergica. Dato il suo ruolo cruciale nel preservare l'omeostasi dei neuroni DA, l'eterodimero D3R-nAChR può rappresentare un nuovo bersaglio per farmaci progettati per supportare la plasticità dei neuroni DA e la sopravvivenza contro i danni tossici in varie patologie, come il morbo di Parkinson. Per studiare i meccanismi di neurodegenerazione nella malattia di Parkinson, i ricercatori hanno concentrato la loro attenzione principalmente sui neuroni DA. Tuttavia, gli astrociti svolgono un ruolo significativo nella vulnerabilità di questi neuroni (Lee et al., 2019) ma a causa della difficoltà nell'ottenere astrociti umani, il loro ruolo in queste patologie è ancora poco caratterizzato. In questa direzione, abbiamo sviluppato un protocollo per differenziare iPSC derivati da controlli sani, da pazienti con mutazione LRRK2 G2019S (LRRK2-PD) e le rispettive linee isogeniche corrette (LRRK2-ISO) in astrociti per indagare il loro possibile coinvolgimento nel PD. Attraverso tecniche di immunofluorescenza e analisi funzionali, abbiamo dimostrato che il nostro protocollo produce costantemente una popolazione funzionale di astrociti maturi. Inoltre, negli astrociti è espresso un set completo di recettori dei

neurotrasmettitori, inclusi i recettori della dopamina D2 (D2R) e dopamina D3 (D3R), che hanno dimostrato di agire come regolatori chiave della neuroinfiammazione. Abbiamo scoperto che gli astrociti derivati da iPSC di pazienti parkinsoniani con la mutazione LRRK2 G2019S sono caratterizzati da una localizzazione alterata del recettore D2R sulla membrana plasmatica, suggerendo che in questi pazienti i difetti nella localizzazione del D2R possono rappresentare un evento molecolare rilevante che porta alla progressione del PD. Il targeting degli astrociti può rappresentare un approccio alternativo attraente per lo sviluppo di nuove terapie per il trattamento dei disturbi neurologici.

1 INTRODUCTION

1.1 Parkinson's Disease

1.1.1 Etiology

Parkinson disease (PD) is the second most common neurodegenerative disorder characterized by severe motor symptoms, including static tremor, bradykinesia, postural imbalance and muscle rigidity. PD affects more than six million people globally, predominantly those over the age of 65 years. The frequency of the disorder is about 1.3 cases per 100000 people younger than 45 years of age, 3100 per 100 000 in those aged 75–85 years, 4300 per 100 000 in those older than 85 years (De Lau, 2006). Similar to other neurodegenerative disease, ageing is the major risk factor, although 10% of people have young-onset PD, defined as a diagnosis between 21 and 50 years of age (Schrag,2006). For later-onset PD, patients are usually diagnosed over 70 years of age (Pagano et al., 2016). The exact cause of PD is still unknown. Both environmental and genetic factors are recognized to affect disease risk and progression. Only approximately 10% of PD cases can be directly attributed to genetic factors (Trinh, 2013). There is also likely a strong contribution of environmental factors in PD. Regarding environmental factors, neuroleptic drugs, exposure to pesticides, smoking, caffeine intake, and manganese toxicity are all reported to increase the risk of developing parkinsonism (Kieburtz, 2013). PD is twice more common in men than in women in most populations. A protective effect of female sex hormones, a sex-associated genetic mechanism or sex-specific differences in exposure to environmental risk factors might explain this male preponderance (Poewe et al., 2017).

1.1.2 Pathophysiology

Recent evidence, highlighting that axonal and synaptic pathologies are among the cardinal features of PD, suggests that synaptic derangement may play a crucial role in the pathogenic processes leading to PD. The cardinal motor symptoms of PD results from the progressive loss of dopaminergic (DA) neurons in the substantia nigra (SN) pars compacta (SNpc) in the midbrain. SNpc neurons form the nigrostriatal DA pathway; thus, loss of SNpc neurons leads to the striatal dopamine deficiency responsible for the major symptoms of PD (Fig.1). Degeneration of DA neurons results in a threshold decrease of approximately 80% dopamine in the striatum, leading to the emergence of motor

symptoms (Blesa, 2014). Nerve cell loss in the substantia nigra correlates with the severity and the duration of motor dysfunction (Ma et al., 1997). This leads to a neurochemical misbalance of the basal ganglia circuits caused by dopamine (DA) loss leading to severe progressive motor disability, reduced quality of life and expectancy. Gross macroscopic atrophy of the brain is not a feature of Parkinson disease; rather neuronal degeneration occurs in only certain types of neurons within particular brain regions. In early-stage disease, loss of DA neurons is restricted to the ventrolateral substantia nigra with relative sparing of other midbrain DA neurons, but becomes more widespread by end-stage disease. The loss of these DA neurons even early in the disease suggests that the degeneration in this region starts before the onset of motor symptoms, which is supported by several recent clinic-pathological studies (Damier et al.,1999; Dijkstra et al., 2014).

The other pathological hallmarks include the formation of Lewy bodies (LB) and Lewy neurites (LN) consisting of neuronal and axonal aggregates of misfolded α-synuclein (α-syn), resulting in dopaminergic denervation of the corpus striatum (Fig.1). α-syn is a presynaptic nerve terminal protein of 140 amino acids. Native α -syn appears unfolded and represents approximately 1% of cytosolic protein in the brain. Results from α -syn knockout mice suggest a role for α–syn as a presynaptic activity-dependent negative regulator of dopamine release. Knock-in and overexpression of α -syn mutants seem to be especially toxic to DA neurons (Cuervo et al., 2004). These outcomes support the conjecture that α–syn plays an important role in the nigrostriatal dopamine pathway.

Proteome studies have shown that LB consists of more than 300 proteins, of which approximately 90 have been confirmed by immunohistochemistry in various post-mortem studies and are associated with α-syn, protein degradation systems, molecular chaperones or axonal damage (Wakabayashi et al., 2013). The relevance of α-syn deposition in PD pathophysiology has been corroborated by the discovery that mutations or multiplications in the α-syn locus (SNCA) are responsible for the onset of some familial forms of PD (Singleton et al.,2003; Ibanez et al., 2004).

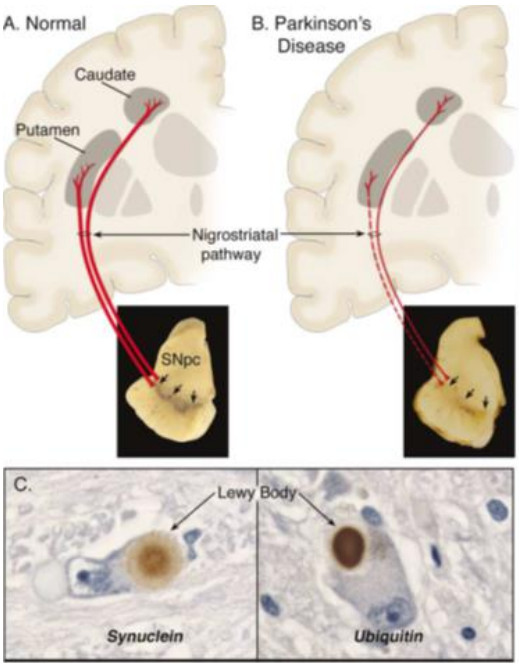


Fig.1. Neuropathology of Parkinson's disease (Dauer 2003)

In 2003, Braak et al. postulated the hypothesis of an associated staging system for PD based on a specific pattern of LB spreading studies (Braak 2003, Braak 2003) (Fig.2). These studies were followed by the dual-hit hypothesis, postulating that sporadic PD starts in two places: the neurons of the nasal cavity and the neurons in the gut (Hawkes et al.,2009). The fusion of these theories is now known as Braak's hypothesis of PD. It has been described that LB deposits show a distinctive topographical distribution pattern (Braak 2003, Braak 2003). In the earliest phase (Stage 1), the cerebral areas involved are the dorsal motor nucleus of the vagus and anterior olfactory structures. Then, (Stage 2) the deposition in brainstem nuclei and adjacent structures, such as caudal Raphe nuclei and the locus coeruleus, starts. Only in the midstage 3 and 4, LB appear in substantia nigra pars compacta in dopaminergic neurons and this phase coincides with the onset of clinical motor signs. Lately, in the final stages (4, 5 and 6) the pathology extends to cortical areas. Remarkably, LB are not restricted to the central nervous system (CNS), but have also been found in peripheral tissue as well as vagus nerve (Beach et al.,2010) the submucosal plexus in the gut (Braak et al.,2006) cardiac sympathetic ganglia and superior cervical ganglion (Del Tredici et al., 2010).

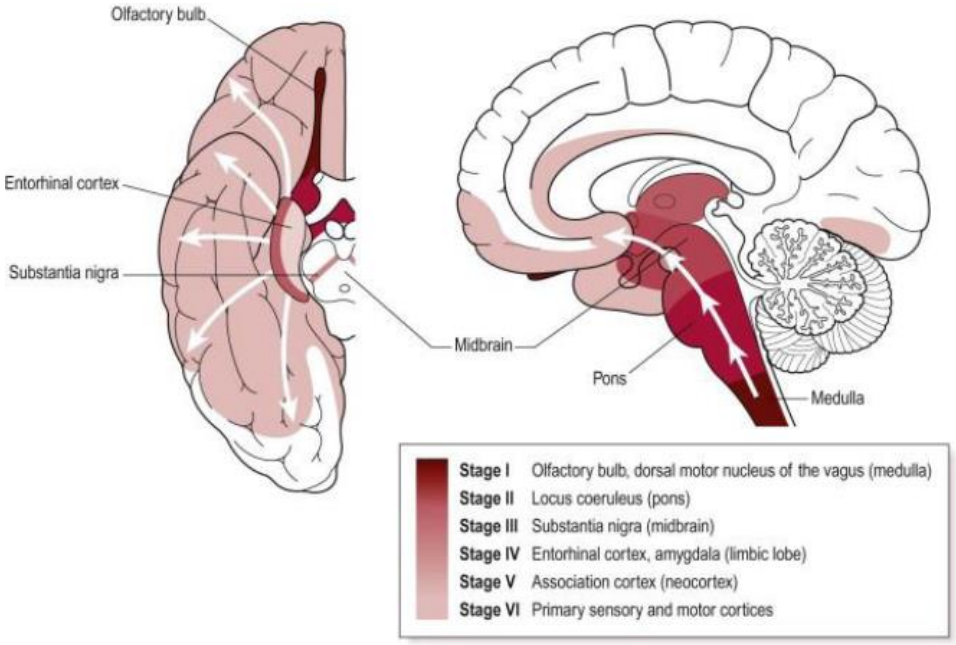


Fig.2.Braak's stadiation (Braak et al., 2006)

From these places, the pathology is hypothesized to spread according to a specific pattern, via the olfactory tract and the vagal nerve, respectively, toward and within the CNS.

1.1.3 Therapy

Parkinson's disease is still an incurable progressive disease, but treatment substantially improves quality of life and functional capacity. DA precursor levodopa (L-DOPA) has remained the gold standard for Parkinson disease and over time, all patients with Parkinson disease will require treatment with this agent (LeWitt, 2016). This drug is particularly effective in the early stages of the disease and achieves therapeutic dose levels in a relatively short time, but its chronic assumption is associated with the onset of severe adverse effects such as motor complications, like fluctuations in motor response and dyskinesias, and hallucinations. After five or seven years, patients treated with L-DOPA progressively lose their pharmacological response starting to display side effects such as wearing on off and dyskinesias, whose resolution request add-on therapies introduction. This includes drugs enhancing synaptic DA levels by reducing its catabolism, such monoamine oxidase-B (MAO-B) inhibitors (selegiline) and catechol O-methyl transferase (COMT) inhibitors (tolcapone,

entecapone, opicapone) to be used in association with L-DOPA (Simola et al.,2010) (Fig.3). Inhibition of COMT in the periphery will further enhance bioavailability and the half-life of L‑ DOPA, which is of particular benefit in patients who have developed motor fluctuations of the wearing-off type (Muller, 2015). Oxidation via monoamine oxidase type B (MAO-B) in glial cells is a major clearance mechanism for synaptically released dopamine, next to presynaptic reuptake via the dopamine transporter, inhibition of MAOB prolongs and increases synaptic dopamine concentrations (Schapira,2011).

The actions of dopamine on striatal medium spiny neurons are mediated via two classes of dopamine receptors. Dopaminomimetics with direct activity to dopamine receptors (dopamine receptor agonist) mainly target the D2 receptor family and were first introduced into Parkinson disease therapy in the 1970s with the ergot alkaloid bromocriptine and have since become an important medical therapy for motor symptoms (Fox et al., 2011). An important advantage of dopamine agonists is their longer half-life than L DOPA, which makes them attractive candidates as adjunct therapies in patients with motor fluctuations. Drawbacks include their potential to induce drowsiness and impulse dyscontrol the latter being possibly associated with their preferential activity at D3 receptors that are located in the ventral striatum, which causes excessive stimulation of the brain reward systems (Connolly, 2014). However, in parallel with disease progression DA agonists lose their efficacy and must be replaced by the canonical L-DOPA therapy.

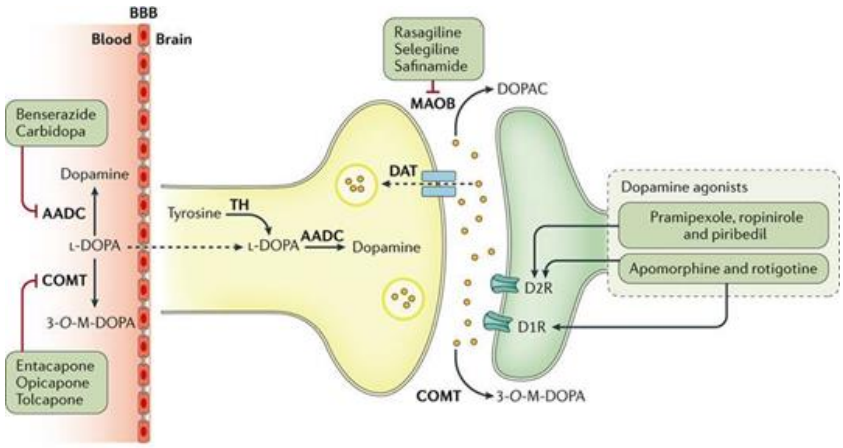


Fig.3 Dopaminergic drug targets in Parkinson disease (Poewe, 2017).

Despite the remarkable effect of dopaminergic therapy on the symptoms of Parkinson disease, there is a clear need for therapies that target other pharmacological systems. (Jankovic, 2012).

The adverse effects of the pharmacological therapies for PD have prompted an outstanding interest in the surgical treatment of PD. These surgical approaches have expanded from early lesion procedures to targeted electrical stimulation of specific areas in the basal ganglia circuitry able to rebalance the activity of the basal ganglia. Thalamic Deep brain stimulation (DBS) is based on the finding that high-frequency (100–200 Hz) electrical stimulation of specific brain targets can mimic the effect of a lesion without the need for destroying brain tissue and involves the implantation of an electrode in brain tissue (Bronstein et al., 2011). In general, ideal candidates have idiopathic PD with an excellent L-DOPA response but motor complications due to long-term medical treatment (Bronstein,2011). An important field of study in PD therapeutic strategies include gene therapy to deliver target genes in specific brain regions using viral vector-based technologies, thus limiting off-target effects. Gene therapy can be used to treat diseases by the introduction of therapeutic genes or by replacing, silencing, or correcting faulty genes. Many different approaches exist, but the primary strategy is the use of engineered non-replicating viral vectors; predominantly various serotypes of recombinant adeno-associated virus (AAV) or lentivirus. The disadvantages of these approaches are related to the fact that gene delivery is irreversible and the amount of gene inserted in target cells cannot be controlled or could be inserted in wrong sites causing in turn tumors. So far, several possible targets for genetic treatment of PD have been identified. These targets can be classified as either disease modifying or non-disease modifying. The non-disease modifying treatments are aimed at ailing parkinsonism symptoms by attempting to normalize aberrant firing in the basal ganglia by expression of either dopaminergic or GABAergic enzymes. These treatments are symptomatic and do not alter the underlying pathophysiological process. Disease modifying strategies revolve around stopping PD-mediated cell death and/or regenerating lost neurons. The most investigated approach has been nigral overexpression of growth factors found to have neuroprotective properties (Axelsen, 2018). Optogenetics is a rapidly emerging technology using targeted expression of light-sensitive ion channels (opsins) or G-protein coupled receptors which enables precise control of neural activity of transfected specific cell populations (Diester, 2011). Chemogenetics relies on G-protein coupled receptors (GPCR) are found in abundance all over the body and contributes to a great number of physiological processes. Researchers have succeeded in engineering GPCRs only responsive to otherwise inert substances (Jorgensen, 2017).

Gene therapy with optogenetics and chemogenetics could prove a viable alternative option for treating symptoms of PD as they provide a more specific intervention as compared to DBS or ablation. Genome editing with the CRISPR-CAS9 technology might be another future form of

personalized gene therapy for known mutations leading to PD (Singh et al., 2017). It remains to be seen what will be the time window in the lifespan of individual patients for editing disease genes in this manner (Yang et al., 2016).

However, despite recent advances in the symptomatic treatment of PD, currently there are no interventions that stop the progression of this disease. Together, these facts highlight the great need to find new potential drug targets and strategies for the treatment of PD.

1.2 Experimental models of PD

One of the major reasons for the lack of understanding of the underlying pathophysiology of PD is the absence of reliable experimental models that recapitulate all features of the human disease (Gunaseeli et al.,2010). Since brain biopsies are impossible, the analysis of this pathology is based on post mortem studies. However, post-mortem studies do not provide information on the causes and stages of the disease since they represent only the final phase of the disease and may have suffered alterations following the various pharmacological treatments. An ideal disease model recapitulating the respective pathophysiology and clinical manifestations accurately, must be highly reproducible should be easy to maintain and easily available in adequate number. The current models reproduce the two most salient changes found in the brains of patients with PD:

the degeneration of DA neurons and the existence of protein aggregates consisting mainly of α-synuclein. Choosing a specific cellular model implies the need to focus on one aspect of the disease while overlook others. Animal models, particularly genetic ones, have often been used to reproduce the effects of mutations observed in patients. These models allow a more complete and integrated vision of the pathology but at the same time are affected by the discrepancies related to the diversity of species and often do not share features found in human pathology. Current animal models lack many essential human characteristics and conventional in vitro models, in turn, are limited in their capacity to provide information regarding many functional and systemic responses (Nikolakopoulou et al., 2020). Recently human induced pluripotent stem cells (iPSC) became an attractive alternative for the study of human diseases, particularly those with genetic causes. Given their inherent self-renewing properties and the potential to differentiate into any phenotypic cell, iPSC are considered a promising model for drug discovery. More notably, the iPSC cells from the patients serve as a true in vitro model of that particular disease and reflect the same pathological features as in in vivo

conditions. Furthermore, because iPSC can be derived from the relevant patients themselves, they could enable personalized disease modeling that would be a central part of precision medicine (Gunaseeli et al.,2010).

1.2.1 Animal models

1.2.1.1 Neurotoxin-Induced PD Models

The investigation of pathogenic and pathophysiological mechanisms of Parkinson's disease relies on experimental models reproducing, in the animal, the pathological and behavioral features of the disease (Noyce et al., 2012). Animal models play a key role in devising novel pharmacological approaches to therapy, in developing new treatment strategies and in understanding the nature of the pathogenic processes involved in neuronal loss.

An ideal animal model of PD should display a progressive degeneration of nigrostriatal DA neurons and show the presence of LB-like protein inclusion as well as other typical disease-related alterations such as oxidative stress and mitochondrial dysfunctions

Common toxin-induced animal models include stereotaxic injections into the SNpc or, preferably, into the medial forebrain bundle that conveys the efferent fibres from nigral cell bodies to the striatum (Blandini, 2012) of 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) and α-syn preformed fibrils (PFFs) (Thakur et al., 2017). The 6-hydroxydopamine (6-OHDA) remains the most widely used tool to induce a nigrostriatal lesion in the rat. 6-OHDA is a synthetic neurotoxic compound selectively destroying DA and noradrenergic neurons in nigrostriatal pathway. As an analog of dopamine, 6-OHDA can be taken up into DA neurons by dopamine transporters (DATs) (Carvey et al.,2005). MPTP primarily causes damage to the nigrostriatal DA pathway with a profound loss of DA in the striatum and SNc (Dauer, 2003).

PFFs derived from the aggregation of recombinant α-syn monomers can initiate the formation of α-syn pathogenic inclusions in neurons from mice. In both mice and rat PFF models, introduction of α-syn inclusions in the striatum caused neuronal dysfunction, mitochondrial damage, and eventually retrograde degeneration of nigrostriatal DA neurons (Duffy et al.,2018).

1.2.1.2 Transgenic PD Models

Increasing evidence suggests that genetics plays a major role in the etiology of PD, as some genes are linked to the development of rare forms of PD. Although the majority of cases are sporadic, the recent discovery that mutations or multiplications in a number of gene loci cause familial forms of PD

has prompted the development of new genetic-based animal models to investigate the biological basis of PD (Dawson et al., 2010). The most diffused mutations causing familial PD are located on the leucine-rich repeat kinase 2 (LRRK2) locus. Nonetheless mutations and multiplications of α-syn gene (SNCA) as well as Parkin, DJ-1 phosphatase and tensin homolog—PTEN-induced novel kinase 1 (PINK1) mutations have been correlated to early onset PD likewise many others susceptibility genes. (Bardien et al.,2011). LRRK2 mice models display mild or not functional disruption of the nigrostriatal DA neurons of the SNc and no LB-like pathology. PINK1 KO mice have an age-dependent, moderate reduction in striatal DA levels accompanied by low locomotor activity, (Gispert et al.,2009). KO models of DJ-1 mice with a targeted deletion of exon 2 or insertion of a premature stop codon in exon 1 show decreased locomotor activity, a reduction in the release of evoked DA in the striatum but no loss of SNc DA neurons and no change of the DA levels (Goldberg et al.,2005).

Three missense mutations of α-syn, encoding the substitutions A30P, A53T and E46K have been identified in familial PD (Vekrellisetal et al.,2011; Schapira et al.,2014). Furthermore, the duplication or triplication of α-syn is sufficient to cause PD,suggesting that the level of α-syn expression is a critical determinant of PD progression. Transgenic mice expressing the A53T or A30P mutation show abnormal motor behavior and granular or filamentous protein aggregates, but fails to induce neuron de generation (Tofaris et al., 2006).

Despite all the benefits biomedical research on animals can bring, several studies show negative or inconsistent results. Animal models occasionally fail to reproduce the complexity of human behavioral disorders and fail to recreate various aspects of human pathology (Kalueff et al. 2007). Moreover, not all the results obtained on animals can be translated directly to humans.

Various reasons can be evoked. First, despite the great similarities, there are differences between a given animal species and humans. The availability of an experimental model mimicking all the major pathological and phenotypic features of PD is a crucial need that remains to be addressed (Bezard et al., 2013). Such a tool would be instrumental for a full understanding of PD pathogenesis, which would lead to the identification of disease-modifying therapies. Various toxic and transgenic models of PD are currently available, all with significant advantages and disadvantages. If we consider toxic models, substantial nigrostriatal degeneration is generally obtained, with good replication of PD motor symptoms although no consistent LB-like formation is detected. On the other hand, transgenic models offer insights into selected molecular aspects of PD pathogenesis, particularly for the familial forms. However, the absence of consistent neuronal damage in the nigrostriatal pathway remains a major limitation for these models (Blandini, 2012). Thus, until a ‘perfect’ model is developed, any

suitable research strategy will rely on the accurate selection of the model to be used based on the specific research needs.

1.2.2 In vitro models

The neuronal dysfunctions, at the cellular and molecular level, that occur in the early non-symptomatic phases of the disease and which precede neuronal death are not yet clear. This is a crucial issue as a limited understanding of pathogenic mechanisms drastically precludes the development of drugs that counteract the progression of the disease. One of the main limitations to our understanding of the early events that occur in DA neurons during the development of PD is the lack of adequate experimental models that recapitulate the key pathological features of the disease. The mechanisms involved in the progression of PD are still unknown.

Cellular models have several advantages (Lopes et al., 2017): are highly proliferative; are high reproducibility, can be easily genetically manipulated, some of them, present all the dopaminergic machinery, for example enzymes for dopamine metabolism and synaptogenesis (Lopes et al., 2017); many of them have human origins and therefore human genetic background.

However, the use of these models is limited by relevant disadvantages: they often require further differentiation steps to have the morphological and/or physiological characteristics of DA neurons; they have oncogenic origin; therefore, their main feature is the high proliferation which is in clear contrast with neuronal feature (Herrup and Yang, 2007; Lopes et al., 2017).

The introduction of human induced pluripotent stem cells (iPSC) technology is an excellent alternative to study disease mechanisms and can be used to develop patient-specific cell lines (Fig.4). Therefore, iPSC allow to overcome some of the obstacles, given either by the use of animal models, ethical issues and the limited availability of post-mortem human brain tissue. More in detail, iPSC give the opportunity to have disease-relevant neuronal subtypes that retain the specific genetic background of the patient (Ferrari et al., 2020). Human induced pluripotent stem cells (iPSC) are generated from somatic cells by reprogramming. The somatic cells reprogramming technology was pioneered by Shinya Yamanaka in 2006 and showed that introduction of four transcription factors (OCT4, SOX2, KLF4 and c-MYC) could convert somatic cells into PSCs (Takahashi, 2006) and then differentiate in any cellular phenotype. iPSC represent a means to derive physiologically relevant cell types from patients and healthy controls, offering promise for the testing of individualized medicine approaches and could provide a promising model for fundamental research and drug screening (Fig.4). Somatic cells from PD patients with SNCA, LRRK2, PINK1, GBA and Parkin mutations have

been successfully induced to iPSC and differentiated in DA neurons and disease-related pathologic phenotypes have been identified in these cells.

The use of genetic PD iPSC offer the promise of addressing the contribution of individual genetic factors and functional relevance of underlying molecular pathways in the development of PD (Kang et al.,2016). Furthermore, patient iPSC-derived neuronal cells offer a direct insight into the early-stage and progressive pathologic alternations in disease, further recapitulating the molecular pathogenesis of disease (Ke et al., 2019). Moreover, the quality of iPSC-based studies depends on the availability of appropriate controls any phenotypes observed in a patient's iPSC-derived cells should only be interpreted via comparison with control cells. Use of gene-targeting strategies based on CRISPR/Cas9 can be an excellent tool for modeling some monogenic diseases or to study the contribution of single or several gene variants to a certain pathology. In this sense, iPSC derived from PD patients carrying a certain mutation can be used not only to model the disease but through the correction of this specific mutation and generation of isogenic cell lines to be used as a control (Musunuru, 2013). This 'isogenic' approach offers the unique possibility to analyze the effects of a single mutation within the same genetic background (Weykopf et al., 2019). The discovery and development of iPSC technology, especially when combined with gene-editing strategies, represents a novel and more reliable platform helpful for neurodegenerative disease modeling, a system that can be employed as well for performing screenings to identify new therapies able to counteract course of these pathologies (Garcia-Leon et al.,2019).

In our laboratory by using a modified version of dual-SMAD protocol developed by Kriks et al (2011), in human iPSC derived from healthy control, we have successfully reproduced the process of midbrain neurogenesis, efficiently generating a neuronal population enriched in TH/DAT- positive DA neurons (~40% of cells) and expressing the correct set of genes and proteins typical of DA neurons. There are presents other neuronal populations such as GABAergic (~ 23%) and glutamatergic (~ 28%) neurons. (Bono et al., 2018). Moreover, our DA neurons release DA in a regulated way under basal conditions and after stimulation with either a non-specific synaptic vesicle releaser such as potassium (K^+) or nicotine. Thus, based on both mRNA expression and the functional interaction between nAChR and D2-like receptor in controlling DA release, it is indicated that DA neurons generated from human iPSC reach the full maturation. Increasing evidence suggests that DA and D2-like receptors are required for neural differentiation during both development and neurogenesis in adult brain (Kim et al., 2006; Yoon et al., 2011; Belinsky et al.,2013). The possibility to monitor DA neurons development during their in vitro differentiation from iPSC represents a powerful tool for investigating the role of

D2-like receptors in these processes, the mRNAs encoding for D2R, D3R, and D4R were detected in the undifferentiated iPSC, suggesting their susceptibility to DA already in the pluripotent state (Bono et al., 2018). This demonstrates that iPSC technology represents a new strategy for the modeling of neurodegenerative diseases, and allows investigating the impact of mutations on the homeostasis of DA neurons and could offer new opportunities for the identification of new drug targets.

