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**Neurodegeneration associated-proteins in human olfactory epithelium: immunocytochemical and biomolecular study in healthy subjects and patients with synucleinopathies**

S.S.D. MED/26 NEUROLOGIA

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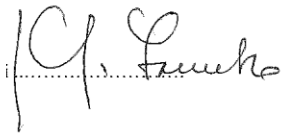

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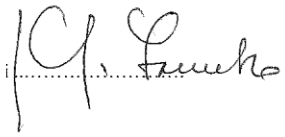

Dottorando:

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“Parlare oscuramente lo sa fare ognuno,

ma chiaro pochissimi”

(Galilei 1793, p.39)

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## List of abbreviations

α-syn: α-synuclein

α-syn RT-QuIC: α-synuclein Real-Time Quacking-Induced Conversion

AD: Alzheimer's Disease

AgD: argyrophilic grain disease

AP: atypical parkinsonism

ALS: amyotrophic lateral sclerosis

AON: anterior olfactory nucleus

ASA: Amyloid Seeding Assay

BSE: bovine spongiform encephalopathy

CAA: cerebral amyloid angiopathy

cAMP: cyclic adenosine monophosphate

CBD: corticobasal degeneration

CJD: Creutzfeldt-Jakob disease

CNS: central nervous system

CSF: cerbrospinal fluid

CTE: chronic traumatic encephalopathy

FTD: frontotemporal dementia

GBCs: globose basal cells

HBCs: horizontal basal cells

HD: Huntington's disease

IgA: immunoglobulin A

IQ-CSF: Improved RT-QuIC assay for CSF

IPTG: isopropyl b-D-1-thiogalactopyranoside

LBD: Lewy body dementia

mAb: monoclonal antibody

MSA: mulitple system atrophy

NAC: non-amyloid component

NACP: non-amyloid component precursor protein

NAD: neuro-axonal dystrophies

NDs: neurodegenerative diseases

NMR: nuclear magnetic resonance

OB: olfactory bulb

OBg: olfactory brushing

OBP: odorant-binding proteins

OE: olfactory epithelium

OM: olfactory mucosa

OMP: olfactory marker protein

ORPs: olfactory receptor proteins

PAF: pure autonomic failure

PCK: pan-cytokeratin

PD: Parkinson's disease

PGP 9.5: protein gene product 9.5

$PrP^{C}$: prion protein cellular isoform

$PrP^{Sc}$: prion protein scrapie isoform

PrPD: prion diseases

PSP: progressive supranuclear palsy

RBD: REM-sleep behavior disorder

rfu: relative fluorescence units

rPrP: recombinant prion protein

rPrP$^{sen}$: recombinant prion protein proteinase K-sensitive

RT-QuIC: Real-time quaking-induced conversion

SCA: spinocerebellar ataxias

sCJD: sporadic Creutzfeldt-Jakob disease

SNc: *substantia nigra pars compacta*

TDP-43: TAR DNA-binding protein 43

ThT: thioflavin T

UPDRS: unified Parkinson's disease rating scale

vCJD: variant Creutzfeldt–Jakob disease

# ABSTRACT

Olfactory impairment is considered an initial disturbance of several neurodegenerative diseases (NDs), including Parkinson's disease (PD) and Alzheimer's disease (AD). In addition, smell impairment precedes a decade, or even longer, the onset of motor or cognitive symptoms.

Olfactory signals are detected by olfactory receptor proteins (ORPs) expressed in the cilia of olfactory receptor neurons (ONs). ONs are the distinctive cellular components of the peripheral olfactory epithelium (OE) and lie in the nasal vault.

ONs axons pass the cribriform plate and reach the olfactory bulb (OB) where the olfactory stimuli are processed and sent to the superior nuclei of the CNS.

Previous studies in AD and other neurodegenerative disorders have shown the presence of β-amyloid deposits in the OB, neurofibrillary tangles, as well as Lewy body pathology. OB represents the brain area earlier involved in the neuropathological process, decades before the development of clinical symptoms. Therefore, OB can be considered a target in the study of neurodegenerative diseases in their early molecular processes. Moreover, the OB of healthy subjects presents deposits of aggregated proteins confirming that these aggregates are deposited in a prodromal disease stage.

Since the OB is an early accumulation site of aggregated proteins and the synapses derive from the ONs, it is possible that the first event of protein aggregation occurs in OE. ONs are directly exposed to the external environment including chemical/physical toxic injuries and such micro-environment predisposes to abnormal protein processing and folding (Sammeta and McClintock 2010).

In addition, ONs and all other mature cell components have a half-life of three months and programmed apoptosis. The neural activity is maintained by a constant cellular turn-over, which is sustained by the basal stem cells. This regeneration process is persistent during the whole life of an individual, albeit with a decreasing rate with aging.

Extensive scientific literature indicates the neuronal damage as the consequence of exposure to toxic injuries leading to neurodegeneration and ONs are a natural model of this noxious process (Lema Tomé, Tyson et al. 2013).

The hypothesis of this pathological pathway is supported by several studies, in which aggregated forms of α-synuclein, tau and β-amyloid are detected in olfactory mucosa (OM) biopsies as well as in autoptic samples of patients with Parkinson's disease (PD), Lewy body dementia (LBD), Frontotemporal dementia (FTD) and Alzheimer disease (AD) (Funabe, Takao et al. 2013) (Saito, Shioya et al. 2016) (Tabaton, Cammarata et al. 1991) (Talamo, Rudel et al. 1989) (Crino, Greenberg et al. 1995) (Arnold, Lee et al. 2010).

In this study, we investigated for the first-time primary ONs sampled *ex vivo* using olfactory brushing (OBg) in normal subjects and patients with different neurodegenerative disorders. Because of its convenient location, OE is easily accessible and can be sampled to obtain the ONs in the tissue outer layer. This sampling method is harmless and non-invasive, bypassing potential artifacts due to *postmortem* specimens as well as avoiding the invasiveness of biopsy procedures.

Recently, we showed that OBg procedure in Creutzfeldt-Jakob Disease (CJD) patients allows efficient OM sampling for the Real-Time Quaking-Induced Conversion (RT-QuIC) assay. We specifically amplified pathological prion protein ($PrP^{Sc}$) providing a diagnostic *intra vitam* test with sensitivity and specificity nearly to 100% (Orrú, Bongianni et al. 2014).

For the purpose of our study, we firstly defined the phenotypic characterization of the human olfactory cells sampled with OBg from healthy subjects.

Distinct antibodies were selected to analyze the olfactory epithelium cells: olfactory marker protein (OMP), neuron-specific class III β-tubulin (TUJ-1), protein gene product 9.5 (PGP 9.5), Pan-Cytokeratin (PCK).

Secondly, we aimed to determine the expression patterns of the major misfolded proteins involved in the main neurodegenerative diseases. In particular, the selected proteins were: α-synuclein, APP/beta-amyloid, tau, and TDP-43.

The identification of the expression patterns of these proteins in the ONs might provide information to understand the abnormal molecular mechanisms in the

initial misfolding species involved in the pathological process. Moreover, in this study, we speculated on the subcellular locale where the protein aggregation may occur.

Furthermore, by demonstrating the constitutive expression of the native NDs-associated proteins in the OE, we could assume that they may represent a potential template for triggering the aggregation process.

Based on the immunocytochemistry analysis, we investigated the α-synuclein expression in patients affected by different synucleinopathies. In fact, α-synuclein misfolding and aggregation mechanisms are involved in the pathogenesis of neurodegenerative disorders such as Parkinson's disease (PD), dementia with Lewy bodies (LBD) and multiple system atrophy (MSA), which are all characterized by α-synuclein fibrils deposition (Spillantini, Schmidt et al. 1997).

Finally, we analyzed the immunocytochemistry results in OM samples tested by α-synuclein RT-QuIC (α-syn RT-QuIC).

# 1. INTRODUCTION

## 1.1 Neurodegeneration and protein aggregation

Neurodegeneration refers to the process of progressive atrophy (degeneration) and/or death of nerve cells, which characterizes neurodegenerative diseases (NDs). "Neurodegenerative diseases" is an umbrella term for a range of neuro-pathological, incurable and debilitating conditions, which result in disturbances in movement (ataxias) and/or mental functioning (dementias).

NDs principally affect neurons in the human brain. Neurons are the building blocks of the nervous system which includes the brain and spinal cord. In NDs, the progressive loss of selectively vulnerable populations of neurons diverges from the confined stationary neuronal loss caused by metabolic or toxic disorders. Neurons do not replicate and when they become disrupted or die, the organism cannot replace them, and their role and functions are lost.

Neurodegenerative diseases can be classified according to primary clinical features (e.g., dementia, parkinsonism, or motor neuron disease), anatomic distribution of neurodegeneration (e.g., frontotemporal degenerations, extrapyramidal disorders, or spinocerebellar degenerations), and principal molecular abnormality. Dementias are responsible for the greatest burden of neurodegenerative diseases, with Alzheimer's disease (AD) representing approximately 60-70% of dementia cases.

NDs are extremely debilitating illnesses, including: Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), spinocerebellar ataxias (SCA), frontotemporal dementia (FTD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), chronic traumatic encephalopathy (CTE), multiple system atrophy (MSA), dementia with Lewy bodies (LBD), and prion diseases (PrPD). Despite the widely different clinical symptoms and the vast variations in incidence that characterize NDs, they have many shared features, such as the chronic and progressive nature, increase of age-dependent prevalence, neural damage, synaptic network disruption and

degeneration of specific brain areas causing brain mass loss (Ross and Poirier 2004).

A wealth of research in recent years has revealed that many of the most common age-associated neurodegenerative diseases result from the transformation and accumulation of specific proteins within the nervous system.