We have recently developed a protocol for the differentiation of human astrocyte starting from iPSC, based on Yan et al., (2013) protocol, which allows to obtain mature astrocytes. from iPSC after 40 days. IPSC cells derived from a healthy subject were cultured in specific medium until the fully differentiated astrocytic phenotype was achieved (Filippini et al.,2020). In particular, by immunofluorescence techniques we then identified the mature astrocytes, positive for the GFAP protein (a typical marker for mature astrocytes), corresponding about 40-45% of all the cells present in culture. In this thesis we will use the innovative human iPSC-technology model for mimicking PD phenotype in vitro that, at present, likely represents the experimental model for recognizing pre-degenerative neuronal dysfunctions. In particular, iPSC derived from two PD patients carrying LRRK2 mutation, their respective isogenic controls in which the mutation has been corrected by gene editing techniques (Reinhardt et al., 2013) and healthy control were differentiated in DA neurons and astrocytes to investigate their possible involvement in PD.

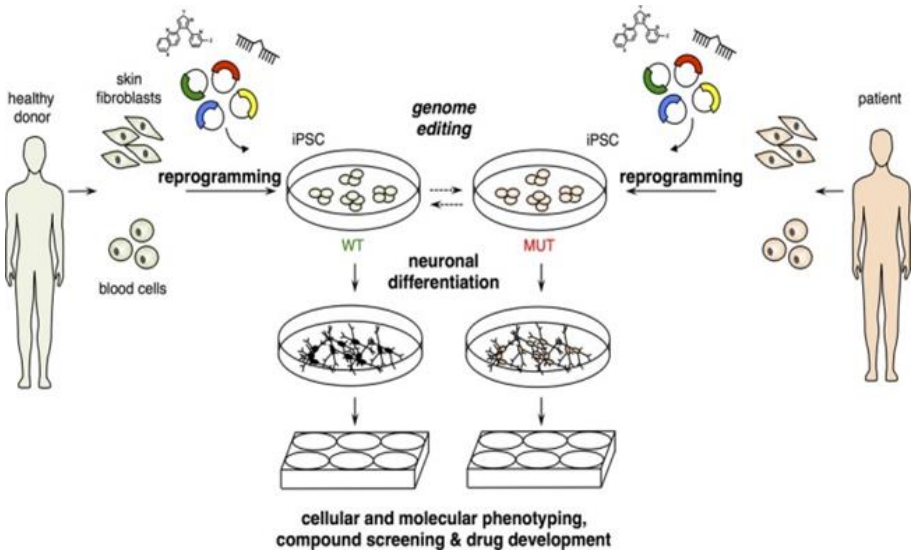


Fig.4: Schematic representation of the potential applications of induced pluripotent stem cells (Weykopf ,2019).

1.3 Genetic bases of PD

There is an increasing list of mutations related to the onset ofPD. They account for more than 50% of early-onset cases and about 2–3% of late-onset forms of PD. Findings of gene mutations linked to the onset of familial PD cases have confirmed their critical roles in the progress of PD.

The mutations of main seven gene associated to PD were α-synuclein, LRRK2, PINK1, Parkin, DJ-1, VPS35 and GBA1 (Fig.5).

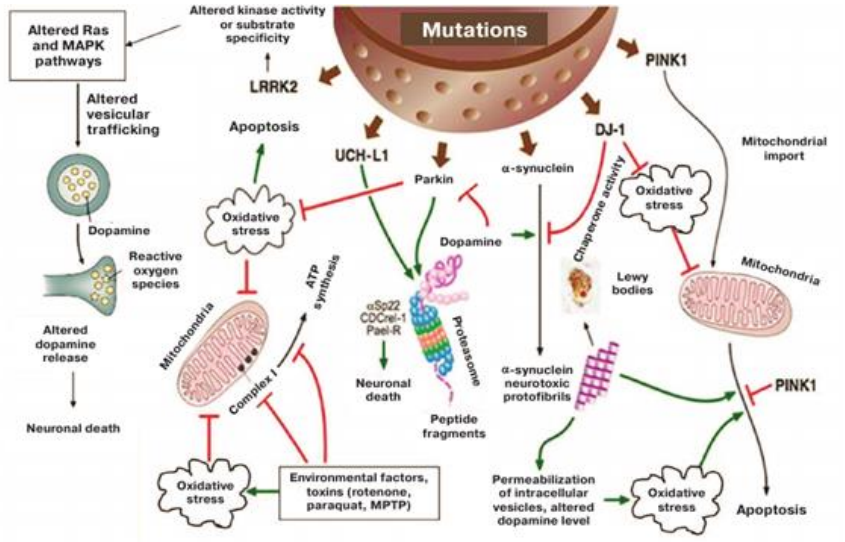


Fig. 5: Pathological changes in cells caused by mutant PD-related genes (Lebedeva 2018).

1.3.1 LRRK2

Leucine-rich repeat kinase 2 (LRRK2/PARK8 gene) is a 2527-amino-acid-long protein with a catalytic core region composed of the ROCO protein family signature ROC-COR bidomain followed by a kinase domain (Fig.6). The ROC domain is named for Ras of complex proteins as it has some homology to small GTPases including Ras. The COR domain is characteristic of the ROCO protein family and is so named because it is C-terminal of ROC. The kinase domain is generally similar to other Ser/Thrtype protein kinases, although it is only close to the LRRK2 homolog. The catalytic core is flanked N-terminally bya leucine-rich repeat domain (LRR) and C-terminally by a WD40 repeat domain which is deemed essential for protein folding, thus controlling LRRK2 function and kinase activity. In its N-terminal region, LRRK2 displays a large number of unusual repeat sequences (Marin,2006). Mutations of LRRK2 represent the highest risk of familial PD, causing autosomal dominant PD. There are several

missense mutations concentrated in the catalytic center of the protein, including R1441C/G/H in the ROC domain, Y1699C in the COR region and G2019S and I2020T in the kinase domain (Greggio, 2009).The dominant, pathogenic mutations described up to date, occur within the enzymatic core of LRRK2 suggesting that modification of LRRK2 activity greatly affects PD onset and progression.

The similarity in PD phenotype and age of onset between homozygous and heterozygous mutation carriers suggests that pathogenic mutations might act by conferring a toxic function on LRRK2 (Tsika, 2012).

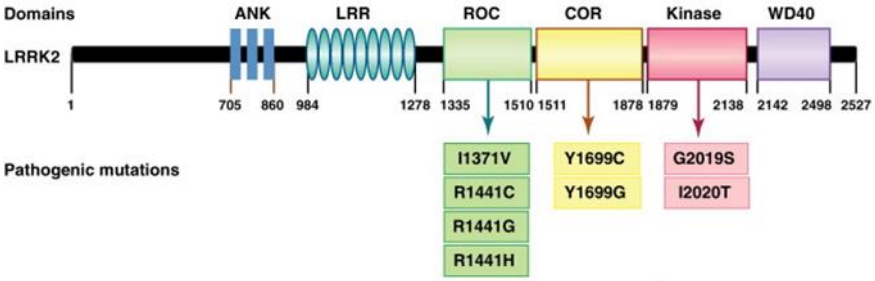


Fig. 6: LRRK2 domains (Dae, 2012)

LRRK2 predominantly exists as a dimer under native conditions, a state that appears to be stabilized by multiple domain-domain interactions. It's possible to suppose to a model of a large protein with a central catalytic GTPase/kinase region surrounded by protein–protein and perhaps protein–membrane interaction motifs, forming homo and possibly heterodimers (Greggio et al.,2008).

The precise physiological role of this protein is unknown but presence of multiple functional domains suggesting involvement in wide variety of functions but the presence of both a ROC/GTPase and a kinase domain suggests that it may play a role in intracellular signaling (Wallings et al., 2015). The presence of two enzymatic domains suggests the potential for an intrinsic regulatory mechanism of LRRK2 activity with parallels to the classic Ras/Raf signal cascade. While GTPase activity does not appear to critically require a functional kinase domain (Smith et al.,2006; Ito et al.,2007), autophosphorylation within the GTPase domain may serve a regulatory function (Kamikawaji et al.,2009; Webber et al., 2011), which implies a complex yet poorly understood bidirectional relationship between the GTPase and kinase domains of LRRK2.

Within cells, LRRK2 associates with various intracellular membranes and vesicular structures including endosomes, lysosomes, multivesicular bodies, the mitochondrial outer membrane, lipid rafts, microtubule-associated vesicles, the Golgi complex, and the endoplasmic reticulum (Cookson, 2010). This distribution could reflect a functional role in multiple pathways (Fig.7).

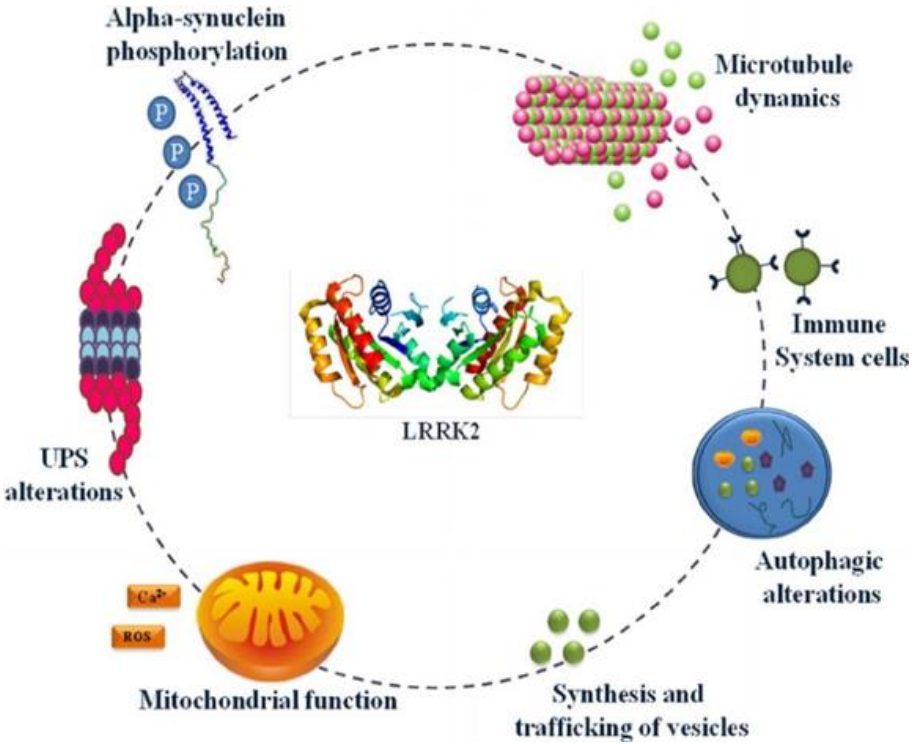


Fig. 7: LRRK2 implication in cellular mechanisms

1.3.2 G2019S LRRK2 mutation

The G2019S and the nearby I2020T mutation are located at the N-terminal portion of the activation loop in kinase domain. The G2019S mutation substitutes a serine for a highly conserved glycine located in subdomain VII of the kinase domain (West et al.,2007) the mutation is situated within this segment of the activation loop and it has been speculated that the glycine residue here imparts conformational flexibility (Shan et al.,2009); therefore, replacement of the glycine with a serine residue may alter LRRK2 dynamics. The penetrance of the G2019S-LRRK2 mutation appears to have a clear age-dependent effect and varies from around 50% at age 50, to ~ 74% at age 79 (Healy et al.,2008); although some patients do not manifest any clinical features even in their 80 (Paisan-Ruiz

et al.,2013). This mutation increases kinase activity by increasing the catalytic rate of the enzyme; it does not enhance substrate affinity (Thomas, 2007). The G2019S mutation facilitates substrate access, thereby leading to a toxic increase in kinase activity. G2019S is the only mutation that consistently shows increased kinase activity (Greggio, 2009) changes in LRRK2 kinase activity appear to be toxic, and seem to induce degeneration of dopamine neurons.

Cellular toxicity, in both the absence and presence of oxidative stress, and the formation of inclusion bodies were observed when overexpressing G2019S-LRRK2 in cell lines and primary neuronal cultures (Heo et al., 2010). These results, and the fact that genetic inactivation of LRRK2 kinase activity showed a protective effect against such a toxic phenotype, suggest that an alteration in LRRK2 kinase activity is potentially involved in the neurotoxic and pathogenic mechanisms of LRRK2-PD. LRRK2 not only inhibits neuronal survival but also impairs dopamine signal. Accumulating evidence suggests that synaptic dysfunction might be an early event in neurodegeneration. LRRK2 and Rab5b, a regulator of endocytic vesicle trafficking (de Hoop et al., 1994; Fischer von Mollard et al., 1994), colocalize and interact on synaptic vesicles (SVs) providing evidence for a role of LRRK2 in SV trafficking (Shin et al.,2008) (Fig.7). In neurons, the vesicle trafficking controls fundamental physiological functions such as neurotransmitter or protein release and uptake, localization of membrane receptors, changes in plasma membrane composition and, not least, organelle biogenesis. In synaptic terminals, LRRK2 silencing leads to a redistribution of vesicles, alters recycling dynamics and increase vesicle trafficking (Piccoli et al.,2011), suggesting that LRRK2 is implicated in the regulation of receptor trafficking.

LRRK2 G2019S mutation impairs dopamine receptor D1 internalization, leading to an alteration in signal transduction (Rassu et al.,2017). In addition, LRRK2 G2019S mutation enhances the kinase activity and results in the impairment of synaptic vesicle trafficking selectively in ventral midbrain neurons, including dopaminergic neurons (Pan et al., 2017). Furthermore, a significant accumulation of D2R was observed in the Golgi apparatus associated with a significant increase in the amount of total protein, suggesting that the LRRK2-G2019S mutation may be involved in altering the localization and / or degradation pathways of D2R (Rassu et al., 2017).

In regard to dopamine signaling, LRRK2 mutations have been shown to affect activity dependent DA neurotransmission and catecholamine release (Tong et al., 2009). This manifests as reduced levels of extracellular striatal dopamine and reduced levels of dopamine metabolites. Cultured dopamine neurons from LRRK2 transgenic mice display markedly reduced neurite complexity (Ramonet et al., 2011) and reduced striatal DAT levels have also been reported. This change, suggests impaired dopamine re-uptake could contribute to altered dopamine levels in LRRK2 transgenic mice.

Therefore, studying LRRK2 pathobiology may also provide clues into the pathogenic mechanisms underlying the sporadic disease. Even if it is widely accepted that LRRK2 mutations are crucial for PD pathogenesis, their precise role in the etiology of PD needs to be further investigated. In particular, the biochemical and molecular neuronal dysfunctions occurring in the pre- symptomatic early stages of the disease that precede neuronal death need to be investigated. This is a crucial issue since a limited understanding of pathogenic mechanisms drastically precludes the development of drugs counteracting the progression of the disease.

1.4 DA neurons vulnerability

The cardinal motor symptoms of PD are caused by the death of DA neurons which are mainly situated in the midbrain. Dopamine is critical for the control of the brain functions, such as motor activity, attention, reward mechanisms and cognition. The effects of DA are mediated by five different receptors, that belong to the G protein-coupled receptor (GPCR) family and are divided into D1-like (D1 and D5) and D2-like (D2, D3, D4) subtypes. This classification is generally based on the original biochemical observations showing that dopamine is able to modulate adenylyl cyclase (AC) activity as a result their activation decreases the cAMP accumulation modulating the activity of PKA and its effectors (Forn et al., 1974; Greengard et al.,1999). Interestingly, increasing evidence suggests that DA receptors can diversify and amplify their repertoire of signaling by forming homo- and hetero-dimers, a property typically shared by the GPCR family that greatly increases their heterogeneity (Carli et al., 2018).

The prerequisite for the study of pre-degenerative defects of PD is to know the physiological properties of human DA neurons, among them, the dopamine D2 and D3 receptors (D2R / D3R) (Collo et al.,2008; Bono et al.,2018), the nicotinic acetylcholine receptors (Collo et al., 2013; Bontempi et al., 2017) have been reported to provide trophic support to DA neurons in mouse cell models.

1.4.1 Dopamine D2 and D3 Receptors (D2R)/(D3R)

The highest levels of D2R are found in the striatum, the nucleus accumbens, and the olfactory tubercle. D2R are also expressed at significant levels in the substantia nigra, ventral segmenta area, hypothalamus, cortical areas, septum, amygdala, and hippocampus (Missale et al., 1998; Gerfen, 2000).

In the substantia nigra, D2R are localized both on dopaminergic neurons, but also on neurons targeted by dopaminergic afferences. In addition to having a dual localization, D2R are a heterogeneous population formed by two molecularly distinct isoforms, named D2S (S=short) and D2L (L=long) generated by alternative splicing of the same gene (Gingrich, 1993). These two alternatively spliced isoforms differ in the presence of an additional 29 amino acids in the third intracellular loop. These variants of the D2R have distinct anatomical, physiological, signaling, and pharmacological properties. D2S has been shown to be mostly expressed presynaptically and to be mostly involved in autoreceptor functions, whereas D2L seems to be predominantly a postsynaptic isoform (Usiello et al., 2000; De Mei et al., 2009).

D2 dopamine receptors seem to be the predominant type of autoreceptors that are involved in the presynaptic regulation of the firing rate, synthesis of dopamine and release of dopamine. Activation of presynaptic D2-class autoreceptors generally causes a decrease in dopamine release that results in decreased locomotor activity, whereas activation of postsynaptic receptors stimulates locomotion. (Wolf and Roth, 1990; Missale et al., 1998). Recent data indicate that D2R also expressed in microglia and astrocytes which are a key negative regulator for neuroinflammation and controlling innate immunity in the central nervous system. (Zhang an Barres 2010; Shao et al.,2013).

The D3R is expressed on DA neurons, both at the somatodendritic level and at synaptic terminals, in the substantia nigra (SN) and ventral tegmental area (VTA), as well as in the ventral striatum in the limbic areas and in the islands of Calleja and cerebellum (Missale et al., 1998). The D3R seem to exert a moderate inhibitory action on locomotion through the involvement of postsynaptic receptor populations (Joseph et al., 2002; Sokoloff et al., 2006).

At the molecular level, D2R/D3R are coupled to the G alpha$_{i/o}$ family of G proteins leading to inhibition of adenylyl cyclase (AC) (Missale et al., 1998). There is now increasing evidence that D2R/D3R are also critical to supporting DA neuron homeostasis (Fiorentini et al.,2015) and are crucially involved in DA neurons development, morphological plasticity and protection, suggesting that an abnormal D2R/D3R function could increase DA neurons vulnerability (Bono el al.,2018). It was recently reported that the D2-like receptor agonists quinpirole and 7-OH-DPAT exert neurotrophic effects on DA neurons in primary mouse mesencephalic cultures, as shown by increased maximal dendrite length, number of primary dendrites and soma area (Collo et al., 2008). These effects required the activation of the PI3K-Erk1/2 and PI-3K-Akt-mTOR pathways (Collo et al.,2012, 2013).

Stimulation of D3R promotes neurotrophic effects and plays a crucial role in triggering key intracellular events with neuroprotective potential (Bono et al., 2018, 2019) and also the activation of

D2R / D3R could promotes the proliferation of neuronal progenitor cells (Kim et al., 2006; Bono et al., 2018). Taken together, these data suggest that the D3R may be crucially involved in the control of DA neurotrophism during development (Diaz et al.,1997; Bono et al., 2018), a property also shared with the D2R (Kim et al., 2006; Yoon et al.,2011), and in counteracting early pathological events that may subsequently result in neurodegeneration.

Interestingly, in animal models of transgenic mice carrying the LRRK2 mutation, impairment in the expression and function of D2R leading to neuronal synaptic dysfunction has been observed (Tong et al., 2009) and also recent data indicate that D2R also expressed in microglia and astrocytes which are a key negative regulator for neuroinflammation and controlling innate immunity in the central nervous system. (Zhang et al., 2010; Shao et al.,2013).

Therefore, it might be useful to investigate the impact of the LRRK2 G2019S mutation on D2R /D3R activity and homeostasis of DA neurons. D2R / D3R expression or defective function may represent an early and pre-degenerative event in patients carrying the LRRK2 mutation with various consequences that likely contribute to making DA neurons more vulnerable.

1.4.2 Nicotinic Acetylcholine Receptors (nAChRs)

The activity of DA neurons is regulated by an integrated interplay between different proteins and receptor systems including the nAChR. nAChRs are a heterogeneous family of ligand-gated ion channels, composed by various α(α2-α7) and β(β2-β4) subunits, which are activated by nicotine (Quick et al., 2011). nAChR, are characterized by an especially high permeability to Na+ and K+, resulting in cell excitation. This excitation may activate voltage gated calcium channels allowing calcium influx into the cell. One of the primary functions of nAChR is to modulate synaptic transmission and synaptic plasticity triggered by other neurotransmitters, resulting in alterations in affective behavior, attention and cognition (Benowitz, 2009).

It is well known that stimulation of nAChR increases DA neuron firing and DA release (De Kloet et al.,2015). Nicotine promotes the morphological remodeling of DA neurons and regulates various genes controlling neuronal morphogenesis (Doura et al., 2010; Collo et al., 2013). Different nAChR subtypes are co-localized with D2-like receptors in DA nerve terminals (Exley and Cragg 2008; Zoli et al.,2015). Epidemiological studies have identified a negative correlation between smoking and the development of neurodegenerative disorders such as Parkinson's disease. These findings have been attributed to the ability of nicotine to act as a neuroprotective agent (Picciotto, 2008).

In the study by Mappin-Kasirer et al., (2020) that involved 30,000 British male doctors, a protective effect of tobacco on PD risk was observed.

There is evidence that nAChR located on DA neurons also provide neurotrophic and neuroprotective support to DA neurons, an effect requiring functional D3R and suggesting the existence of a positive crosstalk between these receptor systems (Bontempi et al., 2017; Bono et al., 2018, 2019).

1.4.3 D3R-nAChR Heteromerization

GPCR have been classically thought to exist as monomeric entities. However, the current view of GPCR organization assumes, in fact, that these receptors may form heteromers by direct interaction with members of the same receptor family and with structurally and functionally divergent families of receptors (Angers et al., 2002; Ferre' et al., 2009). Sometimes heterodimerization is an absolute requirement for the formation of functional receptors may give rise to novel receptors units with unique pharmacological, signaling and trafficking properties (Borroto-Escuela et al., 2014).

Heteromerization often affects the ligand binding properties of interacting receptors and alters the potency of agonists in generating intracellular signals. In addition, heteromerization may generate new binding sites, an observation that may have a significant impact on drug discovery, providing the framework to develop drugs specifically targeting a given heterodimeric pair (Milligan, 2008). By influencing G- protein specificity, heteromerization may be responsible for alterations in the signaling pathways activated by a given receptor. Interacting receptors may also exhibit a specific G-protein coupling and activate peculiar transduction pathways (Gaitonde and González-Maeso, 2017). Heteromerization may also represent a novel mechanism modulating agonist-mediated trafficking, by either decreasing or enhancing receptor desensitization and internalization. Different intermolecular interactions may contribute to receptor dimerization. Either transmembrane and intracellular domains are likely to play a central role in receptor- receptor interactions by the formation of hydrophobic or non-covalent electrostatic bonds respectively (Guo et al., 2003; Lopez-Gimenez et al., 2007; Mancia et al., 2008). DA receptor heteromers have been the most studied complexes so far. It's known that the D3R may form heterodimers with other DA receptor subtypes, such as the D1R and the D2R (Scarselli et al., 2001; Fiorentini et al., 2008).

As previously mentioned, the D3R and nAChRs participate to the control of DA neuron firing and plasticity. In particular, the D3R seems to reduce DA release and regulates the mechanisms of structural plasticity; nAChRs activated by acetylcholine or nicotine facilitate the switch from tonic to burst firing mode, increase the release of DA and modulate neuronal plasticity (Picciotto et al., 2008).

These observations thus underlie the important role of the nicotinic system in the control of DA transmission in both physiological and pathological conditions. Since nAChR and both D2R and D3R are extensively co-localized in both DA neuron cell bodies and nerve terminals (Zoli et al., 2002), the possibility that functional interactions may occur between DA receptors and nAChR has been investigated and clearly demonstrated (Grilli et al., 2009; Bontempi et al., 2017). It is likely that D2R, D3R and nAChR might be associated into different heteromeric complexes that may exert strong control over DA release and DA neuron viability (Bontempi et al., 2017) (Fig.8). Nicotine, in fact, besides controlling DA release, supports DA neuron regeneration and modulates the expression of different genes regulating neuronal morphogenesis (Quik et al., 2006; Doura et al., 2010; Bono et al., 2019).

Has been reported that nicotine provides neurotrophic support to DA neurons by increasing their dendritic arborization and soma size (Bontempi et al.,2017; Bono et al., 2018). This effect is mediated by the α4β2 nAChR subtype and depends on functional D3R, since it was blocked by D3R preferential antagonists and was absent in DA neurons from D3R-KO mice (Collo et al., 2013) suggesting the existence of a positive crosstalk between D3R and nAChR in promoting DA neurotrophism.

Along this line, by using Bioluminescence Resonance Energy Transfer (BRET), we have shown that the D3R directly interacts with the β2 subunit of nAChR to form a heteromeric complex. Interestingly, disruption of D3R-β2 interaction by a cell-permeable interfering peptide abolished the effects of nicotine on DA neuron morphology (Bontempi et al., 2017), suggesting that the D3R-nAChR heteromer represents the molecular unit triggering nicotine-mediated neurotrophic effects.

The Ras-ERK and PI3K-mTORC1 pathways represent key mechanisms for cells to regulate cell survival, proliferation, and motility. In addition to their independent signaling programs that provide compensatory mechanisms, the pathways extensively cross-talk to positively and negatively regulate each other (Mendoza et al.,2011). The PI3K/ERK1/2 signaling pathway has been shown to sustain D2R/D3R-mediated morphological changes in primary rodent neuronal cultures (Alonso et al., 2004; Kumar et al., 2005; Collo et al., 2008). In mouse DA neurons, the engagement of the PI3K-ERK1/2 signaling, associated with the D3R, is required for nicotine-induced structural plasticity (Collo et al.,2008) suggesting that the phosphorylation and activation of ERK1/2 may be the intracellular event associated with the heteromer stimulation and strictly related to neurotrophic effects. The nicotine-elicited activation of MEK-ERK and PI3K-Akt- mediates structural plasticity, because the MEK inhibitor PD98059 or the PI3K inhibitor LY294002, blocked nicotine-induced dendrite growth and soma size increase. These findings indicate that nicotine-induced structural plasticity at mesencephalic

dopaminergic neurons involves α4β2 nAChRs together with dopamine D3R-mediated recruitment of ERK/Akt signaling (Collo et al., 2013). Moreover, it was reported that stimulation of D3R in mouse primary midbrain DA neurons results in the phosphorylation of ERK1/2 pathway, an effect mediated by PI3K. It has also been demonstrated that in iPSC-derived DA neurons, D3R stimulation by quinpirole transiently activated the ERK1/2 pathway. In addition, a chronic treatment with quinpirole led to a significant remodeling of both dendritic arborization and soma size of TH-positive DA neurons, an effect likely dependent on the ERK cascade (Bono et al.,2018).

Moreover, by using the proximity ligation assay (PLA), we identified the D3R-nAChR heteromer in cultured DA neurons and mouse mesencephalic brain sections (Bontempi et al., 2017), as well as in iPSC-derived DA neurons (Bono et al.,2019). The D3R-nAChR heteromer is also involved in neuroprotection of DA neurons. By using glucose deprivation (GD)-induced neurotoxicity, we reported that both D3R agonists and nicotine modulate alpha-synuclein (alpha-syn) accumulation and protect DA neurons against neuronal injury (Bellucci et al., 2008; Bono et al.,2018). More recently, it was reported that nicotine inhibits alpha-syn accumulation in iPSC-derived DA neurons, an effect that was specifically blocked by D3R antagonists (Bono et al.,2018) and was lost in the presence of the specific interfering peptide (Bono et al., 2019), suggesting that, beside the induction of neurotrophic effects, the D3R-nAChR heteromer is the molecular unit involved in neuroprotection and inhibition of alpha-syn accumulation.

These observations suggest that alterations in the assembly and function of this receptor complex, may result in early dysfunctions contributing to the specific vulnerability of DA neurons. The D3R-nAChR heteromer may thus represent a novel target for drugs designed for supporting DA neurons plasticity and survival against toxic damages in various pathologies, including PD.