Protein misfolding, aggregation and accumulation are currently considered crucial events in NDs. These events in NDs provoke cellular dysfunction, synapses defeat and ultimately brain damage. Even though the proteins involved in each ND are different from each other, the central phenomenon of protein misfolding conducive to their aggregation is analogous.

The process of misfolded protein aggregation is commonly defined as amyloid, and the consequent accumulation of such aggregated forms provides further crucial parallelism between the NDs. Moreover, it is thought to be the key event of their onset. Furthermore, the process of protein misfolding, the intermediates generated, the end-products, and other molecular and pathological features are extraordinarily comparable, although the protein aggregates produced are distinctive for each ND (Soto 2003).

The finding that misfolded protein aggregates self-propagate by seeding activity represented an important advance in the understanding of the mechanisms leading to neurodegeneration. In this way, the aberrant forms of the specific proteins spread in the organism, from one cell to another, between tissues, imitating the infectious prion diffusion model. The consequences of such discovery were crucial to the comprehension of the initial mechanisms of NDs and the understanding of the ways in which they evolve. This novel perspective has also proved fundamental in developing experimental strategies for treatment and diagnosis (Soto and Pritzkow 2018).

Hundreds of significant shreds of evidence from *in vivo* and *in vitro* studies, both in human and mouse models, supported the theory that the protein misfolding, oligomerization and accumulation are the key mechanisms that initiate the pathology and its progression (Goedert 2015).

The most frequent aggregated proteins in NDs are: the amyloid precursor protein (APP) peptide amyloid-beta (Aβ) in AD; tau in AD, FTD, CBD, PSP,

argyrophilic grain disease (AgD), CTE; α-synuclein (α-syn) in PD, LBD and MSA; TAR DNA-binding protein 43 (TDP-43) in ALS and FTD; and prion protein ($PrP^{Sc}$) in PrPDs (i.e., Creutzfeldt–Jakob disease (CJD), bovine spongiform encephalopathy (BSE), Variant Creutzfeldt–Jakob disease (vCJD).

Surprisingly, these neurodegeneration-associated proteins do not share common features in terms of primary, secondary or tertiary structure, either in size, expression level, cellular localization, or function. Conversely, all these proteins easily undergo misfolding from their native structure resulting in β-sheet-rich multimers and oligomers in the ill brain. This process goes further without interruptions, until it forms bulky fibrils and tangles (Ross and Poirier 2004).

The amyloids aggregates arrangement is particularly defined, with a size between 100 and 200 Å in diameter, and consists of a cross-β framework, a structure of intermolecular β-sheets array running parallel to the long axis of the fibrils (Fitzpatrick, Debelouchina et al. 2013). Typically, specific dyes, such as Congo red, thioflavin, and their derivatives are used to identify these amyloid species through staining techniques (Rambaran and Serpell 2008).

At first, aggregates of these NDs-associated proteins, deposited in the brain, were considered the neurotoxic agents. However, recent evidences coming from successive investigations suggested a new experimental clue: the triggering species should be the soluble misfolded oligomers, acting as precursors of the fibrillar structures, which in turn would be the actual causes of ensuing neurodegeneration (Caughey and Lansbury Jr 2003).

The species included in the vague and heterogeneous definition of misfolded oligomers extends from dimers to superior protofibrillar structures, to which hundreds of monomers can merge (Rambaran and Serpell 2008).

The process of protein misfolding and aggregation consists of rearranging the secondary and the tertiary structures of the protein and to end, obviously, the quaternary structure. The molecular mechanism resides in the conformational change of the regular protein into numerous sequences of β–strands. Subsequently, hydrogen bonds and hydrophilic interactions then stabilize the conformations acquired. Exposed ‘sticky’ ends from these strands trigger the

incorporation of other regular or not yet completely aberrant proteins into the cross-β polymer.

This misfolding process is highly protean and is in equilibrium between monomers and fibrils. Some of these species are on-pathway intermediates for amyloid fibril elongation, whereas some others could be terminal off-pathway products, causing possible toxic effects. Hence, the identification of the principal oligomeric species in the seeding activity and the achievement of high-resolution information about their structure is very challenging and particularly hard because of their quick interconversion, the vast heterogeneity and their tendency to generate stable higher-order aggregates (Breydo and Uversky 2015).

Jarrett and Lansbury in 1993 proposed for the first time the kinetic model of the protein misfolding and the related aggregation mechanism (Jarrett and Lansbury Jr 1993). According to the theory, the entire process is divided into two principal phases: the initial nucleation, slow and thermodynamically unfavorable, and the fast and facilitated elongation stage. It appears clear that the bottleneck in this process is the formation of a stable seed, which in turn should act as a nucleus for further polymerization that irreversibly and rapidly continues to gobble monomers to feed its growth. Therefore, the bottleneck in this model is the first crucial phase in the formation of the nucleus (Soto, Estrada et al. 2006).

Still unknown are the causes that promote the phenomenon *in vivo* by which the bulky polymers generated start to fractionate in new seeds with ensuing propagation and spread of the aggregation reaction. There are further open issues that need to be clarified in the model regarding the regular protein recruitment into the mounting aggregates, in which the elongation rate notably increases, as well as the propagation grade, and that may also indirectly provoke toxic effects for essential protein removal.

Furthermore, diverse conformational strains may derive from the single molecules, which can assume several organization systems, even though the primary scaffold of the aggregates appears analogous. However, it should be remembered that, despite their similarities, every single ND differs from the others and each of them is characterized by a wide range of distinguishing manifestations.

The main differences within NDs, apart from the crucial hallmark that in each disease are distinctive proteins which preponderantly aggregate, involve the following traits: clinical signs, prevalence, brain area and cell types of affection, genes implicated and risk factors. Also, the cellular location of expression and the aggregation locale change from a disease-associated protein to another.

As a final point, it has been demonstrated that the inner structure and the molecular arrangement of each specific aggregate have distinctive features depending on the protein involved and the affecting disease (Soto and Pritzkow 2018).

The hypothesis formulated to explain this last concept claims that all these particular differences in primary sequence, gene regulation, and expression pattern may influence the misfolding mechanism and the ensuing dynamic of aggregation, thence the cellular toxicity pathway, and in the end, the evolution of the disease in the brain.

## 1.2 Prion paradigm and spread of proteinopathies

The contemporary emerging theory about NDs-associated proteins suggests that they mimic a prion-like mechanism of seeding and spreading.

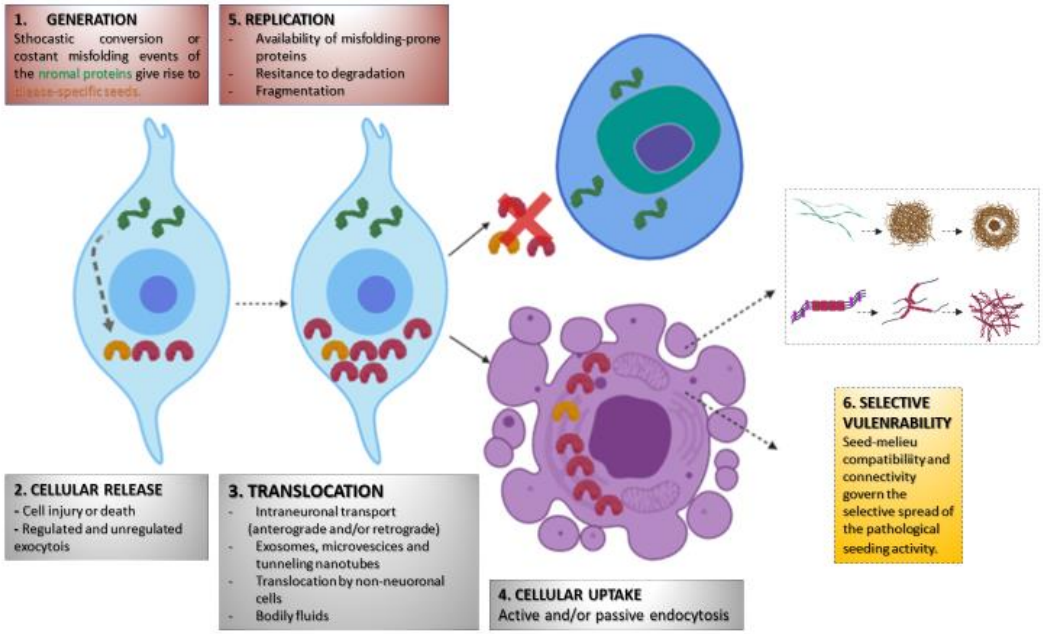


**Fig. 1 Schematic draw representing the main steps leading to the spread of proteopathic seeding activity.** Normal proteins (green) stochastically undergo to misfolding, thereby generating disease-specific seeds (orange). When the formation of the seed occurs, the starting disrupted proteins become inactive and start to self-assemble into multimers. Once the seeds are released, they may move from one location to another by several potential pathways. The local vulnerability for the seeds transfer and replication is selective, depending on the cell type and the recruitment of auxiliary agents. The compatibility of the transport and uptake mechanisms affect the pathological spread (the further propagation of the seeds is halted in the dark blue cell). Also, differences among cells in the expression level, isoform maturation and cellular localization of the starting proteins may modulate the nature and the behavior of seeds, so that the distinct strains produced follow distinct propagation pathways.