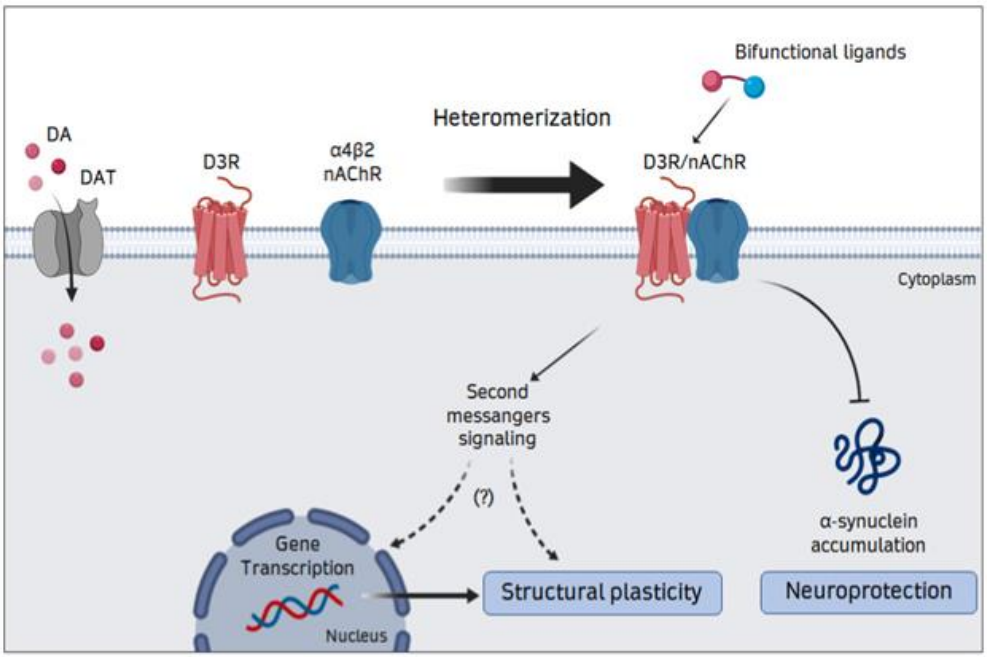


Fig. 8: Representation of the heteromerization between the D3R and the α4β2 nAChR in DA neurons (Bono et al., 2020).

1.5 Neuroinflammation

Glial cells outnumber neurons in the brain and play important roles in the neuroinflammation that accompanies brain damage in neurodegenerative diseases. In PD, dopaminergic neuronal loss is accompanied by inflammatory changes in microglia, astrocytes and innate immune cells. Acute neuroinflammatory response has beneficial effects in the central nervous system (CNS) and prompts repair of damaged tissues; however, when chronically sustained and dysregulated, inflammation can lead to significant tissue and cellular damage (Qin et al., 2007).

In general, neuroinflammation is closely associated with neuronal damage and cell death through many biological mechanisms, such as elevated oxidative stress, glial (astrocyte and microglia) cell activation, excitotoxicity, proapoptotic mechanisms, and mitochondrial dysfunction (Niranjan, 2014). Under neuroinflammatory conditions, activated glial cells release pro-inflammatory and neurotoxic factors that induce neuronal damage and neurodegeneration (Harry and Kraft 2008). Evidence implicating cytokines in nigrostriatal pathway degeneration and from post-mortem analyses indicated that the levels of several cytokines including TNF-α and interleukin 1-beta (IL-1β) are significantly elevated in the area of substantia nigra (Pieper et al., 2008). In addition to these, IL-1α and IL-6 are also involved in the pathophysiology of PD and play a very important role in dopaminergic

neurodegeneration by the modulation of either glial or neuronal cell functions (Allan,2003; Hoffman et al., 2009).

Astrocytes play critical roles for the establishment and maintenance of functional neural networks through refining synapses, coordinating neuronal firing, maintaining the blood-brain barrier, as well as structural and metabolic support and play direct, important, active, and critical roles in mediating neuronal survival and function in Parkinson's disease (Lee et al., 2019). For many years, astrocytes have not been considered therapeutic candidates for neurological drugs but recently, it was demonstrated astrocytes are regulated by immune cells, such as monocytes and T cells, and that they can release neurotoxic factors that cause neurodegeneration (Niranjan, 2018).

The neuroprotective and neurodegenerative functions of astrocytes depend largely on the molecules that they release into and uptake from the extracellular space, also sometimes described as the microenvironment that astrocytes and neurons commonly share (Rappold, 2010). Astrocytes can release and supply neurons with neurotrophic factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), and mesencephalic astrocyte-derived neurotrophic factor (MANF) and basic fibroblast growth factor (bFGF) as well as metabolic substrates for the survival and proper functioning of neurons (Hauser, 2011); Astrocytes also confer neuroprotection by siphoning away excess extracellular excitotoxic agents such as glutamate, potassium, and calcium. On the other hand, it is known that in the presence of neurodegeneration, astrocytes proliferate and express neuroinflammatory cytokines and other toxic molecules that induce dopaminergic neuronal death. Reactive astrogliosis is commonly observed in PD patients (Mirza et al. 2000) (Fig.9).

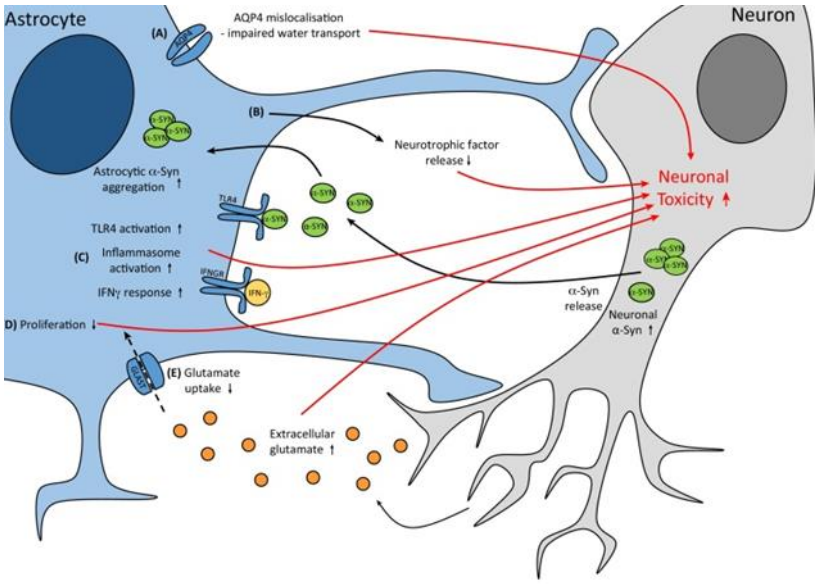


Fig.9: **Dysfunctional Astrocytes Contribute to Neuronal Toxicity.** Astrocyte dysfunction elicits neuronal toxicity via five main mechanisms. (A) Aquaporin-4 (AQP4) water channels are mislocalised away from the astrocyte end-feet, resulting in impaired water transport. (B) The neuroprotective capacity of astrocytes is reduced because of decreased neurotrophic factor release. (C) Inflammatory signaling via the TLR4, IFN-γ, and NLPR3 inflammasome pathways is increased. (D) Astrocyte proliferation is impaired, reducing the capacity of the cells to respond to an insult. (E) Glutamate uptake is reduced, potentially resulting in increased extracellular glutamate and, therefore, neuronal excitotoxicity (Mirza et al. 2000).

Recent data indicate that full sets of neurotransmitter receptor, including dopamine D2 and D3 receptors, are expressed in astrocytes (Zhang Barres; 2010; Montoya et al., 2019; Broome et al., 2020) and they seem to act as a key regulators of neuroinflammation (Shao et al., 2013). There is evidence suggesting that the expression of dopamine receptors (DRs) increase during pathological stimuli (Rangel-Barajas et al., 2015; Talhada et al., 2018; Elgueta et al., 2019). As such, it is of no surprise that the abnormal DA signaling observed in PD could be contributing not only to altered DA neurotransmission, but also to an altered immune response (Brito-Melo et al., 2012; Rangel-Barajas et al., 2015). It was suggested that high DA levels, such as those found in the nigrostriatal pathway under homeostatic conditions, would promote the stimulation of low-affinity DRs (D1R and D2R), thus inducing anti-inflammatory effects. Conversely, pathological conditions that cause depletion of DA levels, such as PD, would induce a selective stimulation of high-affinity DRs, specifically D3R, thereby triggering pro-inflammatory responses, establishing CNS inflammation and consequently, neurodegeneration (Pacheco, 2017). Recently it has been shown that the genetic deficiency of D3R, which displays the highest affinity for dopamine, attenuates neuroinflammation and subsequent neurodegeneration on a mouse model of PD induced by acute intoxication with MPTP (Chen et al., 2013; Gonzales et al.,2013).

The astrocytic D2R modulates innate immunity through αB-crystallin (CRYAB), which is known to suppress neuroinflammation and helps maintaining the immune homeostasis in the CNS. By influencing CRYAB expression, D2R may modulate inflammatory response and maintain balance of immune state. In knockout, mice lacking D2R showed remarkable inflammatory response in multiple central nervous system regions (Shao et al., 2013). More recently, evidence that D2R activation exerts an inhibitory effect on astrocyte-induced NLRP3 inflammasome signaling in primary cultures has been provided (Zhu et al., 2018). NLRP3 inflammasome is a multiprotein complex responsible for the processing of the proinflammatory cytokine interleukin 1 beta (IL-1beta,) implicated in many inflammatory diseases. Secretion of IL-1β by astrocytes contributes towards the destruction of dopaminergic neurons in the brain of PD patients and the initiation of cell pyroptotic death. Interestingly, mice lacking D2R expressions were characterized by increased neuroinflammation in normal conditions and by aggravated dopamine (DA) neuron loss in response to MPTP injections; in both cases, reactive astrocytes and NLRP3 inflammasome activation were observed (Zhu et al., 2018). Thus, it likely that in the substantia nigra a crosstalk between DA neurons and astrocytes occurs, with astrocytes potentially promoting neuroinflammation and DA inhibiting NLRP3 inflammasome activation. Therefore, astrocytic D2R may be a crucial element to control undue inflammasome activation with protective effects on PD while astrocytic D2R dysfunctions could promote abnormal responses and amplify disease progression. Targeting astrocytes thus represent an attractive alternative approach to develop new therapies to treat neurological disorder.

LRRK2 protein is required for various processes and LRRK2 mutations have been associated with abnormalities in DA receptor expression at the plasma membrane in transgenic mice (Tong et al., 2009). Therefore, in PD patients carrying the LRRK2 mutation, astrocyte D2R/D3R dysfunction may represent a relevant molecular event leading to detrimental neuroinflammation and therefore PD progression.

2 AIM OF THE PROJECT

Dopamine regulates various brain functions, including motor activity, emotional response, and positive reinforcement. Changes of DA transmission and DA receptors at both expression levels and signals transduction pathways may be associated with a variety of motor and mental disorders.

One of the characteristics of DA receptors is their propensity to directly interact with other receptor subtypes to form heteromers with peculiar transductional, functional, and pharmacological properties (Bono et al.,2020).

We recently reported that the DA D3R interacts with the nicotinic acetylcholine receptor (nAChR) to form a heteromeric unit (D3R-nAChR) that has been detected both in mouse DA neurons and in human DA neurons derived from induced pluripotent stem cells (iPSC) (Bontempi et al.,2017; Bono et al.,2019). Interestingly, this heteromeric complex exerts both neurotrophic and neuroprotective effects on DA neurons (Bontempi et al.,2017; Bono et al.,2018), thus playing a crucial role in preserving DA neuron homeostasis in physiological conditions and in counteracting neuronal alterations prodromal to neurodegeneration.

On this line, in the first part of my project, a further characterization of the properties of the D3R-nAChR heteromer has been performed, focusing on the role of the D3R within the heteromer and the signalling pathways associated with D3R-nAChR heteromer stimulation and elucidate the consequence of this interaction on DA neuron physiology.

PD is the second most common neurodegenerative disorder worldwide, with prevalence increases with age. Despite the relevance of PD, the pathological cascade triggering neurodegeneration is still far from clear and this represents the main obstacle toward a successful therapy. A genetic background is now recognized in PD and, among the genes associated with PD, LRRK2 mutations with a glycine to serine substitution (G2019S), represents the most frequent mutation in both familial and apparently sporadic PD (Cookson, 2010).

However, the mechanisms by which LRRK2 mutation affects DA neurons viability leading to neurodegeneration are still unknown. Since one of the various functions associated with LRRK2 activity is the regulation of vesicle trafficking (Sanna et al., 2012; Cirnaru et al., 2014; Rassu et al., 2017), in the second part of my project I focused my attention on the impact of abnormal LRRK2 activity on the D3R-nAChR heteromer localization and function. One of the major drawbacks in the study of PD-related mechanisms is the lack of an appropriate experimental model recapitulating all the aspects of the disease. Here we used the innovative human iPSC-technology model for mimicking

PD phenotype in vitro that, at present, likely represents the unique experimental model for recognizing pre-degenerative neuronal dysfunctions. On this line, iPSC derived from two different PD patients carrying the G2019S mutation in the LRRK2 kinase domain, as well as from their isogenic gene-corrected counterpart (Reinhardt et al., 2013) and a healthy control (Bono et al., 2018) were used to generate neuronal cultures enriched in DA neurons to study pathological mechanisms behind LRRK2-induced alterations and the impact of LRRK2 G2019S mutation on DA neurons homeostasis. A defective D3R-nAChR heteromer expression and/or function could represent an early, pre-degenerative event in patients carrying LRRK2 mutation that likely contribute to make DA neurons especially vulnerable. Several lines of evidence indicate that astrocytes might be active players in neurodegeneration associated to PD (Mirza et.,2000; Oksanen et al., 2019). Since LRRK2 is also expressed in astrocytes (Booth et al.,2017) and the expression of dopamine receptor, such as D2R and D3R may been involved in the control of neuroinflammation (Montoya et al., 2019; Broome et al., 2020) we have supposed that in PD patients carrying the LRRK2 mutation, astrocyte D2R/D3R dysfunction may represent a relevant molecular event leading to detrimental neuroinflammation and therefore PD progression. On these bases, in the last part of my project iPSC derived from the two different PD patients carrying the G2019S LRRK2 mutation, as well as from their corresponding isogenic gene-corrected counterpart and a healthy control were differentiated toward astrocytes to investigate their possible involvement in PD. Astrocytes D2R/D3R dysfunction could be a relevant molecular event with detrimental effects for surrounding neurons making the progression of DA neurons degeneration worse.

3 MATERIALS AND METHODS

3.1 Animals

CD1 mice (wild-type) were obtained from Charles River Laboratory (Calco, Italy). D3R knock out mice (D3R-KO) were obtained from Jackson Laboratory (Bar Harbor, ME). Animals were bred and housed in the animal-house facility of the University of Brescia with water and food ad libitum and a 12h light-dark cycle. Animal use was in accordance with the Directive 2010/63/EU. All procedures were conformed to the National Research Guide for the Care and Use of Laboratory Animals and were approved by the Animal Research Ethical Committee of the University of Brescia. All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.2 Primary cultures of midbrain neurons and treatments

Primary cultures of midbrain neurons were prepared from wild-type and D3R-KO by dissecting the ventral mesencephalon from E12.5 mouse embryos, as previously described (Bontempi et al., 2017) Briefly, embryos were then collected and mechanically dissociated at room temperature, and suspended in Neurobasal medium (Gibco, Invitrogen, Carlsbad, CA, USA) containing 2 mM glutamine and B27 supplement (Gibco, Invitrogen). Cells were seeded on poly-D-lysine/laminin-coated plates (1x105 cells/well) at 37°C in a humidified 5% CO2 atmosphere. At the end, we obtain a culture that contains 4% of DA neurons. All pharmacological treatments were performed after 7 days from seeding. Mouse cultures were then treated with nicotine (10 µM) or quinpirole (10 µM) for 72 hours in the presence or absence of interfering peptides able to disrupt the interaction of the heteromeric complex. In particular a cell-permeable mimetic peptide containing the D3R sequence linked to the TAT sequence (TAT-D3R) was thus designed and its scrambled counterpart (TAT-D3R sc) (Bontempi et al., 2017). TAT-D3R (1 µM) peptide or TAT-D3R sc (1 µM) were added 30 minutes before nicotine or quinpirole incubation and every 24 hours until the end of treatment. Cells were than fixed and morphological analyzed by immunocytochemistry.

3.3 Morphological analyses of mouse and human DA neurons

For morphological analyses of DA neurons cultures were fixed using 4% PFA for 10 minutes and incubated in 3% hydrogen peroxidase for 10 minutes to inhibit endogenous peroxidase activity. Cells were then blocked in PBS containing 0,1% Triton x-100 (Sigma Aldrich) and 5% BSA and incubated

with the rabbit anti-TH polyclonal antibody (1:700, Santa Cruz) at 4°C overnight. The next day, cells were incubated with avidin-biotin horseradish peroxidase complex. Staining with peroxidase was performed by incubation in PBS containing 1% 3–3' diaminobenzidine and 0.01% H2O2 (Sigma-Aldrich). Digital images of the immunocytochemical assays were captured with an Olympus IX51 microscope connected to an Olympus digital camera and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA Morphometric measurements were performed on digitalized images using NIH ImageJ software. The morphologic indicators of structural plasticity were: 1) maximal dendrite length; 2) primary dendrite number; 3) soma area. Three slides per treatment group were examined to obtain measurements from at least 30 neurons.

3.4 HEK293 cultures, transfection and treatments

Human embryonic kidney 293 T (HEK293) cells were provided by Open Biosystems. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37 °C in an atmosphere of 5% CO2. Cells were transiently transfected with the GFP-DRD3 vector (D3R) (HEK-D3R cells) (1 μg) (GFP-DRD3 vector was a gift from Jean-Michel Arrang, Addgene plasmid # 24098) or with the pcDNA-alpha4 (0,5 μg) and pcDNA-beta2 (0,5 μg) nAChR subunits (HEK-nAChR cells) (pcDNA-alpha4 and pcDNA-beta2 vectors were a gift from Sergio Fucile, University Sapienza of Rome) or co-transfected with both plasmids (HEK- D3R-nAChR cells)(1 μg tot) using the Arrest-IN reagent (Thermo scientific), according to the manufacturer's instructions. Twenty-four hours post transfection, HEK-nAChR, HEK-D3R and HEK-D3R-nAChR cells were maintained in serum-free medium for 16 hours and then treated. In particular, HEK-D3R cells were treated with quinpirole (10 μM) for different times (5-60 minutes) or with quinpirole (10 μM; 30 minutes) in the presence or in the absence of the PI3K inhibitor LY2940022 (10 μM), added 30 minutes before quinpirole stimulation. In parallel, HEK-nAChR cells were treated with nicotine (10 μM) for different times (0-60 min) or with nicotine (10 μM; 30 minutes), in the presence or in the absence of LY2940022 (10 μM), added 30 minutes before quinpirole stimulation. Finally, HEK- D3R-nAChR cells were treated with nicotine (10 μM) or quinpirole (10 μM) for different times (5-60 minutes) and with nicotine (10 μM; 30 minutes) or quinpirole (10 μM; 30 minutes) in the presence or in the absence of LY2940022 (10 μM), added 30 minutes before quinpirole stimulation. In another set of experiments, primary cultures of midbrain neurons were treated with nicotine (10 μM) or quinpirole (10 μM) for different times (5-14 hours) and analyzed for pERK1/2 by both Western Blot and immunocytochemistry as described below. Moreover, midbrain cultures were treated with nicotine (10 μM; 30 minutes) or quinpirole (10 μM;

30 minutes) in the presence or in the absence of TAT-D3R (1 μM) peptide, TAT-D3Rsc (1 μM) or LY2940022 (10 μM) and analyzed for pERK1/2 by Western Blot. Thus, primary cultures of midbrain neurons were also prepared from D3R-KO mice and treated with nicotine (10 μM) for different times (5-60 minutes), and in the presence or in the absence of the PI3K inhibitor LY2940022 (10 μM) and analyzed for pERK1/2 by both Western Blot as described below.

3.5 Neuronal differentiation from human iPSC lines

iPSC derived from two patients carrying the LRRK2 G2019S mutation (LRRK2-PD; n=2 independent PD patients lines) as well as from gene-corrected iPSC of the same patients (LRRK2-ISO; n=2 independent PD patients lines) were generated by Reinhardt et al (2013). A previously characterized healthy control iPSC line (CTRL; n=1) was used (Bono et al., 2018). Informed consent was obtained from all patients and from healthy donor involved in our study, prior to cell donation (Bono et al.,2018; Reinhardt et al., 2013). iPSC from both patients (LRRK2-PD), from LRRK2-ISO and healthy controls were grown in feeder-free conditions on Matrigel substrate (BD) in StemFlex medium (Thermo Fisher). Cells were differentiated into DA neurons using a modified version of the dual-SMAD inhibition protocol (Kriks et al., 2011; Bono et al., 2018). In particular, starting from day 11, cells were maintained in Neurobasal/B27 medium (Gibco, Invitrogen) supplemented with brain-derived neurotrophic factor (BDNF), ascorbic acid, (glial cell line-derived neurotrophic factor (GDNF), TGF□3 (transforming growth factor type β3), DAPT and cAMP to obtain, at day 50, a neuronal culture enriched in mature, TH-positive DA neurons (40%). Neuronal cultures were exposed to D2R/D3R agonist quinpirole (10 μM), or nicotine (10 μM) for 72 hours followed by morphological analysis. Moreover, iPSC-derived neurons were treated with nicotine (10 μM) or quinpirole (10 μM) for 72 hours in the presence or absence of TAT-D3R (1 μM) peptide or TAT-D3Rsc (1 μM), added 30 minutes before nicotine or quinpirole incubation and every 24 hours until the end of treatment. Cells were than fixed and analyzed by immunocytochemistry. In another set of experiments, neurons derived from LRRK2-PD iPSC (40 day of differentiation) were cultured for 10 days in the absence or in the presence of the GSK2578215A LRRK2 inhibitor (GSK; 100 nM), treated with quinpirole (10 μM) or nicotine (10 μM) for 72 hours and analyzed for morphological, biochemical, and molecular parameters as described below.

3.6 Western blot

Aliquots of total proteins were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis and blotted onto a PVDF membrane (Immobilon-P; Millipore). Membranes were blocked with 5% not-fat milk in 0.1M Tris-buffered saline (pH= 7.4) for 30 minutes and then incubated with the following primary antibodies: anti pERK1/2 (1:1000; Santa Cruz Biotechnology), anti-TH (1:1000; Millipore) and anti-alpha-tubulin (1:500 000; Sigma-Aldrich). rabbit anti-phospho-ser935 LRRK2 (1:1000; Abcam), rabbit anti-LRRK2 (1:10000; Abcam) and anti-alpha-tubulin (1:300,000; Sigma-Aldrich). Blots were incubated with appropriate horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology) and signals were detected by enhanced chemiluminescence (ECL) (GeneSpin). The membranes were scanned and analyzed using gel-pro analyzer software (Media Cybernetics, Bethesda, MD, USA).

3.7 Immunofluorescence analyses

iPSC-derived neurons were fixed with 4% PFA for 10 minutes and blocked in PBS containing 0.1%Triton x-100 (Sigma Aldrich) and 5% bovine serum albumin (BSA; Sigma Aldrich) and incubated at 4°C overnight with the following primary antibodies: microtubule-associated protein 2 (MAP2, 1:500; Millipore), tyrosine hydroxylase (TH, 1:700; Santa Cruz Biotechnology), dopamine transporter (DAT, 1:400; Santa Cruz. Biotechnology), dopamine D3 receptor (D3R, 1:50; Santa Cruz Biotechnology), alpha4-subunit nAChR (alpha4 nAChR, 1:100; Sigma Aldrich), anti-alpha synuclein [MJFR1] (1:200; Abcam) and Receptor binding cancer antigen expressed on SiSo cells (RCAS1, 1:400, Cell Signaling). Cells were then incubated for 30 min at room temperature with appropriate Alexa Fluor 488- Alexa Fluor 647-and Cy3- conjugated secondary antibodies (Jackson Immuno Research). Nuclei were stained with DAPI. Images were acquired by confocal microscopy Zeiss LSM 880 confocal microscope equipped with Plan-Apochromat 63X/1.4 numerical aperture oil objective and the Zen 2.3 Software (Carl Zeiss AG, Oberkochen, Germany) have been used for images analysis.

3.8 Endogenous DA release

At day 50 of neuronal differentiation iPSC-derived cultures (day 50) were washed twice with Krebs Ringer's buffer (KRB), containing 119 mM NaCl, 2,5 mM KCl, 2,5 mM CaCl2, 1,3 mM MgSO4, 1 mM NaH2PO4, 26,2 mM NaHCO3 and 10 mM glucose (pH 7.4). KRB was then added to the cells and immediately collected (T0). Subsequently, cells were treated with KRB (basal), nicotine (10 µM in

KRB) or potassium (K+; 50 mM in KRB) in the presence of the dopamine uptake inhibitor GBR 12935 (30 nM), and the media collected after 30 minutes of incubation (T30). DA content was measured by HPLC, as previously described (Bono et al., 2018). Measurements of dopamine and metabolites were made by HPLC with an electrochemical detection system (ALEXYS LC-EC; Antec Leyden BV) equipped with a reverse-phase column (3-µm particles, ALB-215 C18, 1 × 150 mm; Antec Leyden BV) at a flow rate of 50 µl/min and electrochemically detected by a 0.7-mm glass carbon electrode (VT-03; Antec Leyden BV). The HPLC experiments were done in collaboration with Prof Devoto at University of Cagliari

3.9 Proximity Ligation Assay (PLA)

In situ PLA was performed in neuronal cultures using the Duolink in situ detection kit (Sigma-Aldrich) according to the manufacturer's instructions as previously reported (Bontempi et al., 2017; Bono et al., 2019). Briefly, iPSC-derived cultured were fixed in PBS containing 4% paraformaldehyde (Sigma-Aldrich), incubated with blocking solution (Duolink, Sigma-Aldrich) and then with goat polyclonal anti-D3R (1:50 dilution; Santa Cruz Biotechnologies) and rat monoclonal anti alpha4 nAChR subunit (1:100 dilution; Sigma Aldrich) antibodies (overnight; 4°C). Neurons were incubated with the anti-rat MINUS and anti-goat PLUS probes (Duolink, SigmaAldrich), followed by ligation and amplification reactions, according to the manufacturer's instructions. Neuronal cultures were then incubated with the anti-TH antibody (1:500 dilution; Santa Cruz Biotechnologies) and with the Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:750 dilution; Jackson Laboratories), mounted with 4',6-diamidino-2-phenylindole (DAPI; Duolink, Sigma-Aldrich)-containing mounting medium and analyzed using a Zeiss LSM 880 confocal microscope. Images were examined using Zen 2.3 Software (Carl Zeiss AG, Oberkochen, Germany).

3.10 RT-PCR

Total RNA was extracted from cells at days 50 using TriZol Reagent (Life Technologies). RNA was quantified using the My Spect spectrophotometer (Biomed). For each sample, 1 µg of total RNA was treated with DNase I and reverse-transcribed using High- Capacity cDNA Reverse Transcription kit from Life Technologies (Foster City, CA) according to the manufacturer's protocol using a thermo cycler (Applied Biosystems) at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. RT-PCR was

performed using the DreamTaq Green PCR Master Mix according to the manufacturer's instruction (Thermo Fisher). The following primer pairs were used:

D3R (fw AGCAAACCCTCTCTCCTGAC and rv GAATTCCCTGAGTCCCACCA)
α4-nAChR (fw CGAACGTATGGGTGAAGCAG and rv GCATGTTCACCAGGTCGATC)
GADPH (fw AGGTCGGAGTCAACGGATTT and rv CCATCTTCCAGGAGCGAGAT)

3.11 Differentiation of iPSC-derived astrocytes and treatments

iPSC from PD patients carrying the G2019S LRRK2 mutation (LRRK2-PD; n=2 independent PD patients lines) and their gene-corrected isogenic lines from both patients (LRRK2-ISO; n=2 independent PD patients lines) and healthy controls (CTRL) were grown in feeder-free conditions on Matrigel substrate (BD) in StemFlex medium (Thermo Fisher). Cells were differentiated into astrocytes following a protocol consisting in neural induction, neural expansion and astrocytes terminal differentiation steps. For neural induction, iPSC were cultured for 24h in Stemflex medium (Gibco, Life Technologies), followed by incubation with PSC Neural Induction Medium (Gibco, Life Technologies) in the presence of Y-27632 dihydrochloride (Rock inhibitor; Tocris). When pNSCs reach confluency, they can be further expanded in fact after 7 days, primitive neural stem cells (pNSCs) were disaggregated using Accutase and cultured on Matrigel substrate in Neural expansion medium (NEM) prepared in Neurobasal medium and Advanced DMEM/F12 plus neural induction supplement. For astrocyte differentiation, cells were plated on matrigel substrate in an astrocyte differentiation medium (ADm) composed of advanced DMEM (Euroclone) supplemented with N2 (Life Technologies) and 1% FBS (Sigma Aldrich) in the presence of ciliary neurotrophic factor (CNTF, 20ng/Ml, PeproTech). for 25 days. Cells were passaged every 3–5 days when they reached 70%– 80% confluence. After 3 weeks of maturation (40 days) these cells lost their proliferative capacity. At the end of differentiation, mature astrocytes were cultured in astrocytes medium without CNTF to obtain a culture highly enriched in astrocytes (~45%) and analyzed for astrocytes marker, morphology, dopamine D2R expression, Ca2+waves and ability to sustain neuronal function in astrocytes-neurons co-cultures experiments, as described below. In another set of experiments astrocytes derived from LRRK2-PD iPSC (40 day of differentiation) were cultured for 7 days in the absence or in the presence of the GSK2578215A LRRK2 inhibitor (GSK; 200 nM) and analyzed for the D2R expression. Images were acquired by confocal microscopy Zeiss LSM 880 confocal microscope equipped with Plan-

Apochromat 63X/1.4 numerical aperture oil objective and the Zen 2.3 Software (Carl Zeiss AG, Oberkochen, Germany) have been used for images analysis.