Growing pieces of evidence from recent important studies support this concept of pathology propagation of all NDs-associated proteins. (Walker and Jucker 2015). Proteins that share this propagation prion-like principle consists of α-synuclein, amyloid-β, tau and TDP-43. Alpha-synuclein forms Lewy bodies and Lewy

neurites in PD and Lewy body dementia, as well as glial cytoplasmic inclusions in multiple system atrophy. Amyloid-β results in amyloid plaques and cerebral amyloid angiopathy (CAA) in AD. Tau protein causing neuronal and/or glial tauopathies in AD, chronic traumatic encephalopathy, and other NDs. TAR DNA-binding protein-43 (TDP-43), instead, is the principal protein associated with ALS and frontotemporal dementia (Goedert 2015).

Taken for granted the aberrant misfolding activity of these proteins in the constitution of the related diseases, the pathologic seeds following this principle can be transmitted between cells and tissues.

In support of this hypothesis, cross-sectional examinations have constantly shown that the presence and the distribution of the histopathological lesions do not occur and diffuse casually. Somehow, their incidence follows a precise and outlined pattern in space and temporal course (Braak and Braak 1991)(Brettschneider, Del Tredici et al. 2015).

Data coming from *in vivo* imaging analyses in the last years reaffirm the *postmortem* histopathological findings according to which the neural connectome is the means by which pathological lesions diffuse from one region to another (Iturria-Medina and Evans 2015).

Of course, for the pathology spreading to another district, receptive areas need to be compatible and to some extent favorable for the retention and propagation of the pathogenic agents.

Unfortunately, the mapping of the pathology diffusion in the human brain through the *postmortem* examinations and *in vivo* imaging recounts the dynamic development of the disease in a surrogate way. Indeed, the tools of investigations routinely used to look at the brain lesions to reveal the sites of distribution, missing the information on the activity of the aberrant pathological seeds and the related sequelae (Collinge 2016).

The definition of prion-like recalls the amyloid-prone nature of the NDs-associated proteins. In this regard, it is important to take into consideration that small oligomeric species are also produced by the same proteins. As a consequence, it is reasonable to hypothesize that the molecular species produced may also affect the cells and disrupt their function and homeostasis.

Unfortunately, at the state of the art, there are no assays specific and sensitive enough for the detection of such molecular species *in vivo*. Therefore, we can conclude that even though the presence of proteopathic deposits is a clear claim of the disease, this evidence is not always necessary for the instauration and the progression of the pathology (Jucker and Walker 2018).

All the misfolded protein aggregates own this propensity to the seeding activity, and along with this feature, like the transmissible prion, they acquire the spreading intrinsic capacity of the aggregation process (Soto, Estrada et al. 2006).

In the prion infectivity, after the long polymers have formed, they spontaneously start their fragmentation, triggering the release of new seeds. This mechanism increases the rate of prion propagation, and following the seeding–nucleation model, $PrP^{Sc}$ latently spreads in the brain until it reaches the toxic threshold resulting in cellular dysfunction, damage, and lastly clinical disease. (Soto 2012).

Given the fact that, as mentioned above, all NDs-associate proteins own the seeding aggregation activity, it has been hypothesized that potentially they may also have infectious abilities similar to those of the prions. This point is still strongly debated, even though it is supported by several studies demonstrating the pathological transmission of protein deposits in amyloidosis mice models (Xing, Nakamura et al. 2001).

An increasing number of investigations in cellular and animal models have brought data in favor of this concept providing evidence that NDs in experimental conditions can be transmitted via a prion-like mechanism.

The first NDs-associate protein, whose transmissibility has been demonstrated , according to a prion-like modality, was Aβ (Ganowiak, Hultman et al. 1994). A pioneering study in a transgenic mouse model showed that seeds of amyloid-β in brain extracts are necessary and sufficient to trigger protein aggregation (Jucker and Walker 2013).

Notably, these studies have shown that Aβ, tau, and α -synuclein are able to induce the pathology in cell cultures or animal models inoculated with tissue homogenates of NDs patients or transgenic mice (Goedert 2015) (Soto 2012) (Jucker and Walker 2018). Similar studies showed analogous results with purified, synthetic and/or recombinant proteins components (Jucker and Walker 2018).

Taking into account that tissue homogenates are more transmission-prone, also other cellular aspects probably influence the pathological induction too (Walker and Jucker 2015).

Still discussed is the question of whether the spreading of protein misfolding corresponds to the spreading of disease. In some circumstances, the induction of the pathology is limited to the accumulation of protein aggregates, while in others it matches with tissue lesions and clinical signs, which are typical of each disease (Soto and Pritzkow 2018).

Finally, it must be considered that the transmission of the biological information through the seeding of protein aggregation, is organized at multiple levels (Goedert, Falcon et al. 2014).

At the first molecular level, the conversion of normal proteins elicits the autocatalytic growth of aggregates. When the process is observed at the cellular level, the spreading of the pathology from cell to cell can be noticed, transferring the aggregates between contiguous cells, which in turn spread locally the aberrant deposits. Secondly, at the organ level, this transfer among cells provokes tissue damage, that can be transmitted to distant brain areas. Lastly, at the level of the organism, the transmission of the seeds can induce systematically the protein misfolding process like an infectious disease. Nevertheless, in the NDs, only the molecular, cellular and organ levels replicate the prion nature (Soto and Pritzkow 2018).

## 1.3 Synucleinopathies

The term synucleinopathies derives from the name of the protein α-synuclein, which is the main pathologic agent involved in this subset of NDs. Three major disturbs can be identified in this class of NDs: PD, LBD, and MSA, but also rarer neuro-axonal dystrophies are encompassed in (Goedert, Jakes et al. 2017).

The principal pathological hallmark shared by all disorders included in this disease classification is the presence of pathological aggregates of insoluble, fibrillar α-synuclein in selective populations of neurons, nerve fibers and\or glial cells (Goedert, Jakes et al. 2017).

PD is the second most common NDs after AD and affects approximately seven million people globally and one million people in the USA. It has an estimated prevalence of 0,3 % in the general population and 1% in people aged over 60 (De Lau and Breteler 2006). The most evident and typical symptoms of PD are motor dysfunctions such as: bradykinesia, resting tremor, posture instability, and rigidity. These events are due to low levels of dopamine caused by the selective death of dopaminergic neurons in the *substantia nigra pars compacta* (SNc) (Jankovic 2008).

The progression of the pathology denotes the onset of non-motor symptoms like cognitive impairment, sleep disorders, depression, and sensory symptoms (Chaudhuri, Healy et al. 2006). The principal pathological hallmark of PD is the formation of eosinophilic, intracellular fibrillary inclusions of aggregated α-synuclein and other proteins in the brain stem called Lewy bodies and Lewy neurites (Spillantini, Schmidt et al. 1997).

PD occurs in two principal manifestations: sporadic or familial, and in this inherited form, the genetic autosomal transmission is the causing factor derived from mutations in one of these genes, either in a dominant or recessive way: SNCA, LRRK2, PRKN, PINK1 and DJ-1 (Polymeropoulos, Lavedan et al. 1997) (Selvaraj and Piramanayagam 2019).

Unfortunately, at present, there is no cure for PD; however drugs like L-DOPA, dopamine agonists, and MAO-B inhibitors, as well as surgical treatments like deep brain stimulation, can be used to alleviate symptoms (Connolly and Lang

2014). Studies in animal model have demonstrated that the immunization, either active or passive, is able to reduce the α-synuclein levels. Following these encouraging results, the possibility to start α-synuclein vaccination programs is under examination, and some of them are being evaluated in clinical trials (Masliah 2005) (Sanchez-Guajardo, Annibali et al. 2013).

After AD, LBD is the second most common form of dementia. It affects approximately 15% of all dementia patients (McKeith 2007).

Excluding the core hallmark of dementia, the other crucial symptoms of LBD are fluctuating cognition, parkinsonism, REM sleep behavior disorder (RBD), and sensory hallucinations, particularly visual. For these reasons, patients suffering from RBD are considered affected by one early stage of a synucleinopathy, which will overt definitively in the future.

The early pathology markers of LBD are Lewy bodies in the cortical areas that subsequently, with the progression of the disease, extend largely in the brain.

Notably, PD and LBD have a lot of common features and show an overlapping neuropathological profile in some points that render the defined and net diagnosis very difficult (McKeith, Boeve et al. 2017).

MSA is a rarer disease than the above described NDs. The prevalent symptoms associated with MSA are: autonomic failure, cerebellar ataxia, parkinsonism, urinary dysfunction and corticospinal disorders (Gilman, Wenning et al. 2008).

Currently, the estimated annual incidence rate is 3 new cases per 100.000 people (Bower, Maraganore et al. 1997). The pathology map primarily covers the striatonigral and the olivopontocerebellar areas of the brain, with characteristic α-synuclein cytoplasmatic inclusions in glial cells (Gilman, Wenning et al. 2008).

However, the fibrillary inclusions of α-synuclein are histopathological markers found also in significant number of patients with different NDs. For instance, a fluctuating grade between 7-30% of AD patients is positive for Lewy body inclusions together with the extracellular β-amyloid plaques and tau tangles (Hamilton 2000).

Other forms of synucleinopathies are pure autonomic failure (PAF) and neuro-axonal dystrophies (NAD) (Jellinger 2003).

### 1.3.1 Alpha-synuclein

Alpha- synuclein is a protein consisting of 140 amino acids primarily expressed in the CNS, especially in the presynaptic nerve terminals (Jakes, Spillantini et al. 1994).

Together with β-synuclein and ɣ-synuclein, α-synuclein composes the synuclein protein family which has only been described in vertebrates (George 2001). Originally, it was discovered in human when Uéda and colleagues purified a short peptide from the amyloid β-plaques of AD patients, named it the non-amyloid component (NAC). In this pioneering study the precursor protein of the isolated peptide was also described for the first time, referring to it as the non-amyloid component precursor protein (NACP) (Uéda, Fukushima et al. 1993).