3.12 Astrocytes immunofluorescence analyses

iPSC-derived astrocytes were fixed with 4% PFA for 10 minutes and blocked in PBS containing 0.1%Triton x-100 (Sigma Aldrich) and 5% bovine serum albumin (BSA; Sigma Aldrich) and incubated at 4°C overnight with the primary antibodies against the protein Sox2, a nuclear marker typically expressed in undifferentiated cells, Nestin, a Neural Stem Cells marker, MAP2, a neuronal marker and GFAP protein, specific markers expressed in the mature astrocytes. Primary antibodies were anti-SOX2 (1:100; (Thermo Fisher), anti-Nestin (1:400; Millipore) anti-MAP2 (1:400; Millipore), anti- GFAP (1:900; Dako), dopamine D2 receptor, (D2R,1:50; Santa Cruz Biotechnology) dopamine D3 receptor (D3R, 1:50; Santa Cruz Biotechnology). Cells were incubated for 30 min at room temperature with appropriate Alexa Fluor 488- and Cy3- conjugated secondary antibodies (Jackson Immuno Research). Nuclei were stained with DAPI. Images were acquired by confocal microscopy Zeiss LSM 880 confocal microscope equipped with Plan-Apochromat 63X/1.4 numerical aperture oil objective and the Zen 2.3 Software (Carl Zeiss AG, Oberkochen, Germany) have been used for images analysis.

3.13 Human astrocytes calcium imaging analyses

Astrocytes were plated in a 4 well chamber slide (Ibidi chambers) and after 4 days of culture were incubated in an isotonic saline solution (HBSS, Euroclone), added with the antibiotics Penicillin-Streptomycin (Thermofisher), and subsequently, they were incubated for one hour with a fluorescent dye, Fluo- 4 AM (2.5 µM, Molecular Probes), a lipophilic compound capable of penetrating cells and binding intracellular calcium. At the end of the incubation, the cells were washed in HBSS, to eliminate the excess dye and Sulfinpyrazone (0.1-0.25 mM, Molecular Probes) was added, which is an inhibitor of membrane transporters capable of expelling, by exocytosis, the Fluo-4 AM fluorophore from cells. Cells were then imaged for 5 min using a Zeiss LSM 880 confocal microscope. Recorded data were examined using Zen 2.3 Software (Carl Zeiss AG).

3.14 Neurons/astrocytes co-cultures experiments

Primary cultures of midbrain neurons were prepared from wild-type mice as previously described. Astrocytes were plated on Matrigel (30 days of differentiation) and cultured for 7 days in Astrocytes

medium. Primary midbrain neurons were seeded on a confluent monolayer of astrocytes or on Poly-D-lysine/laminin-coated wells and cultured in Neurobasal medium for additional 7 days. Cells were then fixed with 4% PFA for 10 minutes incubated overnight at 4 °C with anti-TH antibody and then with a biotinylated anti-rabbit antibody (1:700; 30 min at room temperature) followed by a final incubation with avidin-biotin horseradish peroxidase complex. Morphometric measurements were performed on digitalized images using NIH ImageJ software. The morphologic indicators of structural plasticity were: 1) maximal dendrite length; 2) primary dendrites numbers; 3) soma area.

3.15 Statistical Analysis

Each experiment has been conducted independently in each line (LRRK2-PD: n=2, LRRK2 PD-1 and LRRK2-PD-2; LRRK2- ISO: n=2, LRRK2 ISO-1 and LRRK2-ISO-2; CTRL: n =1) and experiments were repeated at least 3 times with superimposable results. Significant differences were determined using one-way analysis of variance (ANOVA), followed by a posteriori Bonferroni's test for multiple comparisons. For comparison between 2 groups, an unpaired Student’s t-test was used. All statistical analyses were per- formed using GraphPad prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

4 RESULTS

4.1 The D3R-nAChR heteromer:

Receptor heteromer complexes are classically defined as single entities in which both the protomers are likely equally essential in inducing a given function; since heteromers represent novel molecular units, their proper pharmacological, signalling and trafficking features, that may differ from those of the two receptors assembled in the complex, need to be investigated (Gomes et al., 2016) Therefore, in the first part of my project a further characterization of the properties of the D3R-nAChR heteromer has been performed. First, the role the D3R within the heteromer has been investigated: while it is likely that stimulation of D3R causes neurotrophic effects "per se" (Collo et al., 2008,2013; Bono et al., 2018) we have demonstrated that α4β2 nAChR gains the ability to induce structural plasticity on DA neurons only when is coupled to D3R. Therefore, when activated, the D3R-nAChR complex is the molecular entity that supports DA neurons remodelling, while the D3R should trigger intracellular signals, for example the PI3K-ERK1/2 pathway, specifically responsible for this effect (Collo et al., 2013; Bontempi et al., 2017; Bono et al., 2018). However, the exact role of D3R has been never investigated. Moreover, the signalling pathway associated with D3R-nAChR heteromer stimulation has been also analyzed. This part of the project has been developed in different cell models: HEK293 cells individually expressing either D3R or nAChR, or co-expressing both the receptors, previously used for studying the direct interaction between D3R and nAChR (Bontempi et al., 2017); primary cultures of mouse DA neurons, physiologically expressing the D3R-nAChR heteromer (Bono et al., 2019) or expressing the only nAChR and derived from D3R knock out (D3R-KO) mice; human DA neurons differentiated from a iPSC of a healthy subject (Bono et al., 2019).

4.1.1 Role of D3R in the D3R-nAChR heteromer

The role of the D3R protomer in the D3R-nAChR heteromer ability in inducing neurotrophic effects was first investigated in primary cultures of mesencephalic neurons, that typically containing 5-10% of midbrain dopaminergic (DA) neurons (Bontempi et al., 2017) and taking advantage of a specific interfering peptides (TAT-D3R peptide), previously designed and characterized for their ability to disrupt the interaction between D3R and the beta subunit of the α4β2 nAChR (Bontempi et al., 2017). In particular, the TAT-D3R interfering peptide has been obtained by linking the cell-penetrating TAT peptide to the 215-225 arginine-rich region of D3R, identified as a region usually involved in

receptor–receptor interactions (Woods, 2005). Primary mouse cultures were stimulated. with the D2R/D3R agonist quinpirole (10 µM) for 72 hours and morphologically analyzed. In particular, DA neurons were identified by TH staining; the average maximal length of the primary dendrite, the number of dendrites and the soma area were used as indicators of structural plasticity (Bontempi et al., 2017). We found that, similarly to nicotine, D2R/D3R stimulation significantly increased the length of the primary dendrite, the dendrite numbers and soma area of TH-positive neurons (Fig.10). Stimulation with quinpirole (10 µM; 72 hours) was next performed in the presence of both the TAT-D3R interfering peptide (1 µM) and TAT-scrambled peptide (TAT-D3R-Sc; 1 µM) (Bontempi et al., 2017), used as a negative control. The morphological analyses of TH-positive neurons have shown that the TAT-D3R peptide, but not its scrambled counterpart, strongly abolished the neurotrophic effects induced by quinpirole thus indicating that the remodeling properties of D2R/D3R agonists are likely due to agonist targeting and activation of D3R only when these receptors are assembled into the heteromeric complex.

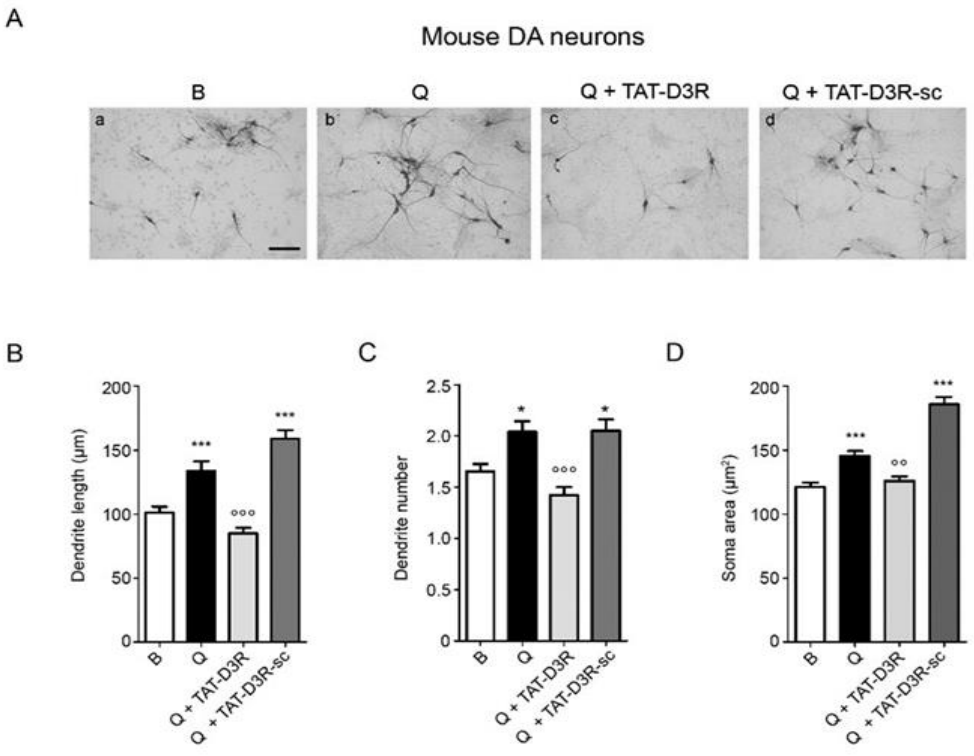


Fig.10: Morphologic effects of quinpirole in presence of interfering peptides in primary colture of neurons. (A) Representative photomicrographs of TH-positive neurons after 72-h exposure to quinpirole (Q) (10 µM) in the absence or in the presence of TAT-D3R (1µM) or TAT-D3R-sc (1 µM) added 30 min before quinpirole and every 24h to the end of treatment. Quantitative analysis of **(B)** maximal dendrite length, **(C)** dendrite number and **(D)** soma area. Bars represent the mean ± S.E.M. ***p < 0.001, **p < 0.01, *p < 0.05 vs basal and °°° p < 0.001, °°p < 0.01 vs Q. Statistical significance of the result was defined by Anova followed by Bonferroni's test.

The ability of D2R/D3R agonist quinpirole in inducing morphological effects through the D3R-nAChR complex was also analyzed in human DA neurons obtained by using the iPSC technology. In particular, DA neurons were differentiated from a healthy iPSC line with a modified version of the dual-SMAD inhibition protocol (Bono et al., 2018; 2019). As previously described, with this protocol, we usually obtain in 50 days of differentiation cultures in which the majority of cells (~ 82%) are positive for the microtubule-associated protein 2 (MAP-2) neuronal marker, and are mainly composed by DA (~40% of cells), GABAergic (~23%) and glutamatergic (~28%) neurons. Moreover, in human DA neurons, the D3R-nAChR heteromer expression has been previously shown by using PLA (Bono et al., 2019). Human neuronal cultures (50 day of differentiation) were incubated with quinpirole (10 µM) for 72 hours and TH-positive neurons were analyzed for morphological remodeling (Fig. 11). We found that quinpirole induced a significant increase of the maximal length of primary dendrite (Fig.11B), number of dendrites (Fig.11C) and soma area (Fig. 11D) as compared to untreated cells. Neurons were then treated quinpirole (10uM; 72 hours) in the presence of the TAT-D3R peptide or its corresponding scrambled peptide (both at 1 µM) and TH-positive neurons morphologically analyzed. As shown in Fig 11 the remodeling properties of quinpirole were almost completely counteracted in the presence of the TAT-interfering peptide, but not of the scrambled one (Fig. 11A-D) thus indicating that as in mouse neurons, D3R is unable to exert neurotropic effects if expressed alone.

Together, these data indicate that in human DA neurons, nicotine and D3R agonists require the D3R-nAChR heteromer as the only molecular effector able to induce neurotrophic responses; neither nAChR (Bontempi et al., 2017) nor D3R, if expressed alone, are able to elicit these effects.

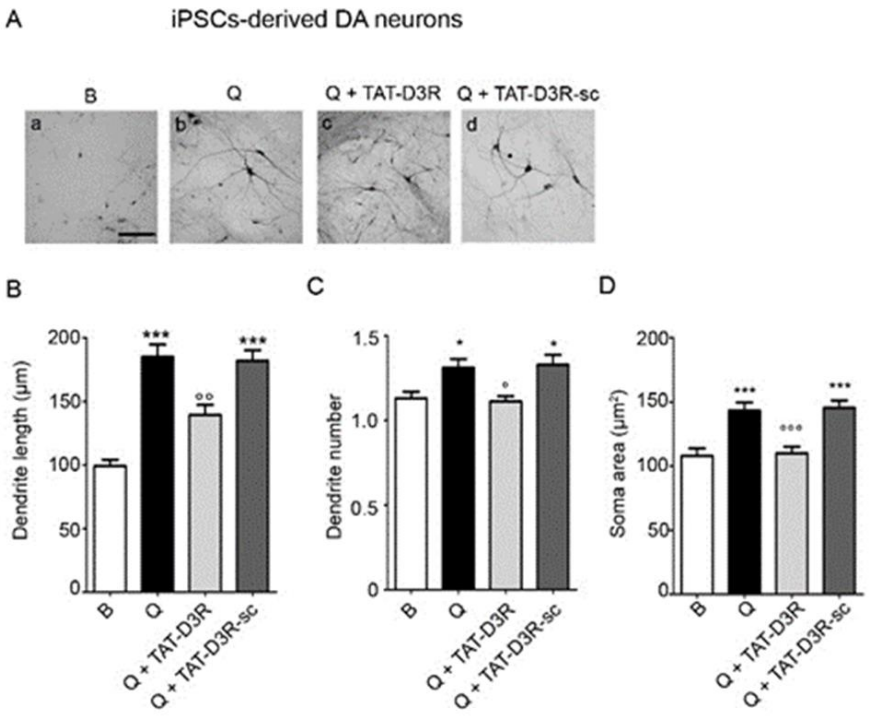


Fig.11: Morphologic effects of quinpirole in presence of interfering peptides in iPSC-derived DA neurons. (A) Representative photomicrographs of TH-positive neurons after 72-h exposure to quinpirole (Q) (10 µM) in the absence or in the presence of TAT-D3R (1µM) or TAT-D3R-sc (1 µM) added 30 min before quinpirole and every 24h to the end of treatment. Quantitative analysis of (B) maximal dendrite length, (C) dendrite number and (D) soma area. Bars represent the mean ± S.E.M. ***p < 0.001, p* < 0.5 vs basal and °°° p < 0.001, °°p < 0.01; p° < 0.5 vs Q. Statistical significance of the result was defined by Anova followed by Bonferroni's test.

4.1.2 D3R-nAChR heteromer signaling properties

Previously published data, carried out in both mouse and human DA neurons, suggested that the PI3K-ERK1/2 pathway is crucially involved in the mechanisms underlying the morphological changes induced by nicotine (Collo et al., 2011; Bono et al., 2018). Therefore, the PI3K-ERK1/2 cascade could be an intracellular signal associated with the D3R-nAChR heteromer and closely related to its ability in exerting neurotrophic effects. Since both D3R and nAChR retain the ability to activate the ERK1/2 pathway, the ability of the D3R-nAChR heteromer in activating this signaling pathway was compared to that induced by D3R or nAChR, individually expressed.

To this aim, HEK293 cells transiently expressing either alpha4beta2-nAChR (HEK-nAChR) or D3R (HEK-D3R) and co-expressing D3R and alpha4beta2-nAChR (HEK-D3R-nAChR) were used. First, HEK-nAChR cells were stimulated with nicotine (10 µM) for 5-60 minutes and analyzed for ERK1/2 phosphorylation

(pERK1/2) using Western Blot. (Fig. 12A). We found that, starting from 5 minutes of stimulation, nicotine resulted in a persistent increase of the pERK1/2 levels that lasted until 60 minutes of stimulation. Activation of ERK1/2 was next analyzed in HEK-D3R cells, incubated with quinpirole (10 µM) for 5-60 minutes. As shown in Fig. 12B, stimulation of the D3R induced a rapid and transient activation of ERK1/2, with a phosphorylation peak at 5 minutes, which rapidly decreased toward basal levels. Finally, HEK-D3R-nAChR cells, in which the ability of D3R to interact with the nAChR was previously demonstrated (Bontempi et al., 2017) were treated with nicotine (10 µM) or quinpirole (10 µM) for 5-60 minutes and analyzed for pERK1/2. As shown in Fig. 12C-D, both nicotine and quinpirole were able to increase pERK1/2 levels, in a long-lasting way, from 5 to 60 minutes of compounds stimulation.

Moreover, treating HEK-D3R-nAChR cells with nicotine (10 µM) or quinpirole (10 µM) for 30 min in the presence of the PI3K inhibitor LY294002 significantly reduced pERK1/2 levels (Fig 12E), suggesting that in HEK293 cells, stimulation of the D3R-nAChR heteromer by both nicotine and quinpirole induces a persistent activation of ERK1/2 that requires the PI3K activity.

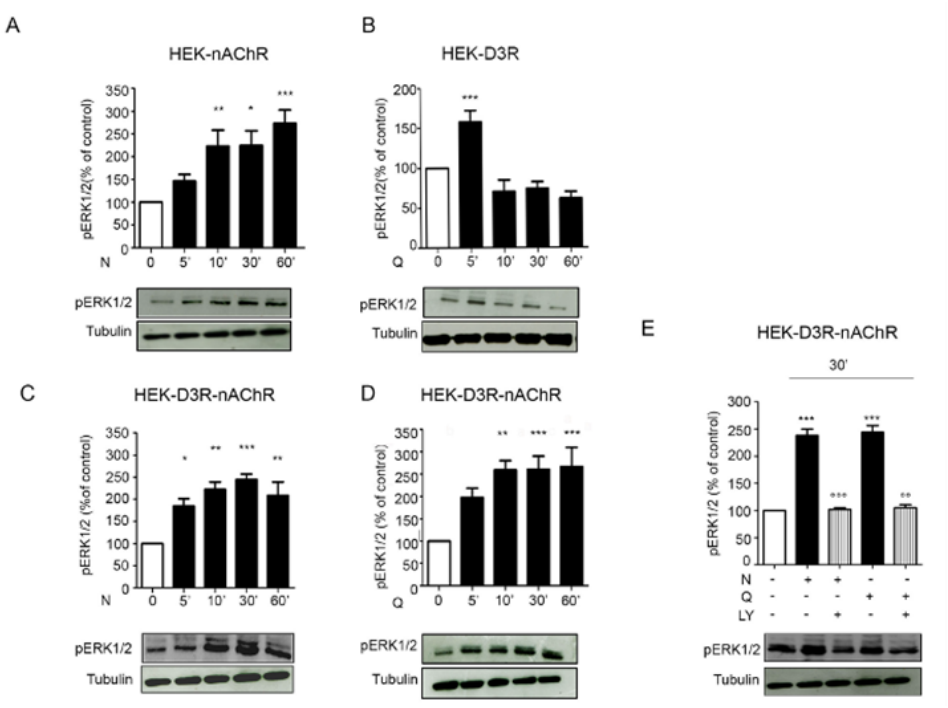


Fig.12: D3R, nAChR and D3R-nAChR-induced activation of ERK1/2 in HEK293 cells. (A) HEK293T cells transiently expressing the alpha4beta2 nAChR subunit (HEK-nAChR) were stimulated with nicotine (N) for different time (5-60 min) and analyzed for pERK1/2 by Western Blot. **(B)** HEK293T cells transiently expressing the D3R receptor (HEK-D3R) were stimulated with quinpirole (Q) for different time (5-60 min) and analyzed for pERK1/2 by Western Blot. **(C-D)** HEK-D3R-nAChR were stimulated with nicotine (10 µM) or quinpirole (10 µM) for different times (5-60 min). **(E)** HEK-D3R-nAChR were exposed to nicotine (10 µM) or quinpirole (10 µM) for 30 minutes in the presence or in the absence of LY294002 (10

μM) added 30 min before nicotine or quinpirole and analyzed for pERK1/2. Upper panel, densitometric analysis of blots (n = 3) with specific levels of pERK 1/2 normalized to the corresponding tubulin level. Lower panel, representative blot (image) of pERK 1/2; Data were statistically analyzed by one-way ANOVA followed by post hoc comparison with Bonferroni test. (***p < 0.001, **p<0.01, *p<0.05 vs 0) (°°° p < 0.001 vs N; °°p < 0.01 vs Q).

The activation of the PI3K-ERK1/2 pathway associated with the D3R-nAChR heteromer stimulation was next analyzed in primary cultures of mouse mesencephalic neurons from both wild type and D3R-KO mice (Fig.13).

Cultures derived from wild-type mice were treated with nicotine (10 μM) or quinpirole (10 μM) for different times (5 min-14 hours) and analyzed for ERK1/2 activation. As shown in Fig. 13 A-B, both the treatments were able to activate ERK1/2 in a persistent way, with elevated phosphorylation levels measured at 60 min, but not at 14 hours. In parallel experiments, neuronal cultures derived from D3R-KO mice and expressing the only nAChR, were treated with nicotine (10 μM) for different times (5 min-14 hours) showing that nAChR stimulation produced a persistent activation of ERK1/2, with a peak level measured at 60 min (Fig 13C).

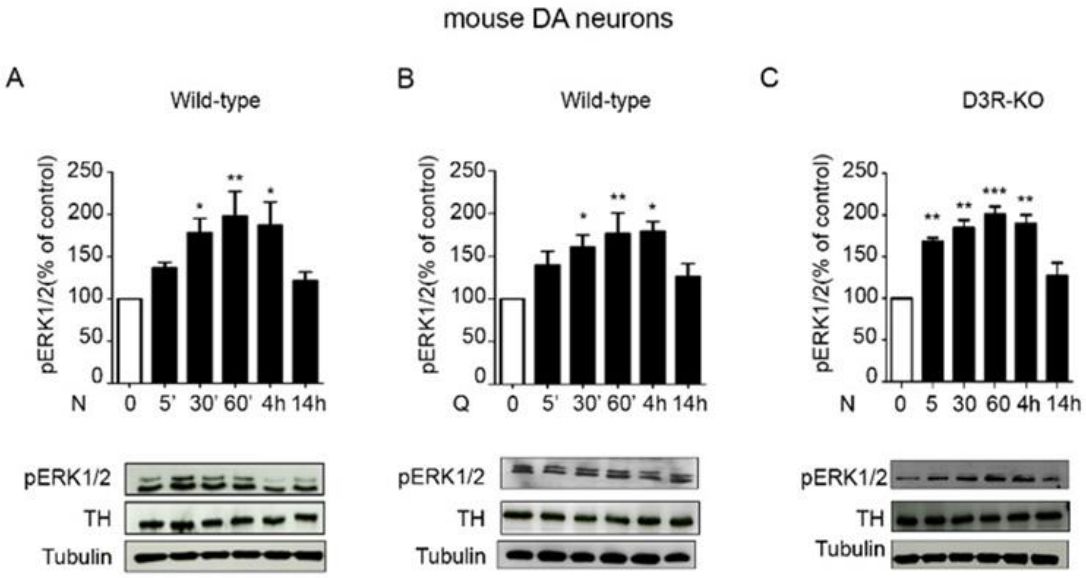


Fig.13: In mouse DA neurons, nicotine and quinpirole induced a progressive ERK1/2 phosphorylation. (A-B) Primary cultures of mouse mesencephalic neurons were stimulated with nicotine (N) (10 μM) **(A)** or quinpirole (Q) **(B)** for different times (0-14 hour) and analyzed for ERK 1/2 phosphorylation (pERK 1/2) by Western Blot. **(C)** Primary cultures of mouse mesencephalic neurons derived from D3R-KO mice were exposed to nicotine (10 μM) for for different times (0-14 hour) and analyzed for pERK1/2. Upper panel, densitometric analysis of blots (n = 3) with specific levels of pERK1/2 normalized to the corresponding TH and tubulin levels. Lower panel, Representative blot of pERK1/2. . Bars represent the mean ± S.E.M. ***p < 0.001, **p < 0.01 *p<0.05 vs 0.

To associate the activation of ERK1/2 with the D3R-nAChR heteromer, neuronal cultures from wild-type mice were treated with nicotine for 30 minutes (10 µM) both in the presence and in the absence of the TAT-D3R or TAT-scramble interfering peptides (1 µM). As expected, since at this time, stimulation of both the only nAChR and of the D3R/nAChR heteromer resulted in a persistent activation of ERK1/2, nicotine-induced pERK1/2 was not prevented by contrasting the nAChR interaction with D3R (Fig 14A). By contrast, phosphorylation of ERK1/2 induced by quinpirole (10 µM; 30 minutes) was significantly prevented by the incubation with the TAT-D3R peptide (1 µM) (Fig. 14B).

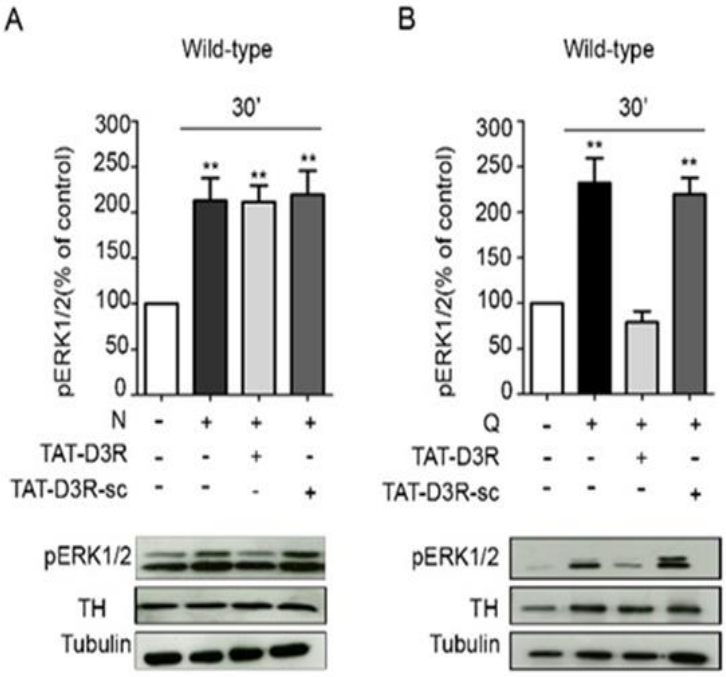


Fig.14: **In mouse DA neurons, nicotine and quinpirole induced activation of ERK1/2 requires the D3R- nAChR heteromer. (A-B)** Primary cultures of mouse mesencephalic neurons were exposed to nicotine (10 µM) (**A)** or quinpirole (10 µM) **(B)** for 30 minutes in the presence or in the absence of TAT-D3R (1 µM) or TAT-D3R-sc (1 µM) and analyzed for pERK1/2 by Western Blot. Upper panel, densitometric analysis of blots (n = 3) with specific levels of pERK1/2 normalized to the corresponding TH and tubulin levels. Lower panel, representative blot of pERK1/2. . Bars represent the mean ± S.E.M. **p < 0.01 vs 0.

Therefore, in mouse DA neurons, both the nAChR and the D3R-nAchR heteromer, when activated, have the ability to activate the ERK1/2 pathway in a persistent way.

The role of PI3K was next investigating. To this aim, wild-type cultures were treated with nicotine (10 µM) or quinpirole (10 µM) for 30 min in the presence of the PI3K inhibitor LY294002 (10 µM) (Fig

15A). The results show that inhibiting the PI3K significantly decreased pERK1/2 levels. In parallel experiments, cultures derived form D3R-KO mice were treated with nicotine (10 µM; 30 min) with or without LY294002 (10uM). As shown in fig 15B, ERK1/2 phosphorylation was not blocked by inhibiting the PI3K.

Together, these data indicate that in mouse DA neurons, D3R-nAChR heteromer activation by both nicotine and quinpirole results in the long-lasting activation of the ERK1/2 pathway that require the PI3K activity.

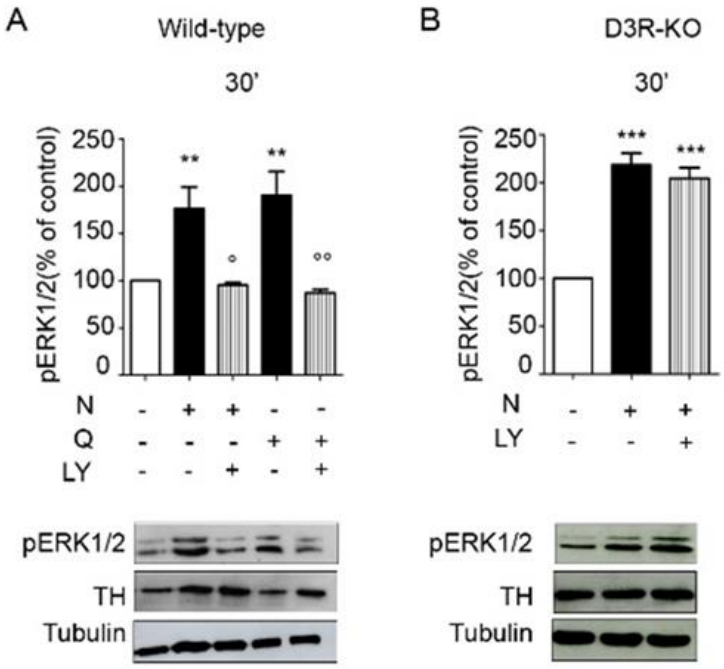


Fig.15: In mouse DA neurons, nicotine and quinpirole induced activation of ERK1/2 that requires the PI3K activity (A-B) Primary cultures of mouse mesencephalic neurons were exposed to nicotine (N)(10 µM) or quinpirole (Q)(10 µM)**(A)** for 30 minutes in the presence or in the absence of LY294002 (10 µM) and analyzed for pERK1/2. **(B)** Primary cultures of mouse mesencephalic neurons derived from D3R-KO mice were exposed to nicotine (10 µM) for 30 minutes in the presence or in the absence of LY294002 (10 µM) and analyzed for pERK1/2. Upper panel, densitometric analysis of blots (n = 3) with specific levels of pERK1/2 normalized to the corresponding TH and tubulin levels. Lower panel, representative image of pERK1/2. Bars represent the mean ± S.E.M. ***p < 0.001, **p < 0.01 vs 0 ; °°p < 0.01 vs Q; p° < 0.5 vs N. Statistical significance of the result was defined by Anova followed by Bonferroni's test.

4.2 Neuronal differentiation from human induced pluripotent stem cells

LRRK2 gene, with a glycine to serine substitution (G2019S), represents the most frequent mutation in both familial and apparently sporadic PD (Cookson, 2010). However, the mechanisms by which LRRK2 mutation affects DA neurons viability leading to neurodegeneration are still unknown However, as previously reported, using iPSC-based studies human DA neurons carrying the G2019S mutation in LRRK2 have been deeply analyzed; interestingly, a specific PD-related DA neuronal phenotype has been described, characterized by reduced neurite outgrowth, increased levels of alpha-synuclein (alpha-syn), alterations in mitochondrial morphology, impaired mitophagy and increased sensitivity to stress (Borgs et al., 2016, Nguyen et al., 2011; Reinhardt et al., 2013; Walter et al., 2019). Since one of the various functions associated with LRRK2 activity is the regulation of vesicle trafficking (Sanna et al., 2012; Cirnaru et al., 2014; Rassu et al., 2017), this protein play a crucial role in both neurotransmitter release and receptor localization at the plasma membrane. On this line, as described before, data obtained in different in vivo and in vitro models suggested that mutant LRKK2 could modify the trafficking of some receptors, including the DA D1 and D2 receptors (Migheli et al 2013; Rassu et al., 2017; Tong et al., 2009). Therefore, our hypothesis is that defects in D3R-nAChR heteromer activity, likely induced by altered expression of the heteromer at the plasma membrane of DA neurons carrying the G2019S LRRK2 mutation, may represent an additional molecular alteration that, by affecting DA neuron homeostasis, could contribute to their peculiar vulnerability (Bono et al., 2017; 2018).

To study the impact of abnormal LRRK2 activity on the D3R-nAChR heteromer localization and function., iPSC derived from two different PD patients carrying the G2019S activating mutation in the LRRK2 kinase domain, as well as from their isogenic gene-corrected–counterpart (Reinhardt et al., 2013) and a healthy control (Bono et al., 2018) were used to generate neuronal cultures enriched in DA neurons to be used as a proper patient-specific model of PD.

4.2.1 Morphological and functional analysis of DA neurons derived from LRRK2 G2019S iPSC

iPSC derived from two different PD patients carrying the G2019S activating mutation in the LRRK2 kinase domain (LRRK2-PD; n=2 independent PD patients lines), as well as from their isogenic gene-corrected counterparts (LRRK2-ISO; n=2 independent PD patients lines) (Reinhardt et al., 2013) and a healthy control (CTRL; n=1) (Bono et al., 2018) were differentiated into DA neurons using a modified

version of the dual-SMAD inhibition protocol, first developed by Kriks et al. (2011). By using this protocol, we usually obtain at day 50 of differentiation, human cultures enriched in TH/DAT positive neurons (~40%) expressing genes and proteins typical of authentic, terminally differentiated DA neurons (Bono et al., 2018). At the end of the differentiation protocol, each line was individually analyzed with superimposable results. Immunocytochemistry analysis showed that cultures derived from control, LRRK2-PD and LRRK2-ISO iPSC included an average of about 40% of MAP2/TH-positive neurons (Fig.16A-B). The morphology of these neurons was analyzed by immunocytochemistry and computer-assisted morphometry using the average maximal length of the primary dendrite, the number of dendrites, and the soma area as relevant endpoints (Bono et al., 2018). As reported in Fig.16C-D and according to previous studies (Weykopf et al., 2019), TH-positive neurons from LRRK2-PD were characterized by reduced dendrite length (Fig.16D), reduced dendritic arborization (Fig.16E), and reduced soma area (Fig.16F) as compared to both LRRK2-ISO and control TH-positive neurons.

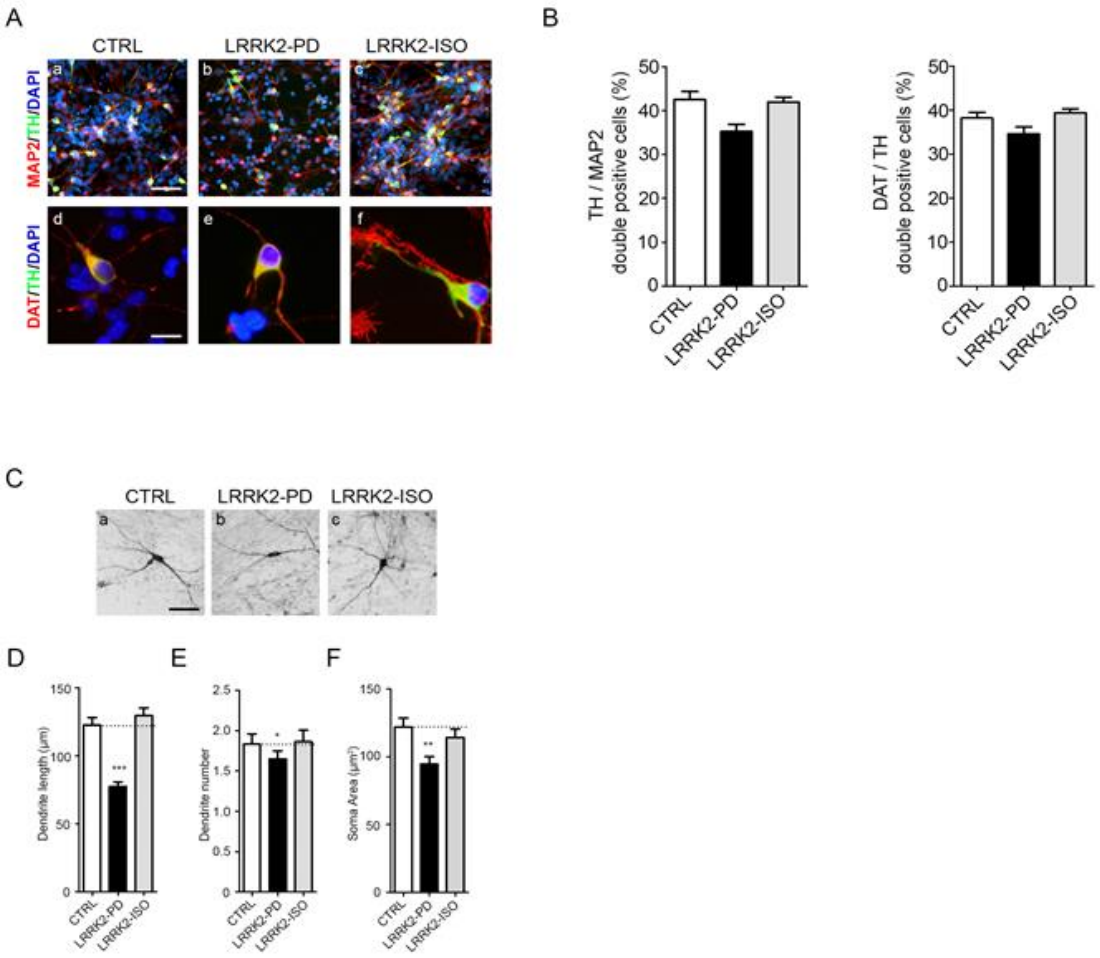
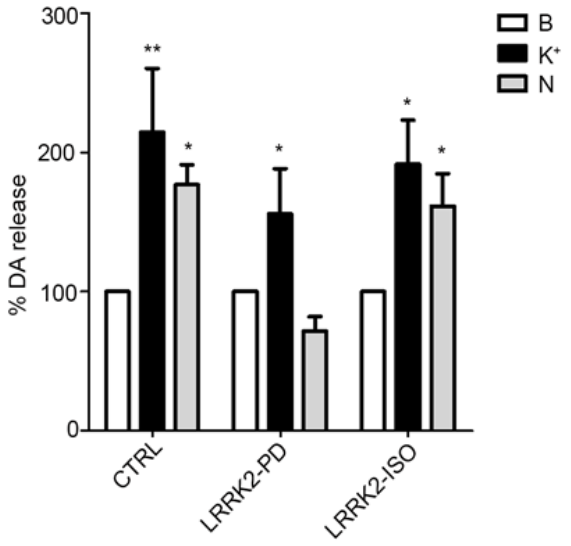


Fig.16: DA neurons derived from LRRK2 G2019S patients are characterized by morphological defects

(A) Representative images of immunofluorescence analyses of MAP2 (red) and TH (green), or DAT (red) and TH (green) co-staining in DA neurons derived from control iPSC (CTRL; panels a and d), from a representative G2019S LRRK2-mutant IPSC line (LRRK2-PD; panels b and e) and the respective LRRK2 G2019S corrected isogenic line (LRRK2-ISO; panels c and f) at day 50 of differentiation. Nuclei were stained with DAPI (blue). Scale bar=100 µm (panel a-c), 25 µm (panels d-f). (**B**) MAP2- and TH- positive cells, or DAT- and TH- positive cells were counted. Bars referred to panel A represent the mean ±S.E.M. of three independent experiments on each cell culture (post hoc Bonferroni's test) (CTRL: n=1; LRRK2-PD: n=2; LRRK2-ISO: n=2) (**C**) Representative microphotographs of TH-positive neurons derived from CTRL (panel a), LRRK2-PD (panel b), and LRRK2-ISO (panel c). (**D-F**) Quantitative analysis of (D) maximal dendrite length, (E) dendrite number and (F) soma area. Bars represent the mean ± S.E.M of three independent experiments. ***p < 0.001, **p < 0.01, *p < 0.05 vs basal. Statistical significance of the result was defined by Anova followed by Bonferroni's test.

We previously reported that TH-positive neurons derived from iPSC are able to synthesize and release DA both under basal conditions and after stimulation with either a non-specific synaptic vesicle releaser such as potassium (K^+) or nicotine, through the activation of nAChR on DA neurons (Bono et al., 2018). On this line, the ability of LRRK2-PD neuronal cultures to release DA was measured and compared to that of their corresponding LRRK2-ISO and control neurons. In particular, neuronal cultures were treated with either K^+ (50 mM) or nicotine (10 µM) and DA content in the culture media was measured by HPLC after a 30-minutes stimulation. Each line was individually analyzed with superimposable results. We found that the basal DA release of LRRK2-PD neurons was undistinguishable from that of both control and LRRK2-ISO neurons (Fig.17). Moreover, treatment with K^+ significantly increased DA release in all cultures (Fig.17). By contrast, nicotine, that stimulated DA release in both control and LRRK2-ISO DA neurons, was inactive in LRRK2-PD neurons (Fig.17). These data suggest that DA neurons, that synthesize and release DA, can be generated from LRRK2-mutant iPSC. Moreover, while LRRK2 dysfunction does not affect DA release both in basal conditions and after non-specific depolarizing stimuli, it abolishes DA release evoked by nAChR stimulation, likely reflecting an altered function of the acetylcholine receptor system.



F**ig.17: Functional release in DA neurons** DA release by DA neurons stimulated either with potassium (K+; 50 mM) or nicotine (N; 10 µM) for 30 minutes was determined by HPLC. Values represent the mean ± S.E.M. of three independent experiments (post hoc Bonferroni's test) (CTRL: n=1; K+: **p=0.0007 vs basal (B), N: *p=0.05 vs B, LRRK2-PD: n=2; K+: *p=0.05 vs B, LRRK2-ISO: n=2; K+: *p=0.0014 vs B; N: *p=0.05 vs B.

Aggregation of alpha-synuclein (alpha-syn), the major neuropathological hallmark of PD (Spillantini et al., 1998) has been observed in various experimental models of both sporadic and familiar PD, including iPSC-derived DA neurons expressing the LRRK2 G20189S mutation (Weykopf et al., 2019). On this line, by using immunofluorescence we individually analyzed alpha-syn levels in the two LRRK2-PD, their corresponding LRRK2-ISO, and control TH-positive neurons. Each line was individually analyzed with superimposable results, showing that the majority of TH-positive LRRK2-PD neurons (Fig.18A panels d-f), but not control and LRRK2-ISO TH-positive neurons (Fig.18A panels a-c or panel g-i, respectively), exhibited strong and diffuse cytoplasmic alpha-syn immunoreactivity. Moreover, co-staining with alpha-syn and Thioflavin-S (Fig.18B-C), a fluorescent marker specific for the fibrillary form of alpha-syn (Rideout et al., 2002), have been performed in TH-positive LRRK2-PD neurons. The results show that Thioflavin S immunostaining was negative, suggesting that mutant LRRK2 not only impairs DA neuron morphology, but also induces spontaneous increase of non-fibrillary alpha-syn.

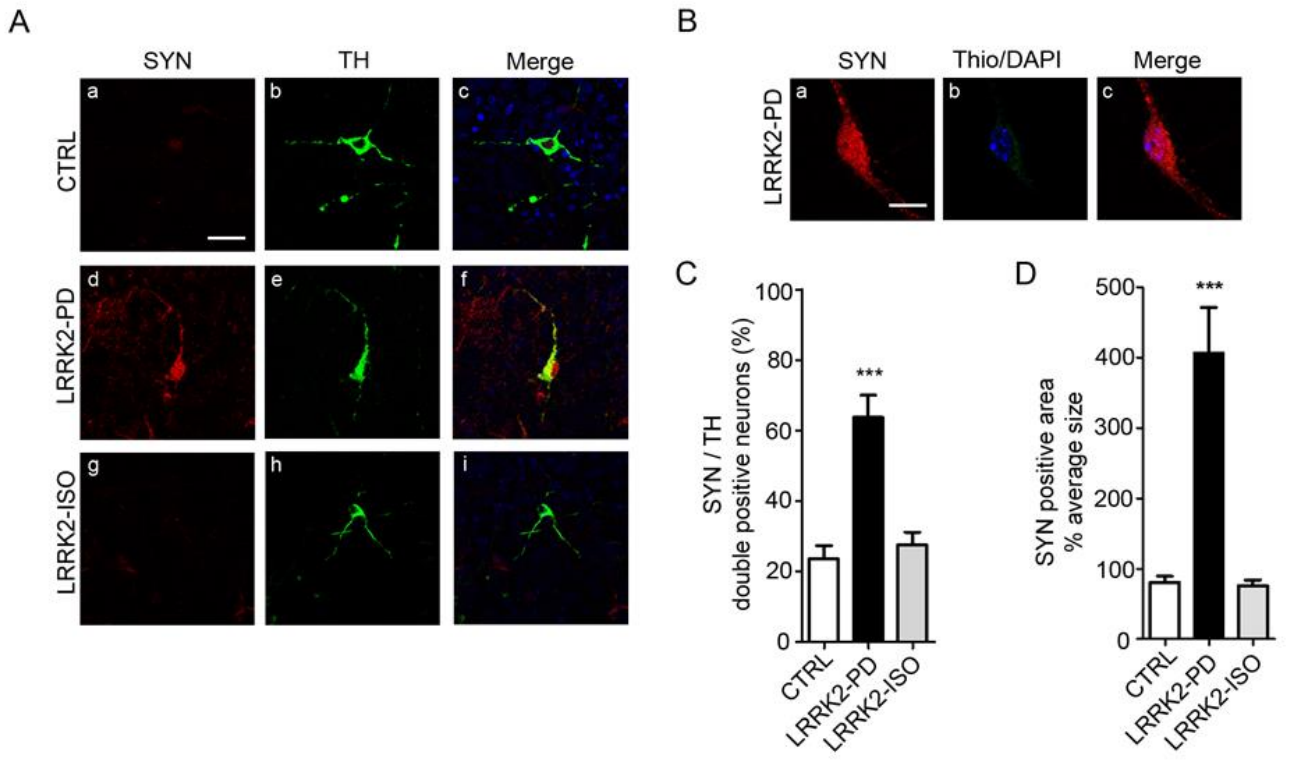


Fig.18: Alpha-synuclein accumulation in DA neurons derived from LRRK2 G2019S (A) Representative images of immunofluorescence analysis of alpha-syn (SYN; red), TH (green) and co-staining (merge) in DA neurons derived from CTRL (panels a-c), LRRK2-PD (panels d-f), and LRRK2-ISO (panels g-i) at day 50 of differentiation. Nuclei were stained with DAPI (blue). Scale bar=50 µm. Images were acquired by confocal microscopy (**B)** Representative images of immunofluorescence analysis of alpha-syn (SYN; red), Thioflavin-S (green) and co-staining (merge) in DA neurons derived from LRRK2-PD. Nuclei were stained with DAPI (blue). Scale bar=20 µm. (**C**) Quantitative analyses of TH-positive DA neurons showing cytoplasmic immunoreactivity of alpha-syn (***p=<0.0001, vs CTRL; post hoc Bonferroni's test); **D**) quantification of the percentage average size of alpha- syn-immunopositive area (***p=<0.0001, vs CTRL; post hoc Bonferroni's test). Bars represent the mean ± S.E.M. of three independent experiments (CTRL: n=1; LRRK2-PD: n=2; LRRK2-ISO: n=2).

4.2.2 DA neurons from LRRK2 G2019S are resistant to the neurotrophic effects of nicotine and D3R preferring agonists

The effects of D3R agonists and nicotine on the morphological characteristics of LRRK2-PD DA neurons, induced by D3R-nAChR stimulation was next investigated. To this aim, the two LRRK2-PD iPSC, their corresponding LRRK2-ISO and control neurons were exposed to the D3R-preferential agonist ropinirole or to nicotine (both at 10 µM) for 72 hours and morphologically analyzed by immunocytochemistry. Each line was individually analyzed with superimposable results. As shown in Fig. 19, in TH-positive control neurons (Fig.19 panels a-c), ropinirole and nicotine significantly increased the maximal length of the primary dendrite (Fig.19B), the dendrite number (Fig19C), and

the soma area (Fig.19D). By contrast, TH-positive neurons derived from LRRK2-PD were almost completely resistant to the neurotrophic effects of both compounds (Fig.19 panels d-f). Ropinirole and nicotine, however, recovered their neurotrophic properties in LRRK2-ISO TH-positive neurons (Fig.19, panels g-i), suggesting a close correlation between LRRK2 G2019S mutation and the inability of both D3R and nAChR agonists in exerting remodeling properties on DA neurons.

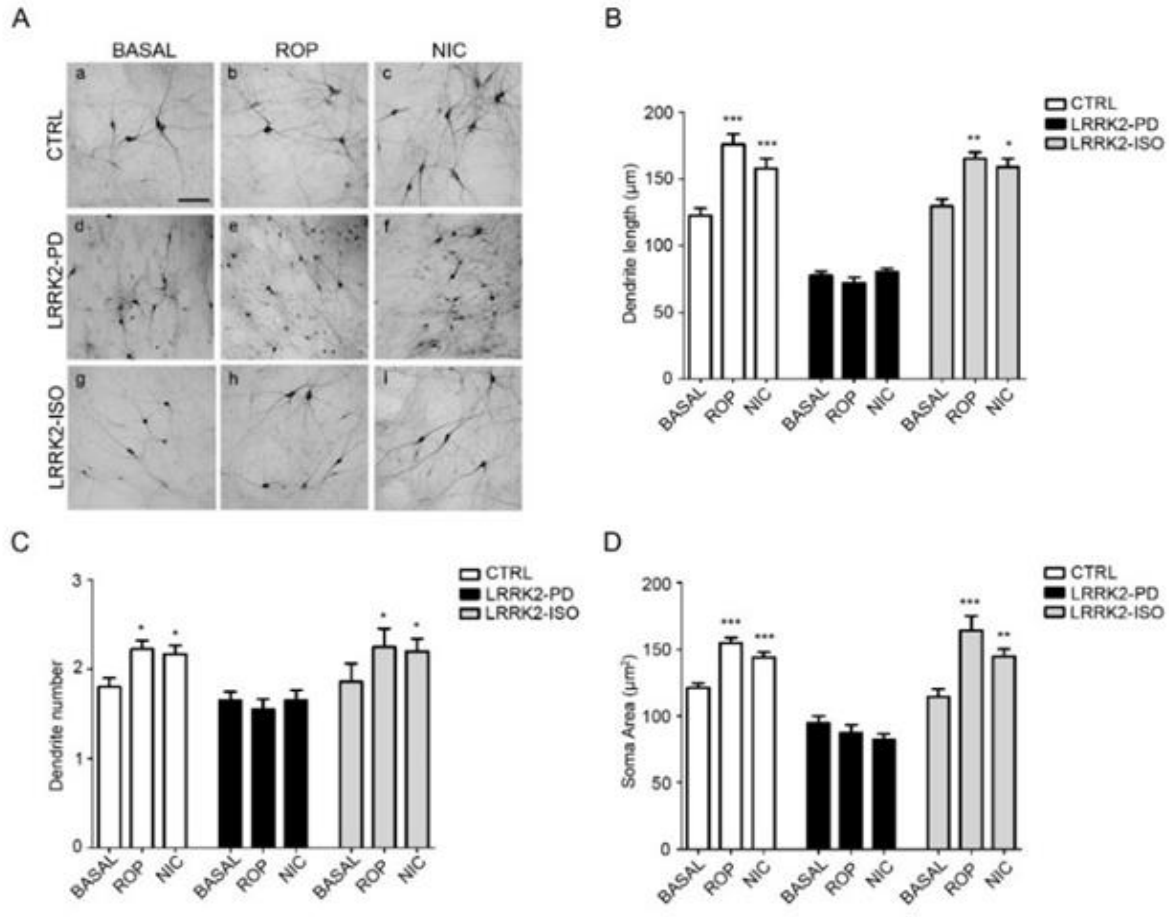


Fig.19: Stimulation of D3R and nAChR fails to promote structural plasticity in LRRK2-PD-derived DA neurons. A) Representative microphotographs of TH-positive neurons derived from CTRL (panels a-c), LRRK2-PD (panels d-f), and LRRK2-ISO (panels g-i) following exposure for 72 hours to ropinirole (ROP; 10 µM) or nicotine (NIC; 10 µM). Scale bar: 100 µm**. B-D)** Effects of ropinirole and nicotine on maximal dendrite length **(B)** (CTRL: p<0.0001, LRRK2-ISO: p<0.0001,) number of primary dendrites **(C)** and soma area **(D)** (CTRL: p<0.0001, LRRK2-ISO: p<0.0001. Bars represent the mean ± S.E.M. of three independent experiments (***p<0.001, **p<0.01, *p<0.05 vs basal; post hoc Bonferroni's test) (CTRL: n=1; LRRK2-PD: n=2; LRRK2-ISO: n=2).

4.2.3 Altered membrane localization of D3R and nAChR in DA neurons derived from LRRK2-PD iPSC

Since mutations in LRRK2 gene have been associated with perturbed vesicle trafficking, a potential molecular consequence of mutant LRRK2 expression could be abnormal receptor localization at neuronal membranes. On this line, we investigated whether aberrant D3R and/or nAChR neuronal localization might underlie the inability of ropinirole and nicotine in exerting neurotrophic effects on DA neurons carrying G2019S mutant LRRK2. The two LRRK2-PD and the corresponding LRRK2-ISO iPSC-derived neurons and control neurons were analyzed by immunofluorescence and confocal microscopy for the cell localization of both D3R and nAChR with superimposable results. Preliminary experiments have been performed in order to define the specificity of the primary anti-D3R antibody in labeling D3R, but not dopamine D2 receptors (D2R) (see below, fig.34), both expressed on dopaminergic neurons and characterized by a high protein sequence homology (Bono et al.,2020).

As shown in Fig.20A, in control and in LRRK2-ISO TH-positive neurons the D3R was detected as small fluorescent puncta mainly localized at the plasma membrane of both soma and neurites (Fig.20A, panels a-d and k-n, respectively). By contrast, an evident decrease of cell surface fluorescence signal was observed in LRRK2-PD TH-positive neurons, that displayed a significant D3R accumulation into intracellular compartments (Fig.20A, panel f-i). Interestingly, D3R co-staining with the RCAS1 Golgi marker (Engelsberg et al., 2003), (Fig.20A, panel j and Fig.20B), indicates that D3R was mainly accumulated into the Golgi compartment.

Analysis of nAChR neuronal localization was carried out with a specific antibody for the alpha4 subunit, the fundamental subunit of neuronal nAChR expressed in DA neurons (Champtiaux et al., 2003; Fiorentini et al., 2015). The results show that the specific alpha4 staining was localized at the cell surface in both control and LRRK2-ISO TH-positive neurons (Fig.20B, panels a-d and k-n, respectively), suggesting that the nAChR was mostly localized at the plasma membrane. By contrast, the alpha4 cell surface staining was significantly decreased in LRRK2-PD neurons (Fig 20C, panel f-i); similarly, to the D3R, the alpha4 nAChR subunit was co-localized with the Golgi marker, RCAS-1 (Fig. 20C, panel j and Fig 20D).

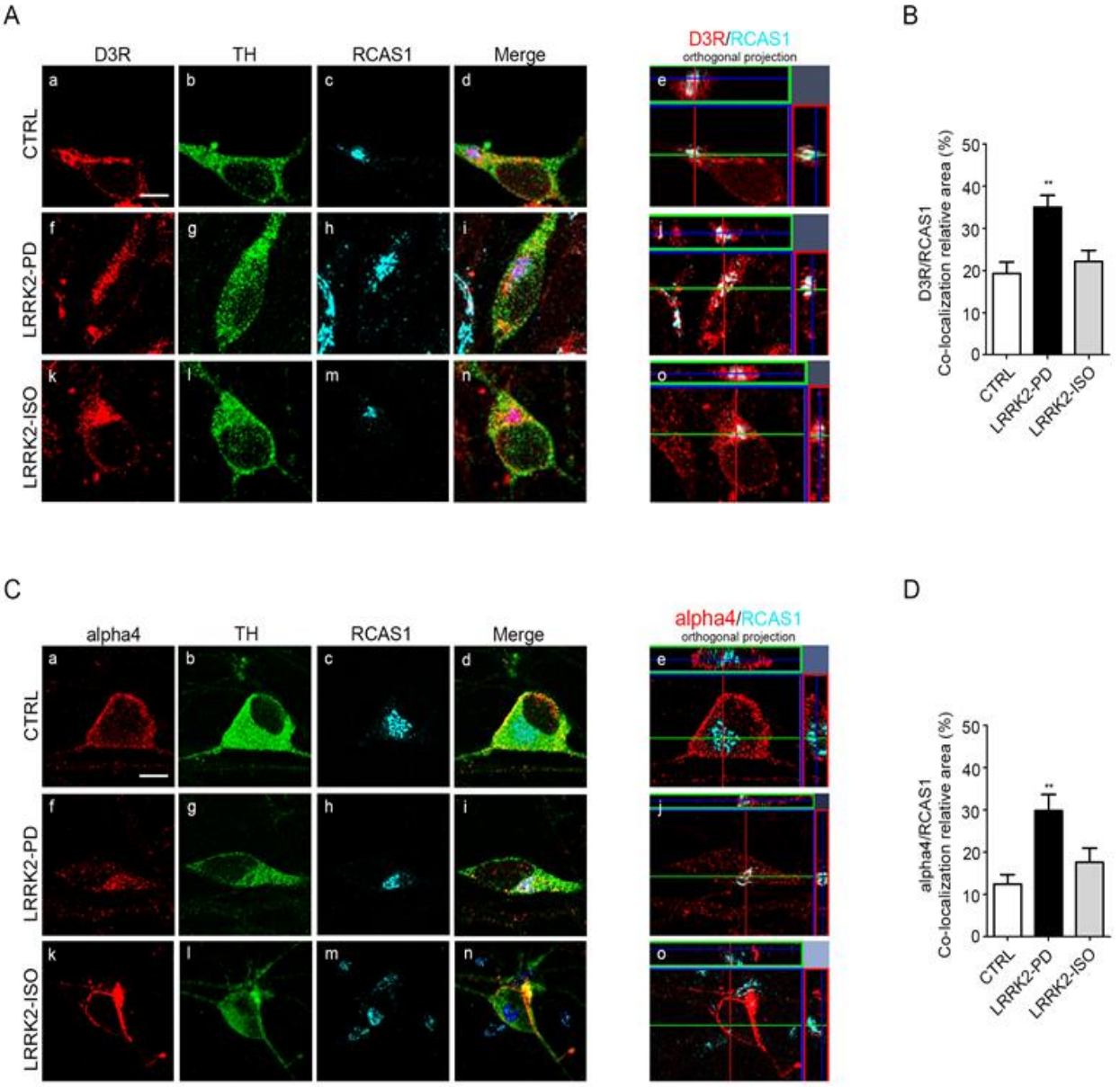


Fig.20: Altered D3R and alpha4 nAChR subunit localization in LRRK2-PD-derived DA neurons. (A) Representative images of immunofluorescence analyses of D3R (red), TH (green), the Golgi marker RCAS1 (cyan) and co-staining (merge) in DA neurons derived from CTRL (panel a-d), LRRK2-PD (panel f-i) and LRRK2-ISO (panel k-n) at day 50 of differentiation. Orthogonal projection of the D3R/RCAS1 co-staining (panel e, j, o) Scale bar=20 µm. **(B)** Quantification of the D3R/RCAS1 co-localization relative area. Bars represent the mean ± S.E.M. of three independent experiments **p < 0.01 vs CTRL. Statistical significance of the result was defined by Anova followed by Bonferroni's test. (CTRL: n=1; LRRK2-PD: n=2; LRRK2-ISO: n=2). (**C)** Representative images of immunofluorescence analyses of alpha4 (red), TH (green), RCAS1 (cyan), and co-staining (merge) in DA neurons derived from CTRL (panel a-d), LRRK2-PD (panel f-i), and LRRK2-ISO (panel k-n) at day 50. Orthogonal projection of the alpha4/RCAS1 co-staining (panel e, j, o). Scale bar=20 µm. **(D)** Quantification of the alpha4/RCAS1 co-localization relative area. Bars represent the mean ± S.E.M. of three independent experiments **p < 0.01 vs CTRL. Statistical significance of the result was defined by Anova followed by Bonferroni's test (CTRL: n=1; LRRK2-PD: n=2; LRRK2-ISO: n=2).