Previous studies should have found the same protein in Torpedo electroplaques and rat brain, then described as a "neuron-specific presynaptic nerve terminal protein localized to the nucleus and presynaptic nerve terminal" and for this called "Synuclein" (Maroteaux, Campanelli et al. 1988).

From a structural point of view, it is possible to divide the α-synuclein protein into three domains. Firstly, the amino-terminus (N-terminus) domain, which interacts with lipids (1-60). Secondly, a central core region, which includes the NAC peptide and that is hydrophobic (61-95). Lastly an acidic carboxyl-terminus (C-terminus) characterized by random coil structure (96-140).

Inside the first two domains, there are imperfect repeats, comprising 11 amino acids each and with a KTKGEV consensus sequence (George 2001).

Within the synuclein family, the only highly conserved sequence is the N-terminus domain, unlike the last two domains (Ulmer, Bax et al. 2005).

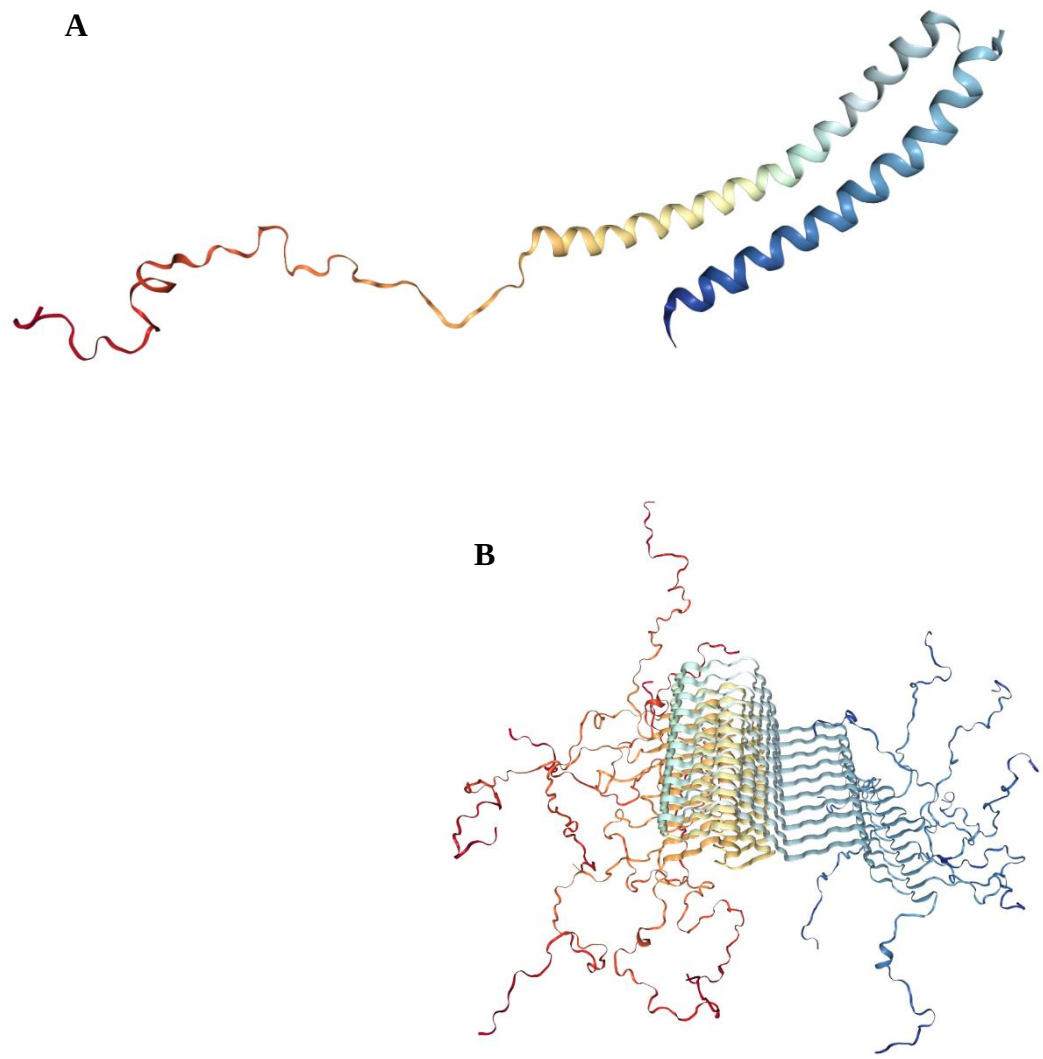


**Fig. 2 A**. **Nuclear magnetic resonance (NMR) prediction of micelle-bound human alpha-synuclein structure.** The N-terminal region (in blue, 1-60) is amphipathic and binds lipids. In the center, there is the hydrophobic region (yellow/orange, 61-95) also called NAC. The C-terminal region (red, 96-140) presents a random coil arrangement. In the N-terminal region there are all the missense mutations associated with familial PD (A30P, E46K, H50Q, G51D, A53T, and A53E).1xq8, from the protein data bank: (Ulmer, Bax et al. 2005). **B.** Atomic-resolution structure by solid-state NMR spectroscopy of a pathogenic fibril of full-length human α-synuclein in a form that induces robust pathology in primary neuronal culture. 2N0A, from the protein data bank (Tuttle, Comellas et al. 2016).

Normally, α-synuclein in the cytosol of cells in physiological conditions has no defined folding, showing just a hint of slight secondary structure (Fauvet, Mbefo et al. 2012).

Nevertheless, it has been advanced that, although α-synuclein is naturally unfolded, in its native state it spontaneously assumes a stable quaternary structure over time. This structure consists of homo-tetramers with a helically folding and it has been proposed that this molecular species does not aggregate (Bartels, Choi et al. 2011).

There are also two hypothetical foldings with α-heliacal conformations, a single extended α-helix or two anti-parallel α-helices, which come to be formed in the N-terminus domain when the protein binds the lipids (Ferreon, Gambin et al. 2009).

Alpha-synuclein preferentially binds the membrane in those points where it is curved because its N-terminal domain promotes that shape (Westphal and Chandra 2013). In several structural studies it has also been observed that α-synuclein forms multimers when it is in contact with cellular membranes (Burré, Sharma et al. 2014).

The proteopathic features of α-synuclein have emerged when it has been isolated as the main fibrillar component of the Lewy bodies and Lewy neurites (Spillantini, Schmidt et al. 1997).

The pathological implications of α-synuclein have been reinforced thanks to the discovery of duplications and triplications (Singleton, Farrer et al. 2003) and six missense point mutations (A53T, A30P, E46K, H50Q, G51D, and A53E) in familial forms of NDs, hence called synucleinopathies. Moreover, variants of SNCA have been associated with sporadic forms of PD in genome-wide association analysis (Meade, Fairlie et al. 2019)

## 1.4 Braak staging and dual hit hypothesis in synucleinopathies

In PD and other parkinsonism, the occurrence of motor symptoms becomes evident only after the death of the 60% of neurons in the *substantia nigra pars compacta* (SNc), harboring the ventral midbrain A9 dopaminergic population, leading to 80% dopamine depletion in the putamen and caudate nucleus of the striatum (Cheng, Ulane et al. 2010).

Since medical practice provides the diagnosis principally from clinical signs, the pathology is evaluated and may be counteracted at the advanced stages. When the disease arrives at the end-stage the dopaminergic neurons are just dying or dead, rich inner the cytoplasm of aggregates with abundant Lewy bodies inclusions.

The motor functions decline over a period of 10 years after the diagnosis in the majority of the patients, when other non-motor manifestations like sleep disorders, dementia and depression may occur (Chaudhuri, Healy et al. 2006).

For that reason, it is reasonable to assume that also different neuronal populations could be involved in the onset and the progression of the pathology. This concept is also reinforced by the disease spreading pattern depicted by Braak staging in a pioneering work (Braak, Tredici et al. 2003).

The hypothesis underlining this pathology diffusion representation stipulates that the aberrant α-synuclein aggregates spread throughout the CNS via the olfactory bulb and/or gastrointestinal tract, entering the *medulla oblungata* and pontine tegmentum at the first two stages of the disease.

From these two peripherical brain districts, as the ongoing pathology spreading in the midbrain progresses, in stages 3-4, the lesions have invaded the *substantia nigra*. In the final 5-6 stages, the pathology brain contamination progress to the neocortex (Braak, Ghebremedhin et al. 2004). These indications suggest that the dopaminergic neurons are not the primary cellular carries of the pathology, but the more susceptible neuronal type to the related damage.

Braak and co-authors advanced the ‘dual-hit hypothesis’ about the pathogenesis of idiopathic PD. According to this “dual hit” theory, an unknown etiopathological agent akin to a slow-virus may enter the nervous system through both the nasal

and intestinal mucosa, eventually resulting in a cascade of events that leads to the pathology in the brain (Hawkes, Del Tredici et al. 2007).

This line of thought may explain the bowel irritability and the olfactory loss, which anticipate the disease up to decades (Braak, de Vos et al. 2006).

Additionally, the successive development of dementia and/or depression in the following stages of the disease also reflects the Braak staiging mapping (Beach, Adler et al. 2009).

Further indications of the olfactory bulb as a possible spreading site of the pathology in the CNS are obtained from neuropathological studies examining the brains of neurologically normal individuals who are found to have incidental Lewy bodies. This study shows that the olfactory bulb is the most frequently affected brain region when only one region is involved in those cases with incidental Lewy bodies (Beach, Adler et al. 2009).

However, it must be pointed out that Braak's hypothesis is frequently debated, and the disease, that is constitutively heterogeneous in forms and evolution, not always follows the pathway delineated by Braak et al. (Burke, Dauer et al. 2008).

Still, on grounds of the theories claiming this thesis, it is important to remark that the spreading of the pathology and the staging progressive course of the disease in PD and parkinsonisms are widely accepted.

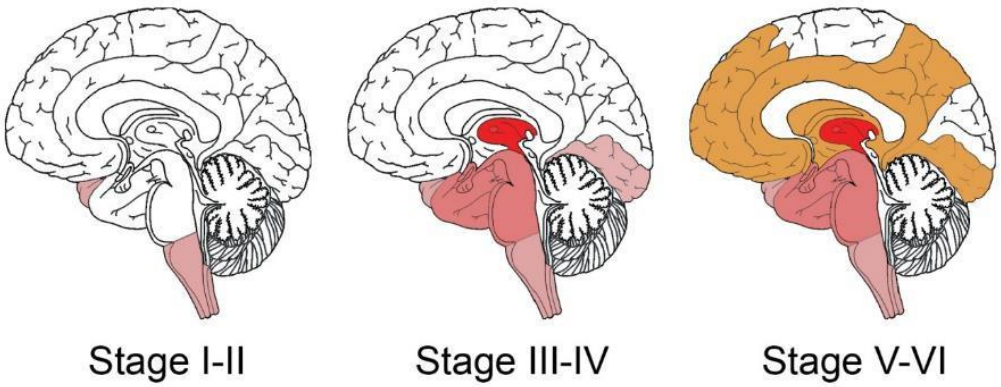


**Fig. 3 Braak staging of Parkinson's disease.** In the first two stages, the pathology spreading is located in the pons and the olfactory bulb. The pathology successively invades the limbic systems. In the final stages, the spreading reaches the neocortex. Taken and modified after Braak et al. 2003.

### 1.4.1 Propagation of synucleinopathies

In synucleinopathies, the aggregates of α-synuclein shows different behaviors depending on the specific disease. In PD and LBD, the typical pathological deposits consist of Lewy bodies and Lewy neurites while in MSA α-synuclein aggregates have the form of glial cytoplasmic inclusions (Goedert, Spillantini et al. 2013).

Following the prion principle, α-synuclein pathology seeded in a specific brain area is propagated along anatomically associated structures (Luk, Kehm et al. 2012) (Luk, Kehm et al. 2012). This mechanism of spreading seems to be driven by selective neuronal transport of the seeding agent (Rey, Steiner et al. 2016). Probably, both the active and passive mechanisms promote the propagation of the α-synuclein seeds. The active axonal transport along specific neural pathways permits the movement of several components such as macromolecules, organelles, and viruses towards and out of the cell body of neurons. Both the anterograde and retrograde transport along axons have been described *in vitro* for Aβ, huntingtin, and α–synuclein, albeit with different transport rates (Brahic, Bousset et al. 2016).

One of the misfolded α-synuclein alternative secretion pathway reported is mediated by the ubiquitin-specific protease 19 (Lee, Takahama et al. 2016). What emerges from these studies is a selective propensity of each cell line and each brain area to the neurodegeneration governed by differential trafficking of the pathological agents.

Even more complex is the understanding of the global process considering the alterations in the host organism and the modifications to the pathological seeds during the course of the disease.

Moreover, the spreading of the pathology throughout the brain is stimulated by the neuronal activity (Bero, Yan et al. 2011)(Yamada and Iwatsubo 2018). Even though the pathology dissemination is mediated by the neuronal connectivity and its activity, the increasing lesions provoke damages to the connectome integrity, as the disease progresses. This event might impair both the relative physiological function and thus spreading pathways (Palmqvist, Schöll et al. 2017).

As a final point, microglia also play a key role in the transport and processing of pathological seeds. Despite that, the modality by which its activation and its state impact on the phenotype of the disease are not completely clear (Asai, Ikezu et al. 2015).

In conclusion, according to the prion principle, other NDs have a time-consuming, silent phase in which the aberrant aggregates propagate, although not already sufficiently to provoke the pathological hallmarks or to be identified by the state-of-the-art analytical methods (Ye, Rasmussen et al. 2017) (DeVos, Corjuc et al. 2018).

## 1.5 Neurodegeneration and olfaction

The indications coming from epidemiology studies suggest the possibility to use the olfactory mucosa as a potential early marker of NDs and other neurological disorders (Godoy, Voegels et al. 2015) (Doty 2009).

Important considerations have to be made about the proposal of olfactory testing as a convenient tool in the differential diagnosis of several NDs (Doty 2009).

The olfactory dysfunctions are assessed in three distinct abilities: detection, recognition, and memory. The capacity to remember odorants is particularly important in elderly patients, especially in AD (Jürgen M. Peters, Thomas Hummel et al. 2003).

Interestingly, the olfactory impairment in AD patients starts in a pre-clinical period of the disease, preceding the onset of the typical motor and cognitive symptoms (Doty 2009).

In addition, olfactory problems are also frequent in AD subjects, such as those who suffer from mild cognitive impairment and those with subjective memory complaints (Jürgen M. Peters, Thomas Hummel et al. 2003) (Doty 2008).

Given the correlation between the olfactory impairment and the disease progression, it is feasible to use olfactory testing as a differential diagnostic tool for major AD. Furthermore, it may have clinical value as an early pathological marker in the diagnosis of incident AD in subjects at risk (Jürgen M. Peters, Thomas Hummel et al. 2003).

Moreover, the apolipoprotein E-4 allele is an increasing risk factor of almost five-fold concerning developing cognitive decline in anosmic subjects without neurological deficits (Doty 2009).

Interestingly, the majority of the AD and PD patients with olfactory dysfunction are unaware of their deficit before the smell test result; nevertheless, up to 90% of these patients already have smell loss at the early stages of the pathology correlating with reduced activation of the central odor processing brain areas, demonstrated also by functional imaging (Doty 2009).

In PD, as already mentioned above, olfactory impairment is a well-characterized and very recurrent non-motor symptom with a prevalence that varies from 50% to

90%. The olfactory loss often emerges as hyposmia at the beginning of the disease (Boesveldt, Verbaan et al. 2008).

This evidence is in agreement with the hypothesis by which the olfactory bulb is the initial site of α-synuclein pathology spreading, successively propagated to the cerebral cortex via the rostral brainstem (Braak, Tredici et al. 2003).

In line with this concept, the presence of Lewy bodies in the olfactory bulb used as a pathological marker corresponds to a sensitivity of 95% and a specificity of 91% in PD deceased individuals. Furthermore, the α-synuclein pathology density grading in the olfactory bulb correlates with the unified Parkinson's Disease rating scale (UPDRS) motor scores (Beach, White et al. 2009).

Other olfactory system areas in which pathological modifications are present include the anterior olfactory nucleus (AON), cortical nucleus of the amygdala, piriform cortex, olfactory tubercle, entorhinal cortex, and orbitofrontal cortex.

The involvement of the AON in the α-synuclein pathology is not surprising, indeed, as this brain region is strictly connected with the olfactory bulb and the presence of the Lewy pathology here is indicated also by neuronal damage (Pearce, Hawkes et al. 1995).

The idea that the α-synuclein pathology spreading inside the olfactory system from the peripheral to the central structures is also a part of the "olfactory vector hypothesis" and shares some conceptual points with the general "dual hit" theory.

Growing pieces of evidence support the idea that the PD pathology is prematurely located in the olfactory bulb and its connected structures, starting from the periphery (Doty 2008).

Besides, tau pathology has been shown in the AON in PD cases. Notably, in these studies, no tau aggregates in the AON of patients have been found (such as those with CBD and PSP), showing absent or slight olfactory impairment. For this reason, it has been proposed that tau protein also contributes to elicit olfactory impairment in PD (Tsuboi, Wszolek et al. 2003).

## 1.6 Anatomy and physiology of the peripheral olfactory system

Olfaction is one of the primitive senses and consists of perceiving and distinguishing odors (Heymann 2006) (Krieger and Breer 1999).

Everyone has a unique capacity of smell, which allows us to perceive and communicate with the environment around us (Wysocki and Preti 2004).

A series of mental processes are in relation to smell. It is known that smell evokes memories and that the memory pathway is linked to the olfaction (Richardson and Zucco 1989). Emotions, such as fear and anxiety, are transmitted through smell and also mating behaviors are provoked by smell (Wyatt 2014).

Anatomically, the nasal cavity, in which the olfactory mucosa allowing olfaction through olfactory receptors is contained, extends from the nares through the external nose and as far back as the posterior nasal apertures or *choanae,* where the nasal cavity connects with the nasopharynx (Agur, Grant et al. 2005).

The nasal cavity is composed of floor, medial and lateral walls, and roof. It is separated sagittally into right and left halves by means of the nasal septum. Each half of the nasal cavity is approximately 5 cm high and 6 cm long. Its shape results narrow transversely given that it is 1.5 cm circa wide at the floor and the same at the roof, (Sinnatamby 2011).

The nasal cavity starts anteriorly with the vestibule, and from there, most of the surface is occupied by the respiratory region, while the restricted and variable olfactory area is principally confined to its postero-superior parts as well as the upper regions of lateral and medial walls (Morrison and Costanzo 1990).

The respiratory mucosa extends anteriorly from the *limen nasi,* where it is continuous with the skin of the nasal vestibule throughout the nose and into the semi-superior part of the nasopharynx. It also extends into the sinuses, through their ostia and prolongs in the epithelia of the nasolacrimal duct and Eustachian tube. In the upper side, it is in continuity with the olfactory mucosa (Jafek, Murrow et al. 2002).