The observation that the mRNA encoding for D3R and the alpha4 subunit of nAChR, measured by RT-PCR, did not change in LRRK2-PD neuronal cultures, individually analyzed, compared to both their corresponding LRRK2-ISO and control neurons (Fig.21), suggests that G2019S mutant LRRK2 impacts on D3R and nAChR trafficking between intracellular sites and the cell surface.

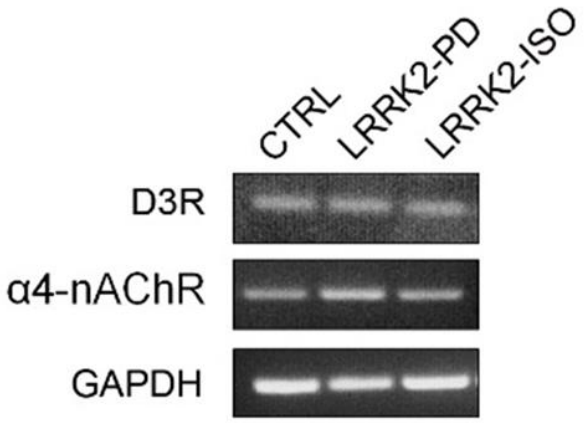


Fig.21: mRNA expression level of D3R and the α4 subunit of nAChR, did not change in LRRK2-PD neuronal cultures. Representative gel figure of the expression of D3R and α4 subunit of nAChR. Expression level was detected by using RT-PCR in DA neurons derived from healthy control (CTRL: n=1), LRRK2-PD (LRRK2-PD: n=2) and LRRK2-ISO (LRRK2-ISO: n=2). Analysis of GAPDH mRNA was used as endogenous control.

Since in DA neurons the D3R and nAChR are assembled into the D3R-nAChR heteromeric complex (Bontempi et al., 2017; Bono et al., 2019), the localization of D3R-nAChR heteromer in LRRK2-PD, LRRK2-ISO and control DA neurons were also analyzed by the "in situ" proximity ligation assay (PLA), with superimposable results (Bellucci et al., 2014; Bontempi et al., 2017). PLA was performed with primary antibodies against D3R and the nAChR alpha4 subunit, since commercial antibodies recognizing the beta2 subunit, the subunit that directly interacts with the D3R, are not recommended for immunofluorescence. Confocal images showed numerous dot-like staining (PLA signal) in TH-positive control and LRRK2-ISO neurons, mostly located at the cell membrane (Fig.22, panels a-c and g-l). Interestingly, in TH-positive LRRK2-PD neurons, PLA signal was not observed, neither at the plasma membrane nor into cytoplasmic sites (Fig.22, panel d-f), suggesting that alteration of vesicles dynamics induced by G2019S mutant LRRK2 not only perturbs D3R and nAChR trafficking to the plasma membrane, but also abolishes their interaction to form the D3R-nAChR heteromer.

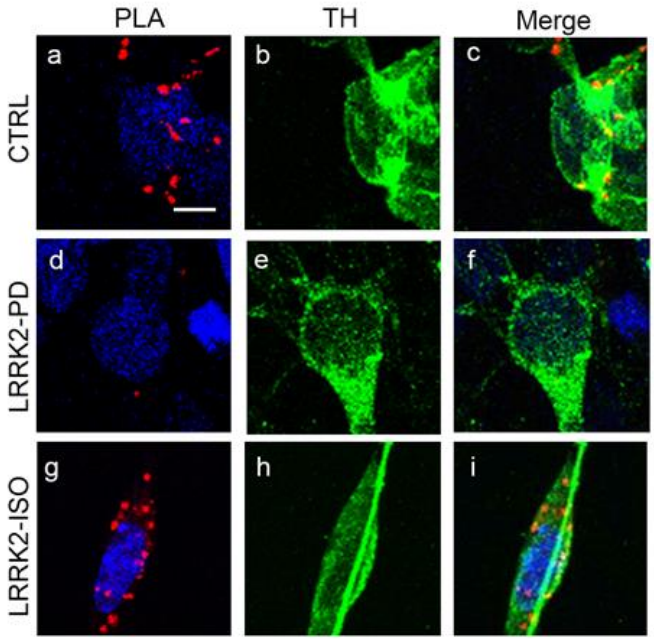


Fig.22: Altered D3R-nAChR heteromer expression in LRRK2 G2019S iPSC-derived DA neurons. PLA was performed with the anti-D3R and the anti-alpha4 nAChR subunit antibodies. Representative images of PLA signals (red spots) in TH-positive DA neurons (green) derived from CTRL (panel a-c), LRRK2-PD (panel d- f), LRRK2-ISO (panel g-i). Nuclei are detected with DAPI (blue) Scale bar=20 µm.

4.2.4 Normalizing LRRK2 activity rescues D3R-nAChR heteromer formation and localization at the plasma membrane and restores the neurotrophic effects of ropinirole and nicotine.

As previous described, the G2019S mutation has been associated with increased LRRK2 kinase activity (Price et al., 2018) and treatment with LRRK2 inhibitors has been shown to rescue neuronal structural defects in iPSC-derived DA neurons (Weykopf et al., 2019). In this study, LRRK2-PD neurons derived from both the two iPSC lines were treated with the LRRK2 inhibitor GSK2578215A (GSK; 100 nM) for 10 days, starting from day 40 of differentiation. The efficacy of treatment was monitored by measuring the levels of Ser935-phosphorylated LRRK2 (phospho-ser LRKK2) by Western Blot. As shown in Fig 23A, LRRK2-PD neurons were characterized by high levels of phospho-ser LRRK2 without substantial alterations in total LRRK2 levels. GSK treatment, that significantly reduced phospho-ser LRRK2 levels, resulted in increased dendrite length, dendrite number and soma size of LRRK2-PD TH-positive neurons (Fig.23B-E), as previously described (Reinhardt et al., 2013; Qin et al., 2017; Weykopf et al., 2019). Notably, GSK treatment also led to a significant reduction of alpha-syn levels in TH-positive LRRK2-PD neurons (Fig.23F-H).

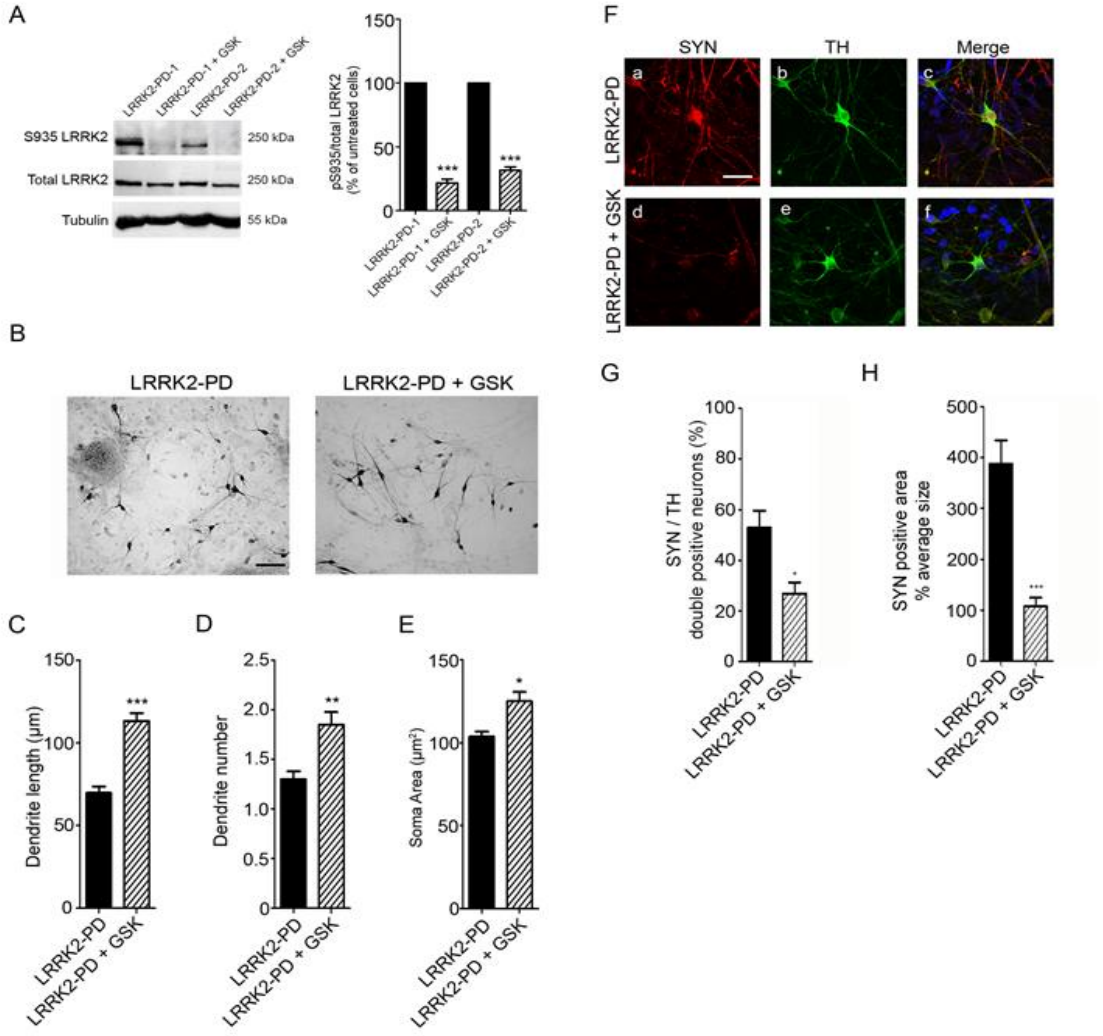


Fig.23: Pharmacological inhibition of LRRK2 kinase activity. LRRK2-PD derived DA neurons (n=2) were treated with the GSK2578215A LRRK2 inhibitor (GSK) (**A)** Representative image of Western Blot analysis of LRRK2 phosphorylation at Ser935 (S935 LRRK2) in untreated and GSK-treated LRRK2-PD neurons. Densitometric analysis of blots with specific levels of S935 LRRK2 normalized to the corresponding total LRRK2 and tubulin levels. Bars represent the mean ± S.E.M. of three independent experiments (***p<0.0001, vs untreated LRRK2-PD; post hoc Bonferroni's test). **B)** Representative microphotographs of TH-positive neurons derived from LRRK2-PD in basal condition (panel a) or after treatment with GSK (LRRK2-PD + GSK; panel b). Scale bar:100 µm. (**C-E)** Morphological analysis of LRRK2-PD and GSK-treated LRRK2-PD (LRRK2-PD + GSK) neurons on maximal dendrite length (panel C), number of primary dendrites (panel D), and soma area (panel E). Bars represent the mean ± S.E.M. of three independent experiments (**F)** Representative images of immunofluorescence analyses of alpha-syn (SYN; red), TH (green) and co- staining (merge) in DA neurons derived from LRRK2-PD in basal condition (panel a-c) or after treatment with GSK (LRRK2-PD + GSK; panel d-f). Nuclei are detected with DAPI (blue). Scale bar=50 µm. (**G**) Quantitative analyses of TH-positive DA neurons showing cytoplasmic accumulation of alpha-syn; **H)** quantification of the percentage average size of the alpha-syn-immunopositive area. Bars represent the mean ± S.E.M. of three independent experiments (***p < 0.001; *p<0.05 vs untreated LRRK2-PD; Student's *t* test).

The effects of GSK treatment on the cellular localization of D3R and nAChR and on the formation of

the D3R-nAChR heteromer in LRRK2-PD DA neurons was investigated by immunofluorescence and PLA, respectively, with superimposable results. As shown in Fig.24, both D3R (Fig.24A) and the alpha4 nAChR subunit (Fig.24B), that showed an intracellular localization in LRRK2-PD DA neurons (panels a-c), were mostly translocated to the plasma membrane because of GSK treatment (panels d-f). Similarly, while PLA staining was undetectable in LRRK2-PD neurons (Fig.24C panels a-c), an intense signal was evident both in the soma and dendrites of LRRK2-PD TH-positive neurons exposed to GSK (Fig.24C panels d-f). Therefore, normalizing LRRK2 activity is sufficient to recover the D3R and nAChR localization at the plasma membrane and to restore the formation of the D3R-nAChR complex and its membrane localization.

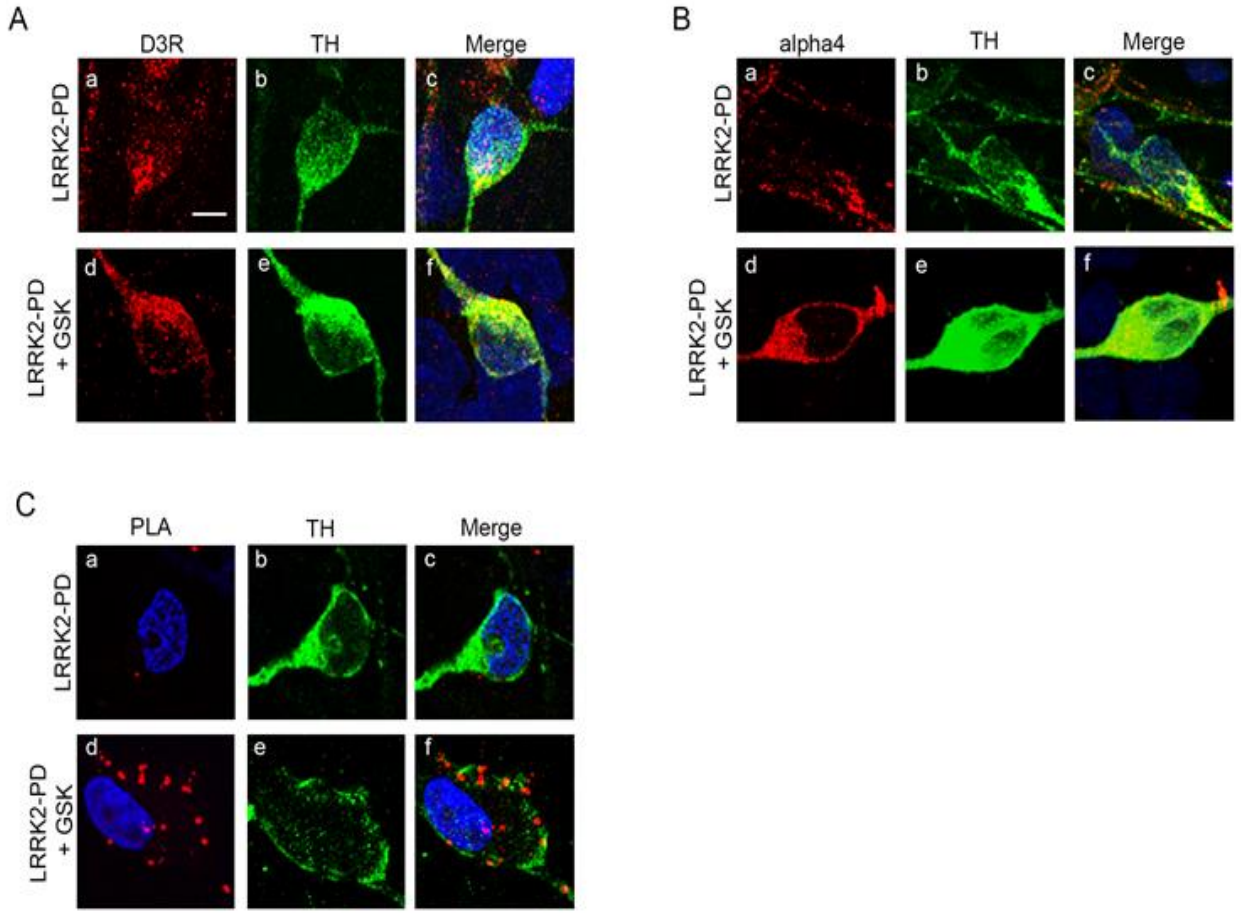


Fig.24: Pharmacological inhibition of LRRK2 kinase activity rescues D3R-nAChR heteromer formation and localization in LRRK2 G2019S iPSC-derived DA neurons. A) Representative images of immunofluorescence analyses of D3R (red), TH (green) and co-staining (merge) in DA neurons derived from LRRK2-PD in basal condition (panel a-c) or after treatment with GSK (LRRK2-PD + GSK; panel d-f). **B)** Representative images of immunofluorescence analyses of alpha4 nAChR (red), TH (green) and co-staining (merge) in DA neurons derived from LRRK2-PD in basal condition (panel a-c) or after treatment with GSK (LRRK2-PD + GSK; panel d-f). **C**) Representative images of PLA signals (red) in TH-positive neurons (green) derived from LRRK2-PD in basal condition (panel a-c) or after treatment with GSK (LRRK2-PD + GSK; panel d-f). Scale bar=20 μm.

To evaluate the functional relevance of these effects, untreated and GSK-treated LRRK2-PD neurons, derived from both the two iPSC lines, were incubated with ropinirole (10 µM) or nicotine (10 µM) for 72 hours and analyzed for morphology by immunocytochemistry, with superimposable results. As shown in Fig.25, while LRRK2-PD neurons were unaffected to these treatments, LRRK2-PD neurons previously exposed to GSK responded to both compounds by increasing primary dendrite maximal length (Fig.25B), dendrite number (Fig.25C), and soma area (Fig.25D). Taken together these data suggest that normalizing LRRK2 activity rescues LRRK2-PD DA neurons from a pathological phenotype.

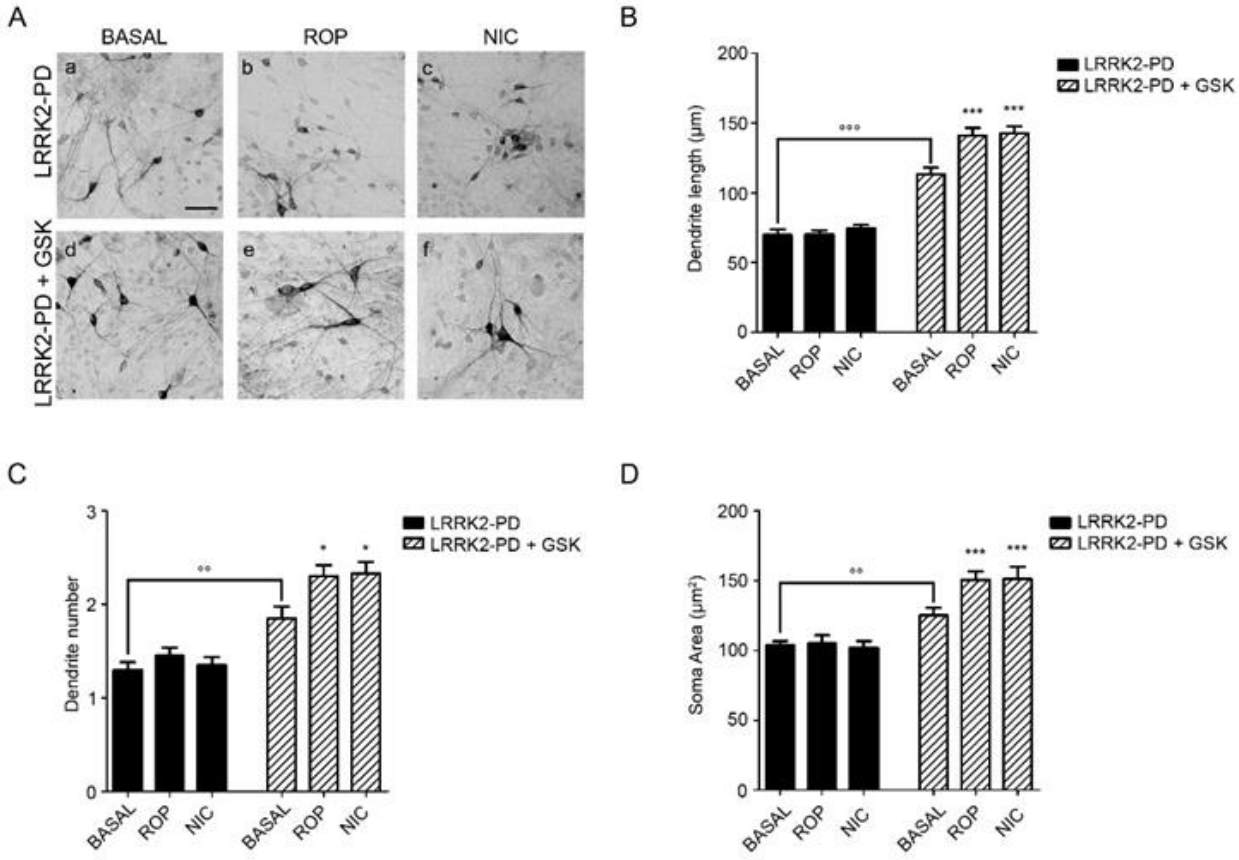


Fig.25 Pharmacological inhibition of LRRK2 kinase activity restores the ropinirole- and nicotine induced structural plasticity in LRRK2 G2019S iPSC-derived DA neurons. (A) Representative microphotographs of TH-positive neurons derived from untreated (panel a-c) and GSK-treated LRRK2-PD (panel d-f) at basal condition or after treatment with ropinirole (ROP; 10 µM) or nicotine (NIC; 10 µM). Scale bar:100 µm; (**B-D**) Morphologic effects induced by a 72-hour exposure to ropinirole (ROP) or nicotine (NIC) on maximal dendrite length (**B**; ***p<0.0001, vs basal), number of primary dendrites (**C;***p<0.05 vs basal), and soma area (**D**; ***p<0.0001vs basal). Bars represent the mean ± S.E.M. of three independent experiments (***p<0.001, *p<0.05 vs basal; post hoc Bonferroni's test; °°°p<0.001, °°p<0.01, °p<0.05 vs LRRK2-PD; Student's *t* test).

4.3 Astrocyte differentiation from human induced pluripotent stem cells

Neuroinflammation is increasingly recognized as an important feature in the pathogenesis of PD (McGeer and McGeer, 2008; Halliday and Stevens, 2011; Wang et al., 2015). However, it remains unclear whether neuroinflammation contributes to nigral degeneration in PD or is merely a secondary marker of neurodegeneration. In the central nervous system, immune responses are mainly mediated by microglia and astrocyte

In particular, several lines of evidence indicate that astrocytes might be active players in neurodegeneration associated to PD (Mirza et.,2000; Oksanen et al., 2019). Since LRRK2 is also expressed in astrocytes (Booth et al., ,2017) and the expression of dopamine receptor, such as D2R and D3R may been involved in the control of neuroinflammation D3R (Montoya et al., 2019; Broome et al., 2020) we have supposed that in PD patients carrying the LRRK2 mutation, astrocyte D2R/D3R dysfunction may represent a relevant molecular event leading to detrimental neuroinflammation and therefore PD progression. On these bases, iPSC derived from the two different PD patients carrying the G2019S LRRK2 mutation, as well as from their corresponding isogenic gene-corrected-counterpart and a healthy control were differentiated toward astrocytes in which investigate abnormalities in D2R/D3R expression as a distinctive feature associated with LRRK2 G2019S mutation.

4.3.1 Differentiation and characterization of human iPSC-derived astrocytes

We have recently developed a protocol for the differentiation of human astrocyte starting from iPSC, based on Yan et al., (2013) protocol, which allows to obtain mature astrocytes from iPSC after 40 days of differentiation (Filippini et al.,2020) (Fig 26). Briefly, iPSC were first incubated with neuronal induction medium (Gibco) for 7 days to obtain primitive neural stem cells (pNSC) and expanded for additional 7 days. NSC were then incubated with DMEM supplemented with FBS and N2 for additional 16-20 days to obtain cultures of mature astrocytes. iPSC (day 0), NCS (day 7) and astrocytes (day 40) were morphological observed and analyzed for the expression of different markers by using immunofluorescence. In particular, the expression of the protein Sox2, a nuclear marker typically expressed in undifferentiated cells, Nestin, a NSC marker, GFAP protein, specific markers expressed in the mature astrocytes and MAP2, a neuronal marker, were analyzed. During the differentiation process from iPSC to astrocytes, cells undergo a dramatic change that can be monitored by a phase contrast microscope. As shown in Figure 27, in fact, iPSC have morphological characteristics typical of stem cells, with a tendency to form high cell density characterized by

defined and translucent edges (Fig.27 panel a). Subsequently, after a few days in culture in the presence of the induction medium, cells, organized in small groups, begin to acquire an elongated phenotype, characterized by some short extensions, typical of NSC (Fig.27 panels b-e). Finally, NCS morphology change toward that typical of astrocytes, characterized by starry morphology (Fig.27 panel f).

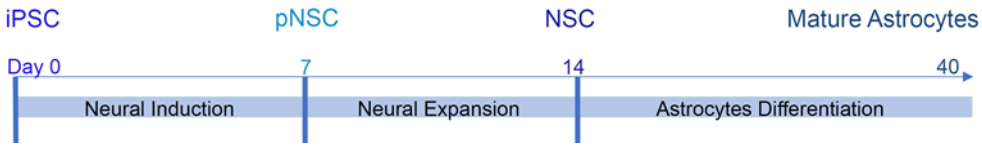


Fig.26 Timeline and culture condition of iPSC differentiation into astrocytes. Timeline representing the different steps of iPSC differentiation into mature astrocytes.

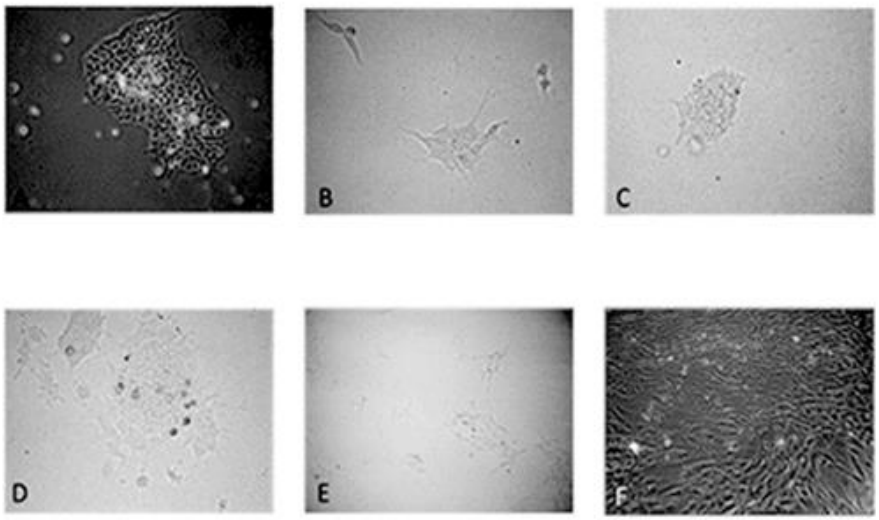


Fig.27 The morphology of cells during neural induction. (A): iPSC. (**B):** iPSC at day 1 of splitting**. (C–E):** The morphology of cells at days 3 (**C),** 7 **(D),** 8 **(E), (F):** mature astrocytes day 40. Image obtain with Bright field microscopy 40X

Immunofluorescence analyses shown that at day 0 (iPSC), a high percentage of cells were positive only for the nuclear marker Sox2 (95%) (Fig.28A panel a). Starting from day NSCs a high percentage of cells (%) expressed Nestin (Fig.28A panel b), but not SOX2 or the astrocytes and neuronal markers, expressed while GFAP and MAP2, respectively. These results suggest that cells derived from iPSC by

neural induction medium possess the NSC phenotype and there is not evident contamination by other cell types, such as neurons.