The main olfactory mucosa has a surface of approximately 10 $cm^2$, lining from the posterior upper parts of the lateral nasal walls, comprising the superior

*concha*, the sphenoethmoidal recess, the upper part of perpendicular plate of the ethmoid, to the roof of the nose arching between the septum and the lateral wall, until the basement of the cribriform plate of the ethmoid bone.

Recent anatomical studies of the peripheral olfactory system have changed the former notion of the olfactory mucosa distribution area. Previously, the neuroepithelium was considered circumscribed to the area of the cribriform plate, superior turbinate, and the opposite superior nasal septum (Escada, Lima et al. 2009). The last discoveries resulting from Leopold's anatomical study extends the olfactory mucosa within the medial and anterior surface of the middle turbinate, either in the lateral or in the medial wall of the nasal cavity (Leopold, Hummel et al. 2000). This new notion does not conflict with Biedlingmaier and Whelan's previous report on the middle turbinate, in which they did not find olfactory sensory tissue. In fact, their studies focused on a more inferior middle turbinate level, where the olfactory epithelium is not present (Biedlingmaier and Whelan 1996) (Leopold, Hummel et al. 2000).

Unlike the rodents, in which there is a clear delineation between the two epithelial types, sensory olfactory epithelium in humans is often intermingled with respiratory tissue along the superior turbinate and septum (Welge-Lüssen and Hummel 2004).The olfactory mucosa consists of a considerably thicker pseudostratified olfactory epithelium (up to 100 μm), which contains approximately 6 million of the distinctive olfactory neurons and an underlying connective *lamina propria* (Lane, Gomez et al. 2002, Jafari 2006).

Four principal types of cells compose the olfactory epithelium: olfactory receptor neurons, sustentacular/supporting cells, basal cells (stem cells) and the microvillar cells (Morrison and Costanzo 1990).

Olfactory neurons are bipolar neurons with a cell body which is generally located in the middle-upper stratum of the epithelium, intermingled with the sustentacular cells and with a density in the tissue of 30,000/$mm^2$ (Escada, Lima et al. 2009) (Moran, Rowley et al. 1982). Their single thin axon spread out from its basal edge, overcomes the *lamina propria* and gathers together the adjacent axons in an unmyelinated nerve fiber encompassed of glial ensheathing cells. In the opposite direction, they present a dendrite that ascends up to the most exterior layer of the

epithelium in which the apical knob branches 10-20 slender olfactory cilia (Bloom and Engström 1952). Olfactory neurons have an elliptical, dark nucleus in the middle of their cell body. Along the membrane they present gap junctions to communicate with the neighbor cells, helping the signal transduction and allowing their common renewal and their maturation (Delay and Dionne 2003). Once the olfactory nerve fibers enter the *lamina propria* joining in *fila olfactoria* they pass through the cribriform plate to establish synapses with the mitral/tufted cells of the olfactory bulb, forming the olfactory glomeruli.

The sustentacular cells are irregular columnar cells separating olfactory neurons and sustaining the tissue structure. They have a large, vertically elongated, euchromatic nucleus that resides in a superior level of the neurons perikaryal layer (Weiler and Farbman 1998). The apical ending of the sustentacular cells gets to the external surface of the epithelium bearings abundant and irregular microvilli. In particular, among that microvilli, the long trailing neurons'olfactory cilia elongate into the mucous layer (Polyzonis, Kafandaris et al. 1979). The cytoplasm of the sustentacular cells is characterized by many mitochondria, granular and mainly agranular endoplasmic reticulum. Proximate to the epithelial surface their plasma membrane has fine microfilaments structure attached to desmosomes in order to give mechanical support to the tissue. Tight junctions are present between sustentacular cells and olfactory neurons (Morrison and Costanzo 1990).

The basal cells are small, round or cone-shaped cells that form a distinct layer in the epithelium, resting on the basal lamina. Since they are stem cells, they represent the source of cellular components replacement for the epithelium renewal. During their commitment, they can differentiate in olfactory neurons or sustentacular cells depending on the particular stimuli received. In this way, they ensure the normal turnover or the regeneration after injuries (Graziadei and Graziadei 1983).

Basal cells are divided into two categories: quiescent horizontal basal cells (HBCs), and the proper globose basal cells (GBCs). While the HBCs are long-lasting progenitors activated exclusively when massive injuries occur to the OE, GBCs are "upstream" stem and progenitor cells, with multipotent stem capacity (Leung, Coulombe et al. 2007) (Krolewski, Packard et al. 2013). However, they

are well-described in rodent and it is known that the sustentacular cell end with "feet" projecting on HBCs (Holbrook, Szumowski et al. 1995) and surprisingly they possess primary cilia that seem to be critical for the activation of the regenerative capacity (Joiner, Green et al. 2015).

According to their name, microvillar cells exhibit at the border of their flask-shaped cell body large, blunt microvilli. Their nuclei lie nearby the external layer of the epithelium, while the basal tip is in synaptic contact with the olfactory nerve fibers that penetrate the *lamina propria* (Moran, Rowley III et al. 1982).

The basement membrane of the olfactory epithelium is generally sharp and homogenous, lying beneath the undersurface of epithelial cells. The *lamina propria* encloses numerous olfactory nerve fascicles and sub-epithelial olfactory glands (Bowman's glands) which secrete mucus into ducts terming on to the epithelial surface (Polyzonis, Kafandaris et al. 1979). It also contains some pigment cells, lymphoid cells and a rich plexus of blood capillaries. Deeper, there is a plexus of large veins and numerous lymphatics. (Krstic 2013).

The Bowman's glands are branched tubule-alveolar structures confined under the olfactory epithelium (Nakashima, Kimmelman et al. 1984). They secrete into the epithelial surface through narrow, vertical ducts releasing defensive substances, lysozymes, lactoferrin, Immunoglobulin A (IgA) and sulphated proteoglycans. However, the primary function of mucus is to bathe the olfactory cilia in order to solubilize and facilitate the diffusion of the odorant molecules and thus their detection from the olfactory receptor proteins (Krstic 2013).

Indeed, the 10-20 elongated terminal cilia emerging from the dendritic knob are the cellular compartment of the olfactory neurons where the sensory function resides, conferred by the olfactory receptor proteins exposed in their membrane surface (Buck and Axel 1991).

The olfactory cilia present in the ONs have a (9 + 2) microtubule configuration that is normally found in motile cilia. Nevertheless, they lack the dynein arms required for movement and thence are rendered immotile. They form an intertwined mat in order to augment the available surface of the OE for odorants by over 40 folds enhancing the global detection capability (Menco 1984) (Jenkins, McEwen et al. 2009).

The structural framework of the cilia is made up of long α- and β-tubulin strands dimers, composing the ciliary axoneme. In this context, the microtubular backbone affords the route for molecular motors, such as kinesins, to transport cargo inside and outside of the cilium (Jenkins, McEwen et al. 2009)

Looking more deeply at the olfactory signal-transduction mechanism, we can say that it begins when an odorant interfaces with the binding site of an olfactory-receptor. In humans, the olfactory receptors genes represent the largest gene family of G protein-coupled receptors. These receptors share a 7-transmembrane domain structure with many neurotransmitter and hormone receptors (Berghard and Buck 1996) (Buck and Axel 1991). An odorant, to be bound by the receptor proteins, must be sufficiently volatile and soluble so that some of its molecules may foremost reach the surface of the olfactory epithelium and then dissolves in the mucous film coating the binding sites.

In the olfactory epithelium there are as many as 1000 different odorant-binding proteins (OBP) whose function is to concentrate and transfer the odorant molecules to the receptor proteins (Buck and Axel 1991).

A single odor, in the recognition process, is broken down into its various detectable components, and each of them is complementary to the binding sites of one discrete receptor. This mechanism allows that each odorant element corresponds to only one type of receptor, among the thousands existing in the epithelium. Therefore, the same receptors could be activated from different scents that contain common odorant components (Berghard and Buck 1996) (Buck and Axel 1991).

Any receptor protein is coupled to a Guanine nucleotide-binding protein (G-proteins), which is activated when the odorant molecule is recognized by the corresponding olfactory receptor binding site. This event triggers the signal cascade leading to cyclic adenosine monophosphate (cAMP) intracellular reactions, which in turn induce the Na+ channels to open. As a consequence of the related ion movement, neuronal depolarization generates action potentials propagated along the axon up to the olfactory bulbs (Buck and Axel 1991).

About 25.000 axons of olfactory neurons converge and establish synapses with each of the 2.000 glomeruli contained in the input layer of one of the two

olfactory bulbs. The glomeruli are rounded structures that receive input only from olfactory neurons expressing a particular receptor protein gene so that the odorant code corresponds to a glomeruli topological map (Buck and Axel 1991).

At this level, the mitral and tufted cells of the olfactory bulb send their axons through the lateral olfactory tract, linking to the secondary olfactory structures (i.e. the anterior olfactory nucleus, the piriform cortex, the medial amygdala nuclei, the peri-amygdaloid cortex, and the entorhinal cortex). Then, from the above-mentioned structures, fiber connections project to associated neocortical areas (tertiary olfactory structures) such as the orbitofrontal cortex and the anterior insula. In addition, it is important to mention that functional imaging recordings revealed that many other brain areas are activated by olfactory stimuli without a direct anatomical link to primary olfactory areas. For example, the pathways also include the cerebellum, which seems to have a role in motor control of olfaction (Freiherr, Wiesmann et al. 2013)(Welge-Lüssen and Hummel 2004).

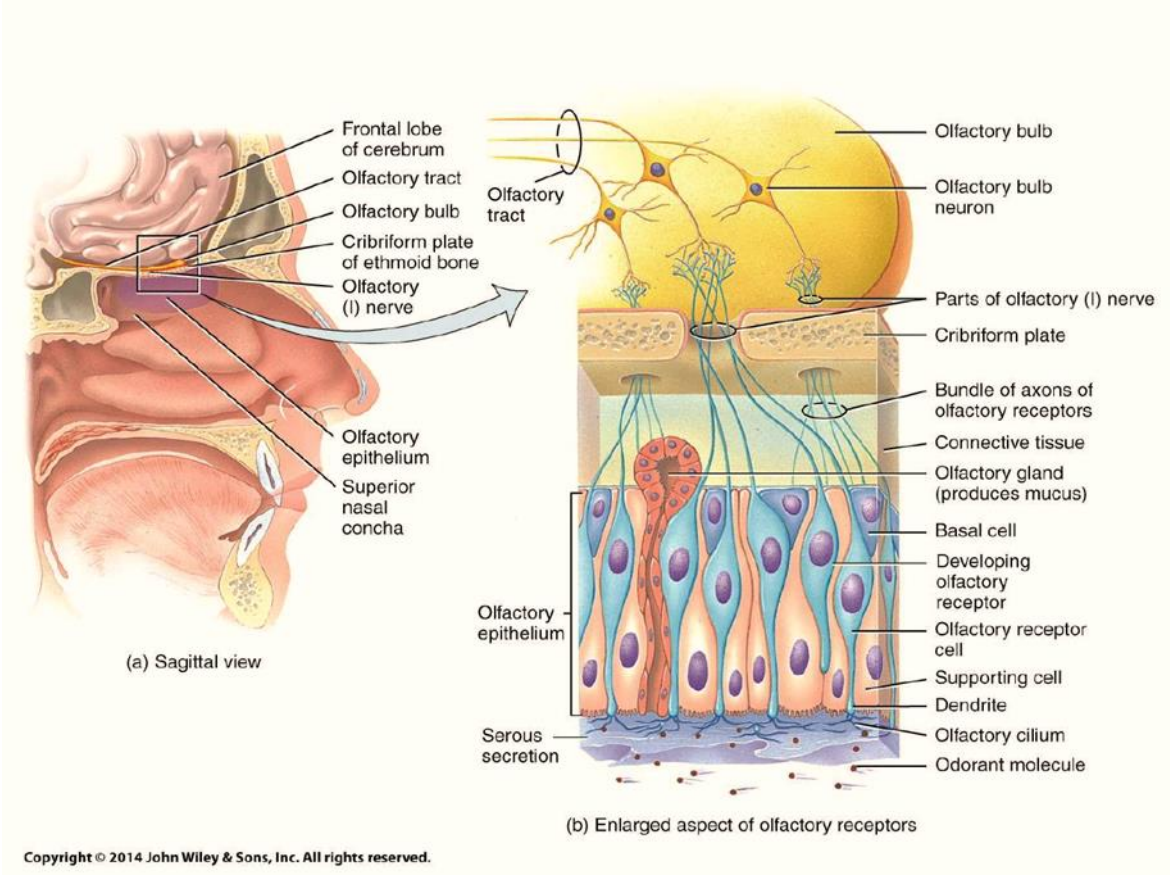


**Fig. 4 Schematic representation of the anatomy organization of the peripheral olfactory system.**

## 1.7 Olfactory neuroepithelium: methodological approaches and application tools

Given the convenient accessibility of its location, its inherent regenerative capacity and tight connection with the CNS, the olfactory epithelium and related neurons have caught the scientific community's attention since the mitotic activity in the basal cells of the olfactory epithelium of adult mice was first reported eighty years ago (Nagahara, 1940). Subsequently, regeneration of olfactory sensory neurons was observed in monkeys after toxic damage (Schultz, 1941). Scientific interest was directed at understanding not only the intriguing physiology of the olfactory system, but also cellular components, including cell culture, in order to exploit its unique neurogenic properties both from a diagnostic and a therapeutic perspective (Takagi, 1971, Graziadei, 1973, J. Attems, 2015, Choi and Goldstein, 2018).

### 1.7.1 Culturing olfactory neurons

Since the scientific community realized the neurogenesis potency of the olfactory neuroepithelium, many efforts have been made to exploit it in order to establish *in vitro* cell systems and different results have been achieved (Moulton, Celebi et al. 1970, Smart 1971, Graziadei and Graziadei 1979, Whitman and Greer 2009, Brann and Firestein 2014).

Therefore, the scientific research on this topic directed towards cell culture technology development, initially using late gestation embryos of rodent model for cell-handling assays (Chuah and Farbman 1983, Calof and Chikaraishi 1989) and then manipulating the olfactory tissue of newborn rats (Gonzales, Farbman et al. 1985, Pixley and Pun 1990).

At first, Pixley and Pun ascertained the neuronal nature of rat olfactory cells in culture assessing their electrophysiological functionality through recordings of electric currents, which were elicited via action potentials evoked by odorous stimuli. Further evidence of their neuronal phenotype was provided by

immunostaining of neuron-specific proteins: neuron-specific enolase, microtubule-associated protein 2, tau protein and synaptophysin (Pixley and Pun 1990).

Unfortunately, the cells obtained with this *in vitro* method could not complete their maturation, as indicated by the lack of immunodetection of the olfactory marker protein (OMP), which is exclusively expressed in mature olfactory neurons (Keller and Margolis 1975, Farbman and Margolis 1980, Miragall and Graziadei 1982).

However, Pixley and Grill developed a new culture system seven years later, plating olfactory cells from adult rat nasal mucosal tissue onto a feeder layer of newborn rat cortical glia (astrocytes) in serum-free conditions. This novel technique, which mimicked a previous strategy with olfactory bulb explants as culture substrate (Chuah and Farbman 1983), enabled them to obtain *in vitro* mature olfactory neurons, as confirmed by the OMP immunolabelling (Grill and Pixley 1997). Although the physiological function of OMP is not yet fully understood (Nakashima, Nakashima et al. 2019), it plays a crucial role in the olfactory signal transduction (Buiakova, Baker et al. 1996). In addition, it is widely accepted as a marker of mature ONs, even though OMP is also detectable in some non-olfactory tissues, which are not classified as classical chemosensory (Kang, Kim et al. 2015).

In the following years, the *in vitro* methodology made important progress, not only in the propagation and differentiation (Liu, Shields et al. 1998)(Cunningham, Manis et al. 1999), but also in the short-time maintenance (Vargas and Lucero 1999) of primary olfactory neurons odorants-responding.

An important study on the improvement of the culture technique using mice olfactory neurons fed onto a cortical astrocytes layer, described a significant enhancement of the survival rate and viable cell density in such conditions. In the same study, the authors reported the immunodetection of novel neuronal markers such as β-tubulin III, NCAM (Neuronal Cell Adhesion-Molecule, CD56) and GAP-43 (Growth Associated Protein 43) (Chen, Dadsetan et al. 2008). Moreover, the mice cells, which had been plated following the aforementioned protocol, completed their maturation process exhibiting the expression of olfactory neuron-

specific markers like OMP, Golf (an olfactory neuron specific-G protein) (Jones and Reed 1989), AC3 ( Adenylate cyclase 3) and CNGA2 (Cyclic Nucleotide Gated Channel Subunit Alpha 2) (Chen, Dadsetan et al. 2008).

Subsequently, research turned its focus on the maintenance and identification of the stem and progenitors' lineage in culture. In fact, it was just known that the olfactory mucosa comprehends cells differentiated at diverse levels originated from neural stem cells, which mature as glial or neuronal cells under the influence of specific growth factors, also in *in vitro* condition (Goldstein, Fang et al. 1998, Sicard, Feron et al. 1998, Murrell, Féron et al. 2005, Guo, Packard et al. 2010, Chen, Tian et al. 2014).

A detailed protocol to isolate stem cells from rodent or human olfactory epithelium has recently been published in the peer-reviewed scientific video journal JOVE (Girard, Devéze et al. 2011). Nowadays, culturing mice olfactory cells is a well-implemented and sound *in vitro* methodology for neuroscientific cellular studies (Gong 2012, Georgiou, dos Reis et al. 2018, Kurtenbach, Goss et al. 2019).

Despite this, the capacity of culturing human samples is far lower compared to the results obtained in the mice model.

In a pivotal study on human, Wolozin and coauthors cultured, for the first-time, olfactory epithelium samples, which had been collected *postmortem* from cadavers (Wolozin, Sunderland et al. 1992). Some years later, olfactory cultures were obtained from human embryonic tissue (Vannelli, Ensoli et al. 1995). Soon after, researchers designed olfactory culture systems from *ex vivo* human samples (Murrell, Bushell et al. 1996, Féron, Perry et al. 1998), but it was only in the 21st century that the methodology of culturing olfactory cells from living subjects became feasible (Gomez, Rawson et al. 2000, Hahn, Han et al. 2005, Borgmann-Winter, Rawson et al. 2009) (Tanos, Saibene et al. 2017).

Despite great efforts, the results of propagating *in vivo* human olfactory neurons from isolated adult olfactory epithelium cells have been modest in terms of neuronal differentiation, especially if compared with the results obtained in animal models.

For example, in the pioneering study by Féron et al. human nasal explants had a 50% efficacy of producing viable culture, and only 50% of this (25% in total) resulted in neurons outgrowth after stimulation with the factor FGF2 (Feron al. 1998).

However, fascinating strategies exploiting the differentiation capability of the olfactory progenitor cells have been designed to obtain commitment of mature neuronal cells. This approach has been implemented mainly in regenerative medicine, such as repair therapies after spinal cord injury (Feron, Perry et al. 2005), and as diagnostic tool for psychiatric and neurodegenerative brain disorders, like schizophrenia (McCurdy, Féron et al. 2006, Evgrafov, Wrobel et al. 2011, Idotta, Tibaldi et al. 2019) and PD (Matigian, Abrahamsen et al. 2010). Lastly, the culturing methodology has been employed also in pharmacology strategies for PD treatment using a trans-differentiation method in dopaminergic neuron-like cells (Alizadeh, Kamrava et al. 2019, Chabrat, Lacassagne et al. 2019).