At the end of differentiation process (40 days) (Fig.28 A panel c), ~45% of cells expressed the astrocytes marker GFAP (Fig.28B), while SOX2, NESTIN and MAP2 were almost totally absent. According to classical taxonomy, astrocytes are divided into two major classes, protoplasmic astrocytes in the gray matter and fibrous astrocytes in the white matter (Miller et al., 1984). Protoplasmic astrocytes have highly branched and complex processes that contact blood vessels and neurons, whereas fibrous astrocytes have elongated, more simple processes that contact blood vessels, oligodendrocytes, and axons at the nodes of Ranvier (Lundgaard et al., 2014). Based on astrocytes appearance, both protoplasmic and fibrous astrocytes were equally expressed in the cultures (Fig.28C).

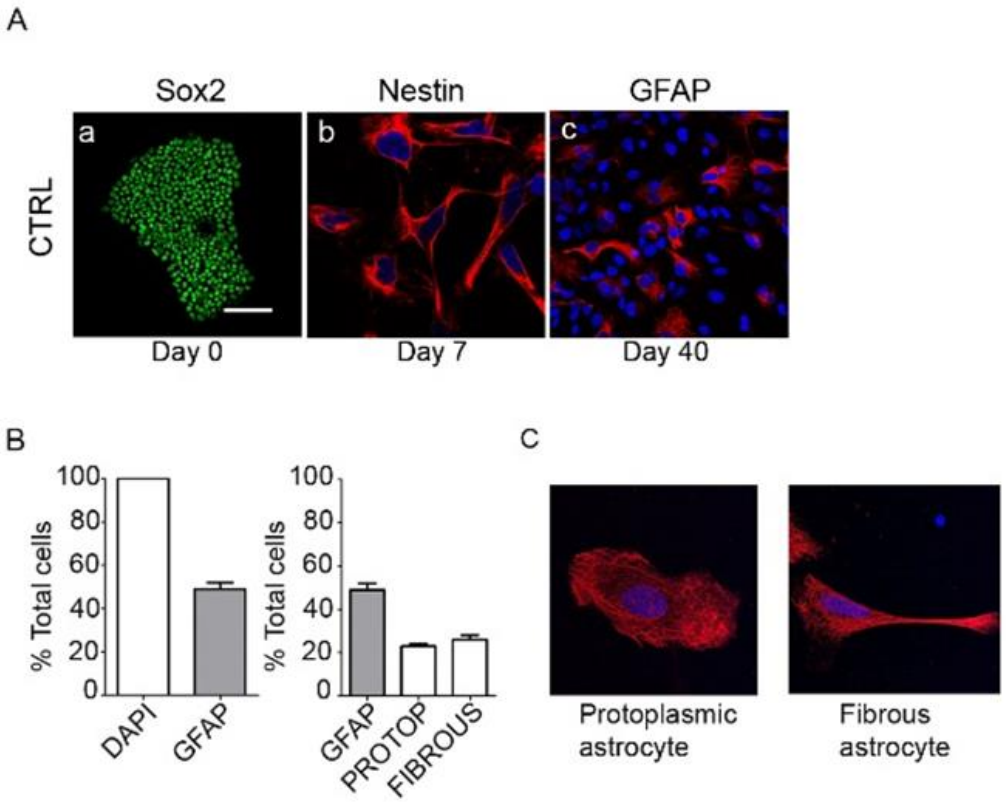


Fig.28: Characterization of human astrocytes derived from healthy control (**A)** Representative immunofluorescence analyses of Sox2 (day 0) (green, panel a), Nestin (red, panel b) (day 7) and GFAP (day 40) (red, panel c) of iPSC-Derived astrocytes. (**B)** GFAP-positive cells were counted. Bars represents the mean ± S.E.M. of three independent experiments (**C**) GFAP-positive cells show 2 different morphologies: protoplasmic cells and fibrous cells

To establish the functional maturity of iPSC-derived astrocytes, two different experimental approaches have been used: first, measurement of the spontaneous intracellular Ca^{2+} waves, as an indirect measure of activity in astrocytes, and measurement of the ability of astrocytes in sustaining the dendritic arborisation of neurons in iPSC-derived astrocytes/mouse dopaminergic neurons co-cultures experiments.

Calcium (Ca^{2+}) wave propagation across astrocytes is important for neuron-glia and glia-glia communication (Scemes, 2006). To measure astrocytes Ca^{2+} waves we used the Fluo4-AM fluorophore, a lipophilic fluorochrome able to bind intracellular calcium and emitting, after excitation, a different fluorescence intensity depending on the calcium levels. In this experiment, Ca^{2+} fluxes were measured in 5 different astrocyte cells. Briefly, viable astrocytes were cultured in an eight-well chamber slide and incubated with Fluo4-AM fluorophore for 1 hour at 37°C. Cells were then analyzed for 5 min using a Zeiss LSM 880 confocal microscope equipped with Plan-Apochromat 63×/1.4 numerical aperture oil-objective. Recorded data were examined using Zen 2.3 Software (Carl Zeiss AG). As shown in Fig.29, astrocytes were characterized by an intense fluorescence detected throughout the astrocytic area, including soma and cell processes. In addition, under basal conditions, each astrocyte shown heterogeneous patterns of Ca^{2+} fluctuations indicating that these cells are functionally active.

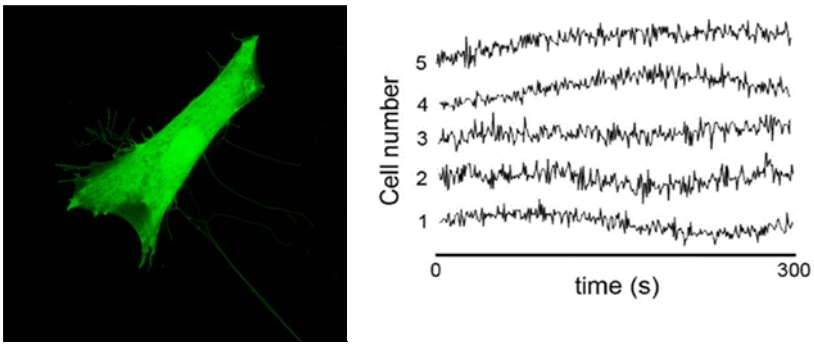


Fig 29: iPSC-derived astrocyte characterization Graph representing single functional astrocyte calcium waves of iPSC-derived astrocytes from healthy control. Calcium wave flux recording for 300s with calcium tracer Fluo4-AM at 40 days (n = 5).

To investigate the astrocytic ability to sustain neurons homeostasis, a co-culture system combining mouse dopaminergic neurons and iPSC-derived astrocytes was developed. To this aim, primary

cultures of midbrain neurons, generated as described above, were seeded on a confluent monolayer of iPSC-derived astrocytes (~40 days of differentiation) or on Poly-D-lysine/laminin-coated wells, used as a control, and cultured for 7 days. Cells were then fixed and incubated with anti-TH primary antibody. Mouse TH-positive neurons were then analyzed for the maximal dendrite length, the number of primary dendrites and the soma area as described. The observation that the TH-positive neurons co-cultured with astrocytes exhibited increased soma area (Fig.30B) and improved both the length of the primary dendrite (Fig.30C) and the dendrite number (Fig.30D) compared to mouse neurons of control strongly suggest the functional ability of human astrocytes in supporting trophism.

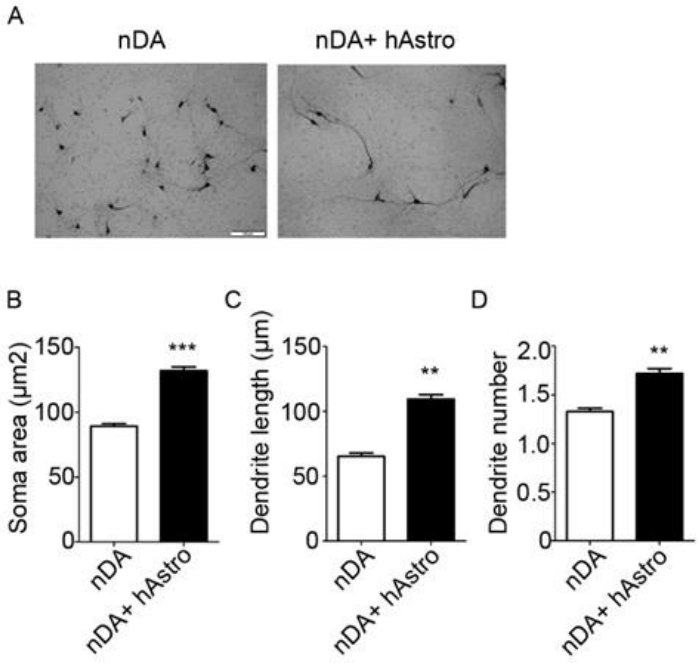


Fig.30: DA neurons (nDA) co-cultured with human Astrocytes (hAstro) derived from healthy control (A) Representative images of TH immunohistochemistry of murine dopaminergic neurons (nDA) cultured alone or co-cultured with astrocyte for 7 days (40 days of differentiation). Quantification (**B)** of soma area (**C)** dendrite length and (**D)** dendrite number of murine dopaminergic neurons (nDA). Bars represent the mean ± S.E.M. ***p < 0.001, **p < 0.01, vs basal(nDA). Statistical significance of the result was defined by Anova followed by Bonferroni's test.

4.3.2 Differentiation and characterization of iPSC-derived astrocytes from LRRK2 G2019S PD

Astrocyte cultures were then generated from iPSC with the LRRK2 G2019S mutation (n=2), their respective LRRK2 G2019S corrected isogenic iPSC lines (LRRK2-ISO; n=2) and compared to astrocyte derived from control iPSC (Fig.30). iPSC (day 0) and mature astrocytes (day 40) were analyzed for Sox2, Nestin and GFAP by immunofluorescence. We found that both the two LRRK2-PD iPSC, their

corresponding LRRK2-ISO and control iPSC were characterized by a robust expression of SOX2, while at day 40, the percentage of cells derived from LRRK2-PD and LRRK2-ISO and positive for the astrocytic marker GFAP were about 45%, comparable to that obtained from control iPSC (Fig.31A). Moreover, as for astrocytes derived from control iPSC, both protoplasmic and fibrous astrocytes were equally expressed in the cultures derived from the two LRRK2-PD and their corresponding LRRK2 ISO iPSC (Fig.31B-C-D) thus suggesting that mutation in LRRK2 does not evidently affect the differentiation process from iPSC to astrocytes.

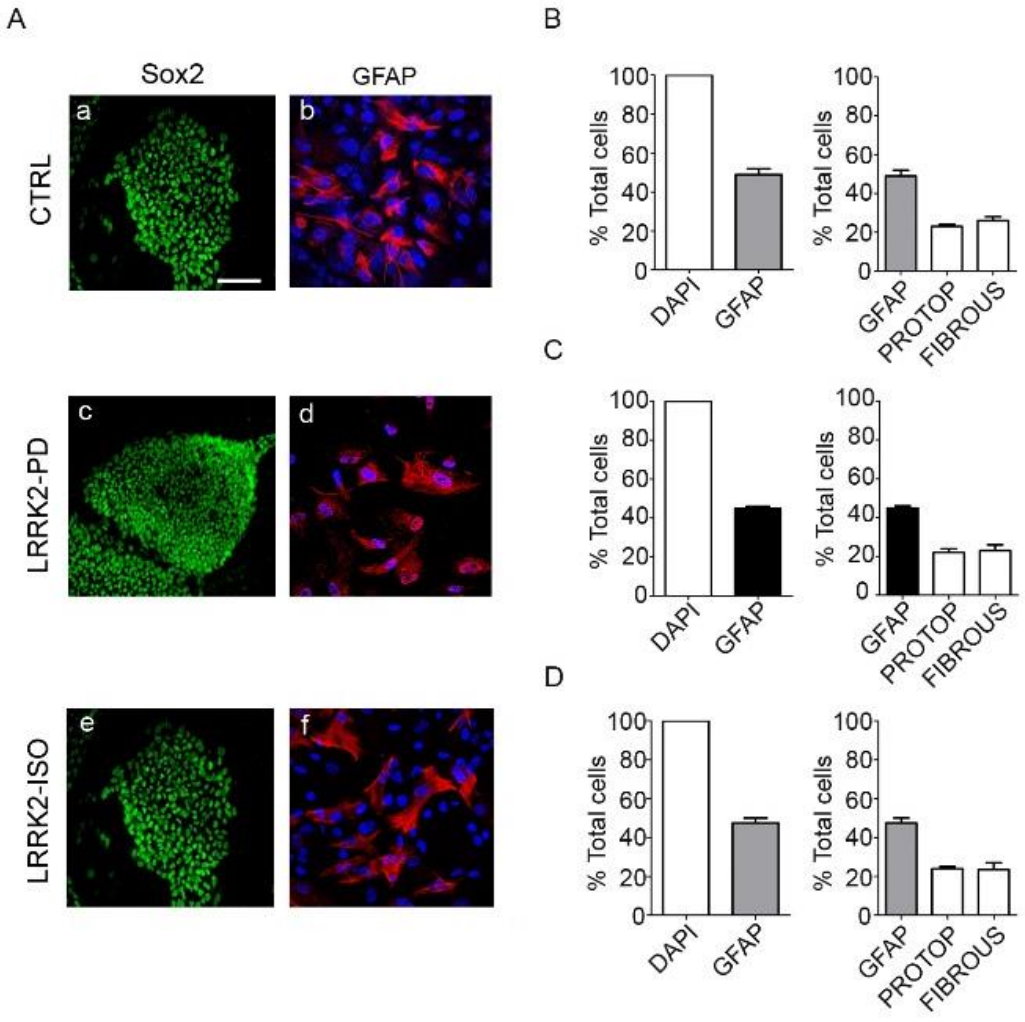


Fig.31: Characterization of human astrocytes derived from two LRRK2 patients (LRRK2-PD) and their gene-corrected isogenic lines (LRRK2-ISO) (A) Representative images of immunofluorescence analyses of Sox2 (green), Nestin (red), GFAP (green) in astrocytes derived from CONTROL (panels a-b) (LRRK2-PD (panels c-d) and LRRK2-ISO (panels e-f) at day 40 of differentiation. Nuclei are detected with DAPI (blue) Scale bar=20 µm (**B**) GFAP-positive cells derived from control were counted. GFAP-positive cells show 2 different morphologies: protoplasmic cells and fibrous cells (**C**) GFAP-positive cells derived from LRRK2-PD were counted. Bars represents the mean ± S.E.M. of three independent experiments GFAP-positive cells show 2 different morphologies: protoplasmic cells and fibrous cells. **(D**) GFAP-positive cells derived from LRRK2-ISO were counted. Bars represents the mean ± S.E.M. of three independent experiments GFAP-positive cells show 2 different morphologies: protoplasmic cells and fibrous cells

4.3.3 Functional characterization of iPSC-derived astrocytes from LRRK2 G2019S PD patients

Astrocyte cultures generated from LRRK2 PD and LRRK2-ISO were then analyzed for their spontaneous intracellular Ca^{2+} waves and for the ability of astrocytes in sustaining the dendritic neuronal arborisation in co-cultures experiments, as described above.

By using the Fluo-4 AM fluorophore, the we found that iPSC-derived astrocytes from both LRRK2 PD and LRRK2-ISO were characterized by similar intercellular Ca^{2+} waves as observed for control astrocytes (Fig.32). Each line was individually analyzed with superimposable results.

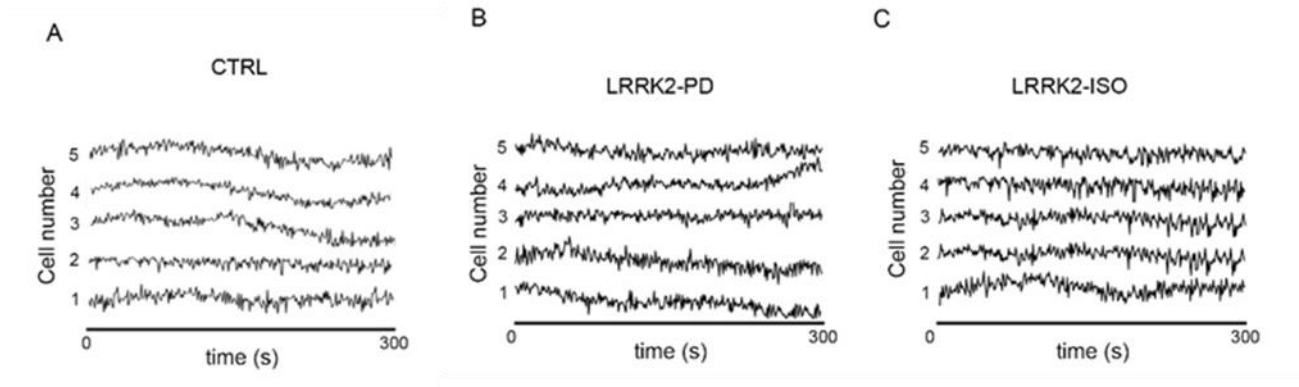


Fig.32: iPSC-derived astrocyte characterization Graph representing single functional astrocyte calcium waves of **A)** iPSC-derived astrocytes from healthy control (CTRL), (**B)** iPSC-derived astrocytes from LRRK-PD **(C)** iPSC-derived astrocytes from LRRK-ISO**.** Calcium wave flux recording for 300s with calcium tracer Fluo4-AM at 40 days (n = 5).

Interestingly, the morphological analyses of mouse TH-positive neurons co-cultured with mature astrocytes derived from both the two LRRK2-PD iPSC showed that both the dendrite branching and soma area were significantly reduced as compared to neurons co-cultured with astrocytes derived from LRRK2-ISO and control iPSC (Fig.33B-C-D).

Together, these data suggest that astrocytes carrying the LRRK2 mutation, that are numerically and apparently morphologically comparable to that derived from control astrocytes, are functionally compromised inn their ability to support neuronal trophism.

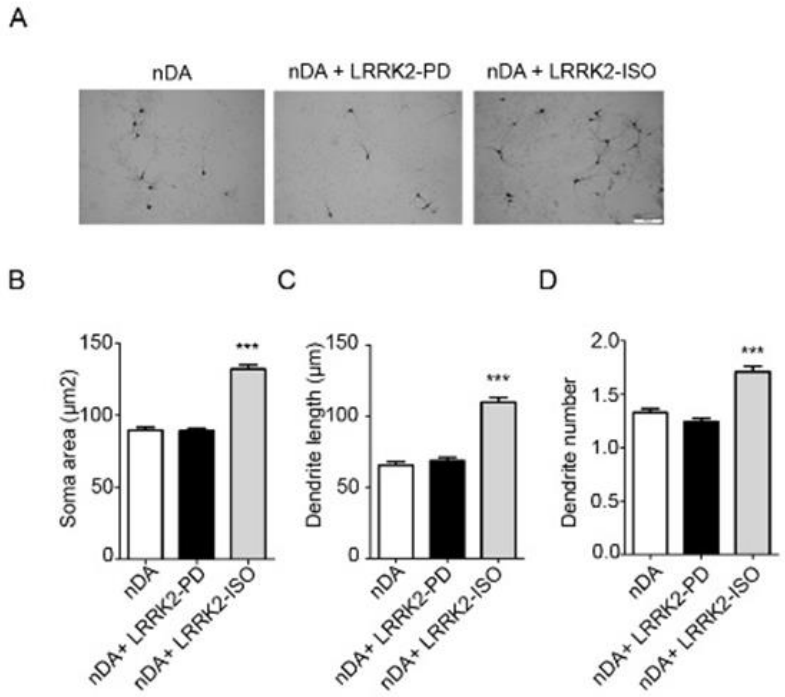


Fig.33 DA neurons co-cultured with human astrocytes-derived from two LRRK2 patients (LRRK2-PD) and their gene-corrected isogenic lines (LRRK2-ISO) (A) Representative images of TH immunohistochemistry of murine dopaminergic neurons (nDA) cultured alone or co-cultured with astrocyte for 7 days (40 days of differentiation). Quantification (**B)** of soma area (**C)** dendrite length and (**D)** dendrite number of murine dopaminergic neurons (nDA). Bars represent the mean ± S.E.M. ***p < 0.001, **p < 0.01, vs basal. Statistical significance of the result was defined by Anova followed by Bonferroni's test.

4.3.4 Dopamine D2/D3 receptors expression and localization in iPSC-derived astrocytes

As previous report D2R and D3R expressed on astrocytes has been described as key negative regulators of neuroinflammation (Zhang and Barres, 2010).

On this line, astrocyte cultures generated from LRRK2 PD and LRRK2-ISO were analyzed for the expression and localization of D2R and D3R compared to control astrocytes. Since previous data has been shown that D3R could be involved (Montoya et al., 2019), a set of preliminary experiments have been performed in order to determine whether both the D2R and D3R are expressed. The expression and localization of D2R and D3R was performed in mature iPSC-derived astrocytes (day 40) from control iPSC by using immunofluorescence and selective anti-D2R or anti-D3R antibody, previously tested on different models (Fig.34).

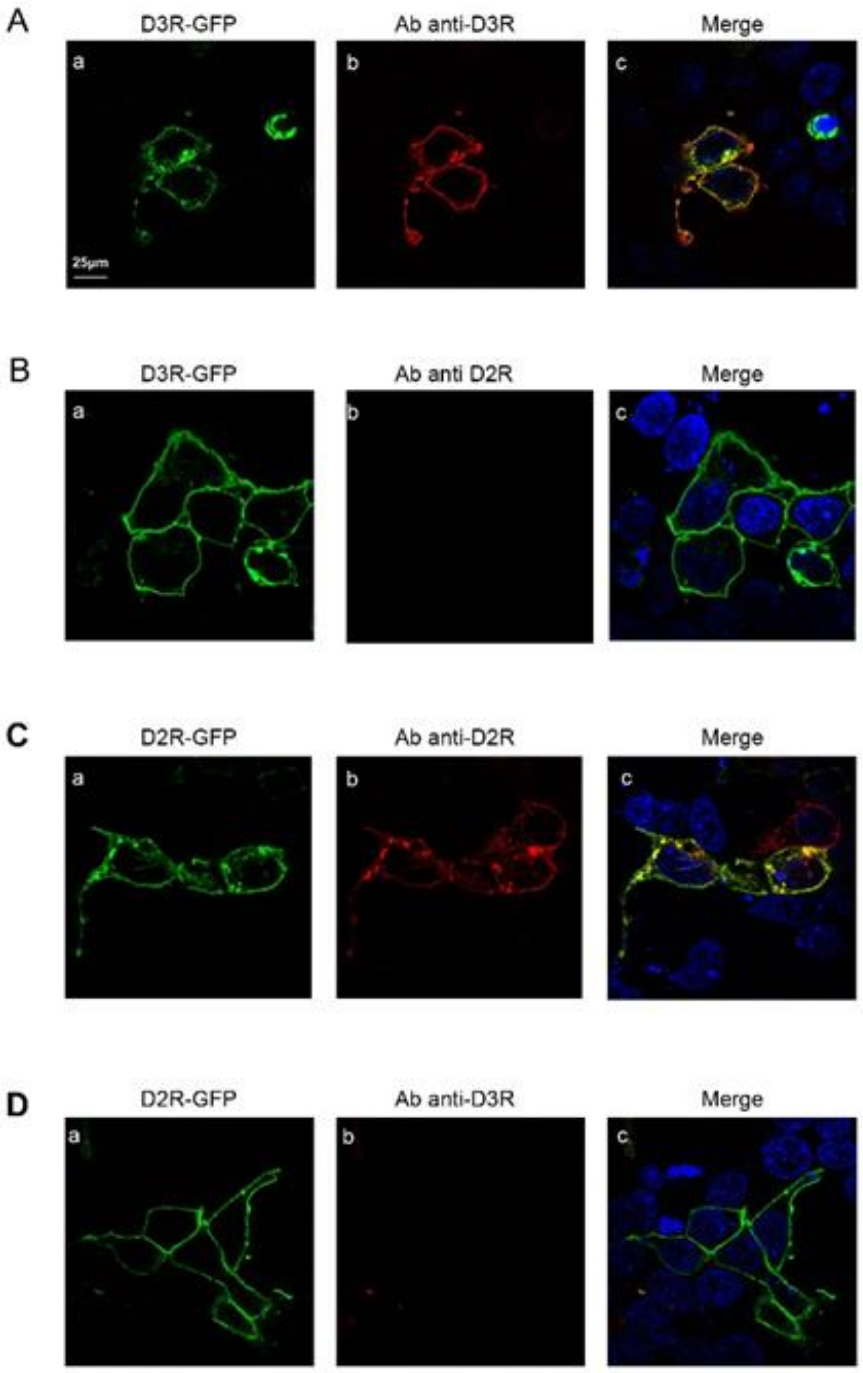


Fig.34 Characterization of the anti-D2R/D3R polyclonal antibody. (A) Immunofluorescence analysis of D3R (red) in HEK293 cells expressing the D3R-GFP (green, panel a) using the anti-D3R (panel b) polyclonal antibody. (B) Immunofluorescence analysis of D3R (red) in HEK293 cells expressing the D3R-GFP (green) using the anti-D2R polyclonal antibody (red, panel b). **(C)** Immunofluorescence analysis of D2R (red) in HEK293 cells expressing the D2R-GFP using the anti-D2R (red, panel b) polyclonal antibody. **(D)** Immunofluorescence analysis of D2R (red) in HEK293 cells expressing the D2R-GFP using the anti-D3R (red, panel b) polyclonal antibody. Nuclei are detected with DAPI (blue).

As shown in fig.35, in human astrocytes derived from healthy control, D2R is the only DA receptor subtypes mainly detected as small fluorescent puncta mainly localized at the plasma membrane of cells (Fig.35 panels a-c)

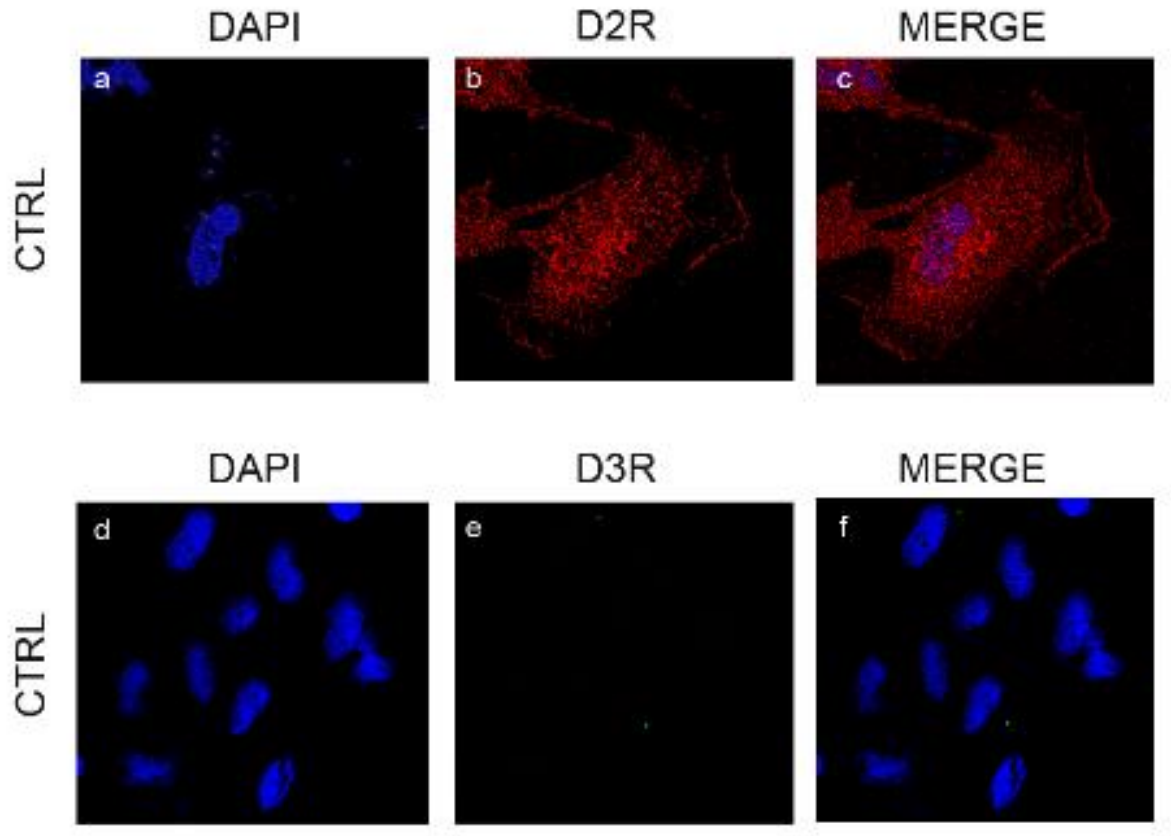


Fig.35: D2R but not D3R is selectively expressed in astrocytes. Representative images of immunofluorescence analyses of DAPI (blue), D2R (red) and co-staining (merge) in astrocytes derived from CTRL (panel a-c). Representative images of immunofluorescence analyses of DAPI (blue), D3R (green) and co-staining (merge) in astrocytes derived from CTRL (panel d-f).

Analyses of D2R localization in astrocytes derived from LRRK2-PD iPSC was next performed (Fig.36 panels d-f), showing a remarkable reduction of the receptor localization at the membrane sites, with a receptor accumulation into cytoplasmic compartments, a distribution completely reverted by correcting the LRRK2 mutation, as show in astrocytes derived from LRRK2-ISO (Fig.36 panels g-i). Each line was individually analyzed with superimposable results.

These results thus indicate in astrocytes, as in neurons, the LRRK2 G2019S mutation likely affects the ability of D2R to properly trafficking to the plasma membrane.

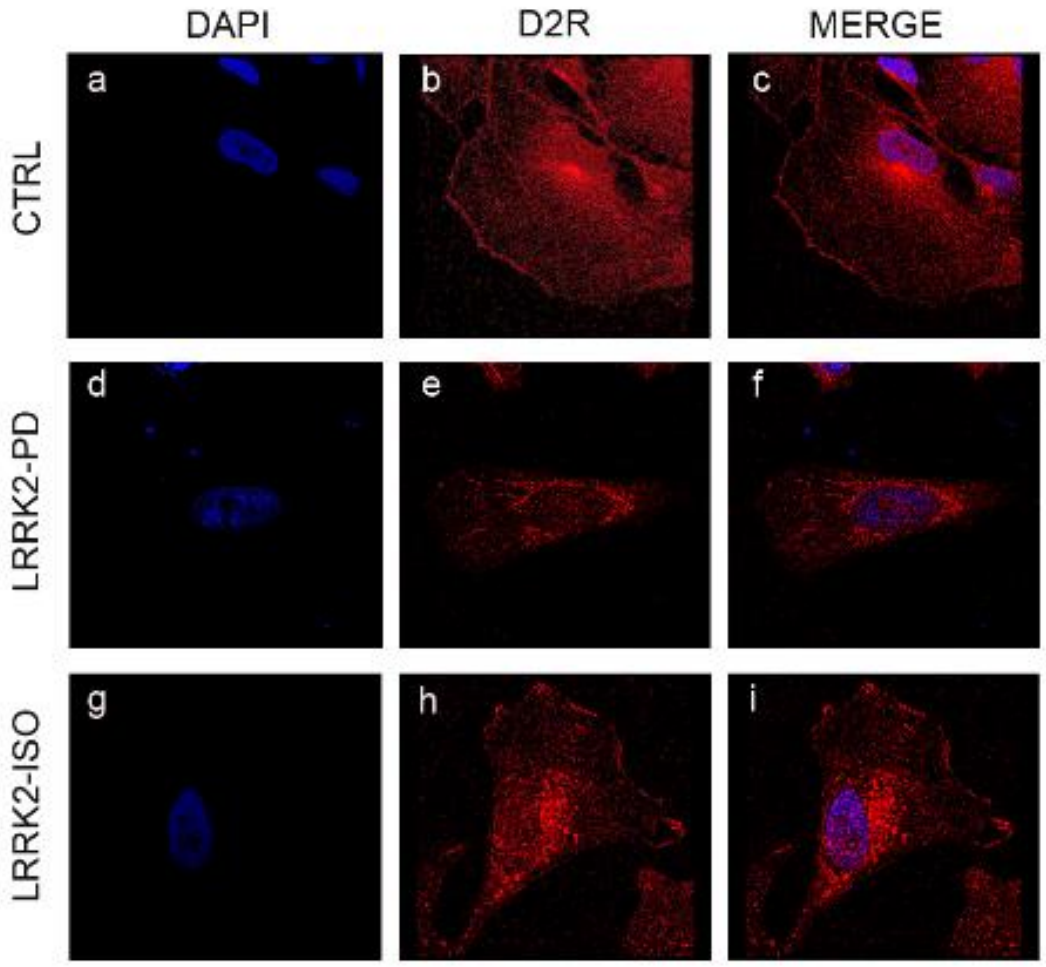


Fig.36 Altered D2R localization in LRRK2-PD-derived astrocytes. (A) Representative images of immunofluorescence analyses of DAPI (blue), D2R (red) and co-staining (merge) in astrocytes derived from CTRL (panel a-c), LRRK2-PD (panel d-f) and LRRK2-ISO (panel g-i) at day 40 of differentiation

LRRK2-PD-derived astrocytes were then treated with the LRRK2 inhibitor GSK2578215A (GSK; 200 nM) for 7 days, starting from day 30 of differentiation and the effect of GSK on the membrane localization of D2R was subsequently analyzed by immunofluorescence. As shown in Fig.37, we found that in both the astrocytes cultures carrying the LRRK2 mutation, normalizing LRRK2 activity is sufficient for recover the physiological D2R expression at the plasma membrane sites.

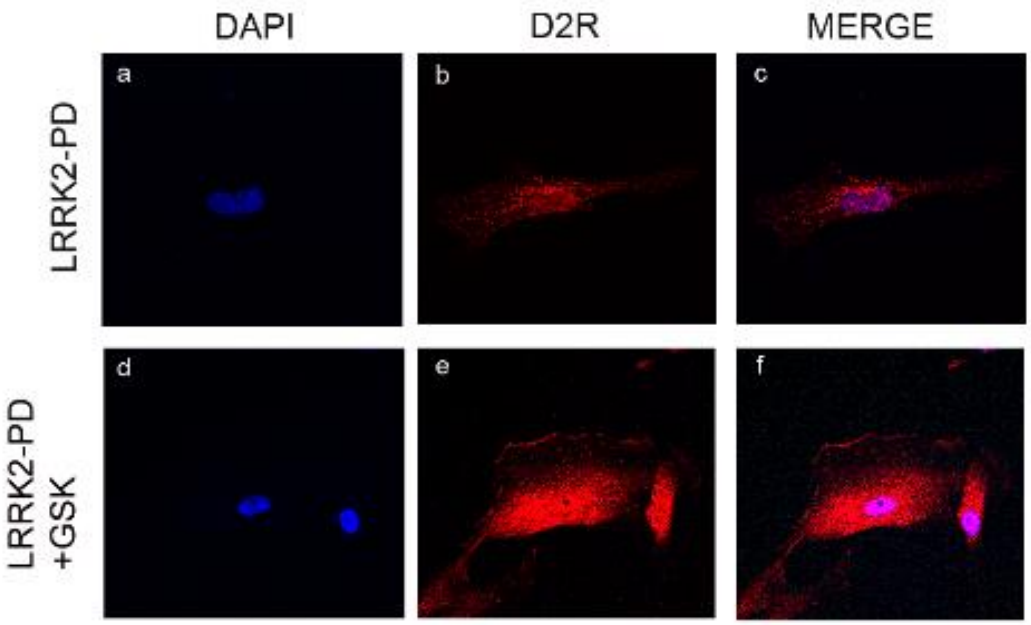


Fig. 37 Pharmacological inhibition of LRRK2 kinase activity rescues D2R localization in LRRK2 G2019S iPSC-derived astrocytes. **A**) Representative images of immunofluorescence analyses of DAPI (blue), D2R (red) and co-staining (merge) in astrocytes derived from LRRK2-PD (panel a-c) or after treatment with GSK (LRRK2-PD + GSK; panel d-f).

5 DISCUSSION

Dopamine regulates various brain functions, including motor activity, emotional response, and positive reinforcement. Changes of DA transmission and DA receptors at both expression levels and signals transduction pathways may be associated with a variety of motor and mental disorders. Interestingly, increasing evidence suggests that DA receptors can diversify and amplify their repertoire of signaling by forming homo- and hetero-dimers, a property typically shared by the GPCR family that greatly increases their heterogeneity (Carli et al., 2018). Identifying receptor heterodimers and decoding their characteristics is a critical step for understanding their contribution to the pathogenesis of psychiatric and neurological disorders. Moreover, heterodimers may represent the targets for drug discovery.

The D3R that belongs to the family of G protein-coupled receptors (GPCRs) is preferentially expressed in DA neurons and is involved in the regulation of neuronal development, in promoting structural plasticity and in triggering key intracellular events with neuroprotective potential. Interestingly, there is evidence that nAChR located on DA neurons also provide neurotrophic and neuroprotective support to DA neurons, an effect requiring functional D3R and suggesting the existence of a positive crosstalk between these receptor systems. Thus, D3R-nAChR heteromerization might shed light on unknown mechanisms modulating DA neuron function. On this line, we have provided evidence that in mouse and human DA neurons the D3R directly interacts with the nAChR (Bontempi et al., 2017) to form the D3R-nAChR heteromer that represents the molecular effector of nicotine-mediated neurotrophic and neuroprotective effects (Bontempi et al., 2017; Bono et al., 2019). Interestingly in the first part of my project, morphological experiments performed in both mouse and human DA neurons have shown that incubating cells with quinpirole in the presence of specific interfering peptides disrupting the D3R-nAChR heteromer affected the ability of quinpirole in mediating neurotrophic effects. The use of cell-permeable interfering peptides is becoming a crucial tool in the study of receptor heteromerization. Interfering peptides have been used to disrupt the properties of heteromeric complex. In fact, morphological remodeling of DA neurons induced by quinpirole, as the length of dendrites, soma area and dendrite number were lost in the presence of interfering peptides that disrupt D3R- nAChR interaction. Therefore, a first main result of this study is that the D3R-nAChR heteromer, but not the single D3R or nAChR protomers, is the functional unit mediating the effect of both D3R and nAChR agonists.

Heteromer is defined as a macromolecular complex composed of at least two receptor units with biochemical properties that are demonstrably different from those of its individual components. Heteromerization often affects the ligand binding properties of interacting receptors and alters the potency of agonists in generating intracellular signals. As shown before, D3R-nAChR heteroreceptor complex is the functional unit mediating DA neurons morphological remodeling induced by quinpirole and nicotine. Therefore, it is clear that activation of D3R or nAChR has a different effect on the physiology of DA neurons than when nAChR and D3R are coupled to form a complex. Therefore, in this study, several "in vitro" models, including transfected HEK293 cells, mouse DA neurons and human iPSC-derived DA neurons, were used to investigate the signaling properties of the D3R-nAChR heteromer. When we talk about structural plasticity, we are considering the development of long-term changes (Lüsher and Malenka, 2014). A short-term change can be triggered quickly after a stimulus and can be rapidly downregulated. Conversely, long-term modifications require the transcription or down-regulation of specific genes in order to induce and maintain changes in the physiology of neurons that can last for days, weeks or longer (McClung and Nestler, 2008). The ERK pathway is one of the main intracellular pathways involved in short- and long-term neuronal changes (Kelleher et al., 2004; Sun and Nan,2017).

Thus, we investigated the kinetics of ERK signaling after D3R-nAChR heteromer stimulation by quinpirole and nicotine. The engagement of the PI3K-ERK1/2 signaling, associated with the D3R and previously found to be required for nicotine-induced structural plasticity, has been analyzed (Collo et al., 2013). We found that in HEK-nAChR cells, nicotine stimulation resulted in a persistent increase of the pERK1/2 levels; by contrast, in HEK-D3R cells quinpirole induced a rapid and transient activation of ERK1/2, with a phosphorylation peak at 5 minutes. In HEK293 cells expressing the D3R-nAChR heteromer, stimulation with nicotine or quinpirole resulted in a persistent activation of ERK1/2. The ability of these compounds to induce a persistent activation of the ERK1/2 pathway was also observed in mouse neuronal cultures. Interfering peptides were used to associate ERK1 / 2 activation with the D3R-nAChR heteromer. Indeed, treatment with the TAT-D3R peptide abolished the long-lasting ERK activation induced by quinpirole in mouse neuronal cultures that co-express D3R and nAChR while as expected, stimulation of both nAChR and heteromer D3R-nAChR resulted in persistent activation of ERK1 / 2, nicotine-induced pERK1 / 2 was not prevented by counteracting the nAChR interaction with D3R by using TAT-D3R peptide. This result suggests that stimulation of nAChR but not D3R, within the D3R- nAChR complex induces a persistent activation of ERK signaling that is not sufficient to obtain a neurotrophic effect. However, it is possible that others D3R-dependent

pathways are affected by the coupling with nAChR. On this line we have investigated a possible crosstalk with the PI3K pathway because, it has been reported that D3R-driven morphological effects could be mainly triggered by the activation of PI3K pathway (Collo et al., 2008). Neuronal cultures were treated with nicotine or quinpirole in the presence of the PI3K inhibitor LY294002 and we found that the persistent activation of ERK1/2 induced by the D3R-nAChR heteromer, but not by the nAChR, requires the activation of the PI3K pathway. These data are further confirmed by the use of neuronal cultures expressing the only nAChR, obtained from mice knock out for the D3R (D3R-KO). Treating the cells with nicotine results in persistent activation of ERK 1/2, and also with LY294002 incubation, pERK 1/2 was not blocked by inhibiting PI3K. These results indicate that nicotine and quinpirole-induced morphological effects in dopaminergic neurons involve nAChR along with dopamine D3R-mediated recruitment of ERK / Akt signaling.

Together, the results obtained in this study further elucidate the molecular and biochemical mechanisms underlying the neurotrophic effects of nicotine and quinpirole induced by the activation of the D3R-nAChR heteromer. Since its crucial role in preserving DA neurons homeostasis, the D3R-nAChR heteromer may represent a novel target for drugs designed for supporting DA neurons plasticity and survival in various pathologies, including PD.

We have provided evidence that the heteromeric complex D3R-nAChR is crucially involved in morphological plasticity and neurons homeostasis, thus indicating that abnormal D3R-nAChR function may be associated with the vulnerability of DA neurons.

Along this line, in the second part of my project through the use of iPSC technology we studied the impact of the properties of the heteromeric complex D3R-nAChR in PD patient-derived DA neurons with LRRK2 G2019S mutation in order to reveal possible pre-degenerative molecular alterations that could contribute to the pathogenesis of diseases involving the DA system such as PD. The iPSC technology represents the most useful strategy to model PD using patient-derived cells in order to disclose, in each patient, a hierarchically relevant pre-degenerative events that precede DA neurons death. Among the familiar forms of PD, the G2019S mutation in the LRRK2 gene, that encodes for multidomain protein is the most frequently observed. Has been shown that the LRRK2 G2019S mutation to enhance kinase activity and selectively impair vesicle trafficking in ventral midbrain neurons, including dopaminergic neurons (Pan et al., 2017). Two different iPSC lines derived from PD patients with the G2019S LRRK2 mutation (LRRK2-PD), their respective G2019S LRRK2 corrected isogenic lines (LRRK2-ISO) (Reinhardt et al., 2013), and iPSC derived from a healthy subject (control) (Bono et al., 2018) were differentiated into DA neurons. Each LRRK2-PD line, as well as their

corresponding LRRK2- ISO lines, was individually analyzed with results almost totally superimposable. At the end of the differentiation process, we get about 40% of TH positive DA neurons (Bono et al., 2018; Bono et al., 2019) and this percentage was similar in LRRK2-PD, LRRK2-ISO, and control neurons, thus indicating that this mutation does not interpose with the quantity of DA neurons derived from these iPSC.

LRRK2 is strongly bound to the cytoskeleton and, in particular, to microtubules. LRRK2 can interact or regulate the phosphorylation of different structural and regulatory components of the cytoskeleton in fact LRRK2 has been implicated in the growth of neurites, in the trafficking of membranous loads along the axons and in cell morphology. Therefore, understanding these cytoskeletal associations can provide information on the pathogenesis of PD (Biskup et al., 2006; Caesar et al., 2013). Morphological analysis of DA LRRK2-PD neurons revealed that these cells are characterized by shortened neurites, reduced dendritic branching, and small soma area, compared to both LRRK2-ISO and control neurons. Similar results have been previously observed in several studies (Weykopf et al., 2019), implying that the LRRK2-PD neurons used in this study are a good tool to investigating the additional features associated with the G2019S LRRK2 mutation.

From a functional point of view, our results shown that DA neurons synthesize and release DA under basal conditions in both LRRK2-PD and control and LRRK2-ISO neurons. Furthermore, treatment with a non-specific synaptic vesicle liberator such as potassium (K^+) significantly increased the release of DA in all cultures thus indicating that the proteins involved in DA synaptic loading and release are functionally preserved.

By contrast, nicotine, which stimulated DA release in both control and LRRK2-ISO neurons, was unable to release DA in LRRK2-PD neurons. LRRK2 dysfunction does not affect DA release either in basal conditions or after nonspecific depolarizing stimuli, but abolishes DA release evoked by nAChR stimulation, possibly reflecting an altered function of the acetylcholine receptor system. Alterations in DA release and impairment of D2 autoreceptor function were observed in transgenic mice expressing the R1441C LRRK2 mutant (Li et al., 2009; Tong et al., 2009).

PD is characterized by aggregation of α-synuclein (αSyn) in Lewy bodies (LBs), Lewy neurites (LNs) and glial cytoplasmic inclusions (Spillantini et al.,1998). Pathological inclusions enriched in alpha-synuclein in DA neurons are typically associated with PD (Benskey et al., 2016). A strong interaction between the LRRK2 gene and α-syn were described. Along this line, our data showed that DA neurons with the LRRK2 G2019S mutation show numerous alpha-synuclein aggregates, visualized as inclusions in neurons, furthermore using the thioflavin S protein fibrillar probe it was found that there is a

greater alpha-syn immunoreactivity in DA LRRK2 -PD neurons which, however, do not show a fibrillar morphology, typical of the pathological structures observed in PD (Rideout and Stefanis, 2002). This suggests that the LRRK2 mutant not only alters the morphology of the DA neuron, but also induces a spontaneous increase in non-fibrillar alpha-syn indicative of non-pathological aggregates.

However, it remains to be clarified whether the G2019S LRRK2 mutation may drive or have minor effects on neuronal alpha-synucleinopathy (Henderson et al., 2018).

Our previous data in both mouse and human DA neurons suggest that the D3R-nAChR heteromer, but not the single D3R or nAChR protomers, is the functional unit mediating the neurotrophic effects induced by both nicotine and D3R-agonists resulting in increased dendrite length, dendritic arborization and soma area. We found that LRRK2-PD neurons, but not LRRK2-ISO or DA control neurons, were almost completely insensitive to the neurotrophic effects of nicotine and agonists that prefer D3R. Our experiments suggest that this condition was mainly due to decreased plasma membrane levels of both nAChR and D3R that accumulated in the Golgi compartment. In DA LRRK2-PD neurons the PLA signal, an index of D3R-nAChR interaction, was totally absent not only on the plasma membrane, but also in cytoplasmic sites, suggesting that in these neurons D3R and nAChR are individually blocked in the Golgi compartments and their heteromerization does not occur. The lack of a PLA signal could mean that individual receptors are misfolded within the Golgi vesicles. Indeed, it is known that the dimerization / oligomerization of G protein-coupled receptors takes place within discrete intracellular compartments, such as ER and Golgi, before trafficking to the plasma membrane as preformed mature complexes (Bulenger et al., 2005; Herrick- Davis et al., 2006). Our results are in line with what has been reported in the literature where it has been reported that the LRRK2 G2019S mutation increases the kinase activity and causes the selective impairment of synaptic vesicle trafficking (Pan et al., 2017). Furthermore, impairment of expression and function of D2R, due to altered trafficking between Golgi and plasma membrane has been observed in both R1441C LRRK2 transgenic mice (Tong et al., 2009) and in SH-SY5Y cells overexpressing the G2019S mutant LRRK2; in these cells, the trafficking properties of DA D1 receptors (D1R) were also impaired (Rassu et al., 2017).

LRRK2 mutation increases the kinase activity by increasing the catalytic rate of the enzyme (Thomas, 2007), changes in LRRK2 kinase activity appear to be toxic and appear to induce degeneration of dopamine neurons (Greggio, 2009) but treatment with LRRK2 inhibitors has been shown to rescue neuronal structural defects in iPSC-derived DA neurons (Weykopf et al., 2019). On this line DA neurons derived from LRRK2-PD were treated with the LRRK2 inhibitor GSK2578215A and this was

sufficient to recover the morphological defects and acquire a denser and more complex axonal arborization in response to both the nicotine and the D3R agonist, typical of these neurons and crucial for their physiological activity (Matsuda et al.,2009) and significantly reduce the increase in alpha-syn immunoreactivity, as previously reported (Weykopf et al., 2019). Furthermore, the localization on the plasma membrane of D3R and nAChR and the heteromer D3R-nAChR was restored and the sensitivity of the heteromer D3R-nAChR to respond to neurotrophic stimulation effects was recovered.

In PD, dopaminergic neuronal loss is accompanied by inflammatory changes in microglia, astrocytes and innate immune cells. Astrocytes have been implicated as potentially exerting both neurotoxic and neuroprotective activities in Parkinson's disease. Astrocytes play an important role in maintaining brain homeostasis, and their functions include metabolic support of neurons, modification of synapse signaling, recycling of neurotransmitters and blood brain barrier regulation (Lee et al., 2019).

On this line we have recently developed a protocol for the differentiation of human astrocyte starting from iPSC derived from healthy subject (Filippini et al., 2020). In particular, after 40 days of differentiation, we produced a population with 40-45% of cells expressing GFAP, a marker that identifies mature astrocytes.

The quality of our iPSC and iPSC-derived astrocytes was routinely assessed by expression of cell-specific markers. In particular, pluripotency markers Sox1 were expressed in undifferentiated cells while the neural stem cell (NSC) marker Nestin was expressed by the NSC population. For astrocytes differentiation, we used a 40-day differentiation protocol leading to a pure astrocytic population composed by >40% of cells expressing GFAP. Moreover, according to classical taxonomy, astrocytes are divided into two major classes, protoplasmic astrocytes in the gray matter and fibrous astrocytes in the white matter (Miller et al., 1984) our astrocytes shown heterogeneity of morphology between fibrosis and protoplasmic.

Astrocytes process information in the brain via calcium (Ca2+) signals that can modulate neuronal communication. Calcium (Ca^{2+}) wave propagation across astrocytes is important for neuron-glia and glia-glia communication (Shemes et al., 2006). On this line, astrocytes were analyzed for their capacity to propagate intracellular Ca^{2+} waves. We found that astrocytes derived from heathy control display intracellular Ca^{2+} fluctuation under basal conditions. Moreover, by quantification of dendrite branching, we found that murine dopaminergic neurons co-cultured with iPSC-derived astrocytes

exhibit longer and a higher number of dendrites compared to neurons cultured alone. Overall, these results indicate an optimal quality and functionality of iPSC-derived astrocytes.

Current knowledge of the mechanisms underlying PD pathology derives primarily from animal models that do not truly recapitulate human disease or from cellular models that focus primarily on DA neurons. The contribution of astrocytes to disease progression is most likely underrepresented. In the third part of my project astrocytes derived from two PD patients carrying the G2019S mutation in the LRRK2 gene and their respective corrected isogenic lines were successfully generated using our previously described protocol and characterized. Each LRRK2-PD line, as well as their corresponding LRRK2- ISO lines, were individually analyzed with results almost totally superimposable. Furthermore, as for the astrocytes derived from healthy subject, we obtain that 45% of the cells express GFAP in both astrocytes derived from LRRK2-PD and LRRK2-ISO with heterogeneity of morphology between fibrous and protoplasmic.

To evaluate the functionality of the astrocytes we observed spontaneous fluctuations of Ca^{2+} and we noticed that the astrocytes derived from LRRK2-PD patients and their corrected isogenic lines LRRK2-ISO showed a heterogeneous pattern of intracellular fluctuation of Ca^{2+} under basal condition. Taken together, these data support the successful generation of functionally equivalent astrocyte-like cell populations, which represent a continuous source of human astrocytes for subsequent analyzes.

By quantification of dendrite branching, we found that murine DA neurons co-cultured with astrocytes derived from LRRK2-ISO exhibit longer and a higher number of dendrites compared to neurons cultured alone. Further, using a co-culture model based on mouse DA neurons and patients' iPSC-derived astrocytes; we found morphological alterations similar to those of neurodegeneration, such as short and few neurites and a reduced soma area (Fu et al., 2005; Garrido et al., 2011).

As previously seen our data shown that astrocytes carrying the LRRK2 G2019S mutation may have a reduced neuroprotective capacity and may contribute to the neurons vulnerability (Di Domenico et al., 2019).

Recently, it has been reported that various receptors and transporters of neurotransmitter are expressed in glial cells (Zhang, 2010). More interestingly, previous reports showed that the dopamine D2 receptor (D2R) and dopamine D3 receptor are expressed in astrocytes and are involved in the modulation of inflammatory response and in maintaining balance of innate immunity in CNS (Shao, 2013). Extensive experimental evidence indicates that inflammatory processes are instrumental in neuronal cell death in PD and LRRK2 seems to have a prominent role in inflammatory cells. In these cells, LRRK2 plays an important role in the control of DRs trafficking likely by a modulation of vesicle

trafficking (MacLeod et al., 2013; Yun et al,2015). On this line, the expression of D2R and D3R was examined in our iPSC-derived astrocytes from healthy control, PD patients and their corrected isogenic lines. By contrast, to some data shown in the literature on animal models, we observed an absence of D3R expression in human astrocytes derived from healthy controls. D3R deficiency could be due to differences in experimental models and the biological samples investigated (cell lines, animal tissue, and human samples), as well as differences in methodologies. Instead, the immunofluorescence analysis showed that the iPSC-derived astrocytes from control line and LRRK2-ISO expressed the D2R that is mainly localized on the plasma membrane. By contrast, iPSC-derived astrocytes from LRRK2 G2019S mutated patients exhibit a remarkable reduction of the D2R localization at the plasma membrane. Therefore, our preliminary data likely suggest that LRRK2 G2019S mutation could be associated with defective D2R localization in astrocytes. In fact, has been reported that LRRK2 is involved in the control of protein trafficking (Lee, 2019) and LRRK2 mutations has been associated with abnormalities in DA receptor expression at the plasma membrane in both transgenic mice (Yan et al., 2015) and human neurons derived from iPSC. LRRK2 mutation increases the kinase activity by increasing the catalytic rate of the enzyme (Thomas, 2007), changes in LRRK2 kinase activity appear to be toxic (Greggio, 2009) but treatment with LRRK2 inhibitors has been shown to rescue neuronal structural defects in iPSC-derived DA neurons (Weykopf et al., 2019). On this line astrocytes derived from LRRK2-PD were treated with the LRRK2 inhibitor GSK2578215A and this was sufficient to recover the localization on the plasma membrane of D2R.

In conclusion the iPSC technology represents the most useful strategy to model PD using patient-derived cells. By using this technique, IPSC derived from healthy control and patients with LRRK2 mutation were differentiated in mature astrocytes. This population was fully characterized, showing the expression of key molecular marker, and functionally and expression of dopamine receptor. More recently, evidence that D2R activation inhibits the activation of the NLRP3 inflammasome in primary astrocyte cultures has been provided (Zhu et al., 2018). The NLRP3 inflammasome is a multiprotein complex responsible for the activation of caspase 1 and secretion of the proinflammatory cytokine interleukin 1 beta (IL-1beta), which uncontrolled production may be detrimental. Interestingly, mice lacking D2R expressions were characterized by increased neuroinflammation in normal conditions and by aggravated dopamine (DA) neuron loss in response to MPTP injections; in both cases, reactive astrocytes and NLRP3 inflammasome activation were observed. Thus, it likely that in the substantia nigra a crosstalk between DA neurons and astrocytes occurs, with astrocytes potentially promoting neuroinflammation and DA inhibiting NLRP3 inflammasome activation. Therefore, astrocytic D2R may

be a crucial element to control undue inflammasome activation with protective effects on Parkinson's disease (PD) while astrocytic D2R dysfunctions could promote abnormal responses and amplify disease progression.

DA receptors are susceptible to change under pathological conditions and rearrangements of intracellular signal pathways might occur that worsen the symptoms in aging diseases or neurodegenerative process. The better understanding of the changes that occur in the DA receptors and their functional responses during pathological conditions is crucial for the development of novel and efficacious therapeutic approaches in neurodegeneration, neurotoxicity and neuroinflammation. Being the most numerous glial cell type in the CNS, astrocytes have great impact on the brain environment and may constitute a very potent treatment target.

6 REFERENCES

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