### 1.7.2 Olfactory biopsies

The initial attempts to perform human olfactory mucosa biopsies have been made for electron microscopy analysis in anatomy studies (Douek, Bannister et al. 1975, Polyzonis, Kafandaris et al. 1979). It was only in 1982 that a detailed protocol was published and through which, using a properly developed instrument and technique, it was possible to perform a safe biopsy of the olfactory epithelium in living human being (Lovell, Jafek et al. 1982). Since then, the technique has been continuously developed (Garcia, Rossaneis et al. 2020) for the evaluation of olfactory diseases and dysfunctions, as well as for scientific research intents in human, given the fact that olfaction is not adversely affected by this procedure if correctly performed (Lanza, Deems et al. 1994). Other applications of the biopsy technique included studies of olfaction in aging (Trojanowski, Newman et al. 1991, Paik, Lehman et al. 1992), the effects of drugs and pollutants (Dalton, Jaen et al. 2010, Sammeta and McClintock 2010, Werner, Nies et al. 2018) and the evaluation of therapeutic regimens for olfactory disorders(Jafek, Murrow et al.

2002). Despite the valuable information obtained with this technique, this never became a routine procedure because of its invasiveness and the pain that it causes to the subject, but rather is considered a research practice performed with extreme caution and discretion in specific cases.

Taking advantage of the minimum area of tissue which is necessary to obtain a whole intact piece of olfactory mucosa and considering that it is suitable for immunohistological examinations, biopsies both in human and in animal models have been performed to attain immunophenotypic characterization. In this regard, an investigation on mice biopsies identified the olfactory neurons with the general neuronal marker PGP 9.5 (Protein gene product-9.5), a neuronal marker selected also in the analysis performed for this thesis. In the same samples, instead, the globose and horizontal basal cells were characterized on the grounds of the different levels of Sox2/Pax6 (Sex determining region Y-box-2/Paired box-6) and Cytokeratin 14 respectively, while the neuronal progenitors showed a modulation of Mash1 (Mammalian aschaete scute homolog-1) and Neurog 1/NeuroD 1 (Neurogenin-1/Neurogenic differentiation factor-1)(Guo, Packard et al. 2010).

Very recently, harvested biopsies of olfactory mucosa have been used for the at-the-state-of-the-art gene expression analysis through single-cell RNA sequencing, resulting in the cellular profiling of proteins selected also for the phenotypic characterization conducted and depicted in this thesis (Section 4.1), like the neuronal marker TUJ-1 and the OMP (Durante, Kurtenbach et al. 2020).

As described in Section 1.6, the olfactory mucosa lies also in the middle turbinate (Leopold, Hummel et al. 2000), and its biopsies contain viable neural progenitor cells capable of generating neuronal cell cultures. For these reasons, a technically simpler biopsy of the middle turbinate can be applied to propagate *in vitro* neural progenitor cells (Wrobel, Mazza et al. 2013). From the same area, it is also possible to attain precious sampling of the olfactory mucosa by the innovative and less invasive procedure of the olfactory brushing (OBg).

### 1.7.3 Olfactory brushing in the diagnosis of brain disorders

Olfactory brushing (OBg) is a novel and non-invasive procedure for sampling olfactory mucosa (OM) from the middle turbinate (Leopold, Hummel et al. 2000). Originally, we showed that OM sampling has a diagnostic application in human prion disease. Indeed, OM samples obtained from patients suffering from Creutzfeldt-Jakob Disease (CJD) and tested with the real-time quaking-induced conversion (RT-QuIC) assay provided a diagnostic accuracy of nearly 100% (Orrú, Bongianni et al. 2014).

In this thesis study, we have used the aforementioned procedure, described in detail in Section 3.4, in order to obtain all the precious OM samples both from the healthy subjects of control and the synucleinopathies patients.

## 1.8 Novel prion and prion-like diagnostic *in vitro* assays

Regarding the analytical tests employed in this thesis for the study of the prion-like seeding activity of the α-synuclein, we implemented an *in vitro* assay on the ground of the RT-QuIC detection system for prion aggregation.

### 1.8.1 Real-time quaking-induced conversion

The real-time quaking-induced conversion (RT-QuIC) reaction is an analytical technique developed through the combination of the base of the assay designed by Kocisko and colleagues (Kocisko, Come et al. 1994) and features of the Amyloid Seeding Assay (ASA) designed by Colby and colleagues (Colby, Zhang et al. 2007).

RT-QuIC was described for the first time in 2010 as an original technique to detect a minimum quantity of $PrP^{Sc}$ (Atarashi, Satoh et al. 2011) (Wilham, Orrú et al. 2010).

Initially, the technique uses a recombinant PrP (rPrP) as substrate for the conversion reaction. Indeed, it exploits the ability of $PrP^{Sc}$ to induce the misfolding $PrP^{C}$ so that it aggregates forming $PrP^{Sc}$ fibrils.

In particular, any $PrP^{Sc}$ present in the sample mixture binding the recombinant substrate induces a conformational change resulting in the formation of fibrils.

The mechanism for generating these structures is defined as the lag phase and may be necessary for up to 30 hours because it evolves. In the following phase, the fibrils formed start to aggregate and in turn bind, thioflavin T, finally emitting fluoresce.

The RT-QuIC reaction is monitored in real-time because of the presence of Thioflavin T (ThT) in the buffer, which is an amyloidotropic fluorescent dye. During the RT-QuIC reaction, ThT binds to the amyloid and amyloid-like fibril species generated by the prion seeded misfolding of the rPrP substrate.

Actually, the RT-QuIC mixture with the addition of biological samples is loaded in a 96-wells plate, and then the reaction is triggered following several intermittent shaking cycles at constant temperature incubated in a plate-reader. The advancement of the reaction is revealed in real-time by the detection of the fluorescence emitted from ThT bound to amyloid structures. The kinetics of the ongoing aggregation is monitored and its graphical representation describes a characteristic sigmoidal curve. (Wilham, Orrú et al. 2010) (Atarashi, Sano et al. 2011).

Currently, the RT-QuIC assay has been proposed as the first specific *in vivo* diagnostic test for sporadic CJD (sCJD) (Atarashi, Sano et al. 2011).

Still, Orrù *et al*. (Orrú, Groveman et al. 2015) achieved an improvement of the technique in the analysis of the cerebrospinal fluid (CSF) some years later, the so-called second-generation RT-QuIC for CSF (IQ-CSF) assay which introduced a truncated form, proteinase K-sensitive, of hamster recombinant PrP ($rPrP^{sen}$, aa 90–231) as substrate and changed few experimental conditions. The results obtained with the IQ-CSF assay showed higher analytical and diagnostic sensitivity, reducing testing times (Bongianni, Orrù et al. 2017).

Moreover, our research group designed a protocol for RT-QuIC analysis on olfactory mucosa samples obtained by nasal brushing. This method has proved to have a comparable specificity and enhanced sensitivity if compared with CSF analysis, making an important step further in the intravital diagnosis of the prion diseases. (Orrú, Bongianni et al. 2014).

### 1.8.2 Alpha-synuclein seeding activity detection

Recently, RT-QuIC technology has been exploited for a novel assay able to detect, with high sensitivity and specificity (95-100%), small amounts of α-synuclein aggregate in CSF (Fairfoul, McGuire et al. 2016) (Shahnawaz, Tokuda et al. 2017) (Groveman, Orrù et al. 2018) (Sano, Atarashi et al. 2018).

However, this test has thus far only been assessed in definite clinical cases and/or by the classical neuropathological examination confirmed cases, despite the clinicians' requests to obtain indications for the discrimination of the uncertain

cases between PD and the various forms of atypical parkinsonism (AP) (van Rumund, Green et al. 2019).

Preliminary studies showed that a significant percentage of MSA and PD olfactory mucosa samples obtained through the nasal brushing procedure we designed, gave a positive RT-QuIC outcome for the α-synuclein seeding activity with high efficiency (De Luca, Elia et al. 2019).

Interestingly, also some samples of CBD and PSP patients clinically diagnosed resulted in RT-QuIC positive assay (Shahnawaz, Tokuda et al. 2017).

The TEM analysis of the final α-synuclein real-time quacking-induced conversion (α-syn RT-QuIC) products obtained from MSA and PD samples showed their own structural characteristics eventually making the identification of the starting pathology possible, using this discrimination approach (De Luca, Elia et al. 2019).

The aforementioned study demonstrated the presence of molecular species with α-synuclein seeding activity in the olfactory mucosa samples of patients suffering from diverse synucleinopathies (PD and similar neurodegenerative parkinsonisms).

On the basis of these results, the combination of CSF and OM α-syn RT-QuIC tests may be a feasible strategy to improve the global diagnostic accuracy of these diseases and to obtain an early pre-motor diagnosis tool.

# 2. AIMS OF THE THESIS

This study has been designed and conducted in order to pursue the following aims:

1) the acquisition of "normative values" of olfactory-related proteins in the nasal mucosa, collected by OBg procedure;

2) the definition of the constitutive expression patterns of the major NDs-associated proteins in the olfactory neuroepithelium of healthy subjects;

3) the description of the α-synuclein expression patterns in patients with synucleopathies;

4) the comparison between the immunocytochemical results of α-synuclein expression patterns and those obtained by α-syn RT-QuIC technology.

# 3. MATERIALS AND METHODS

## 3.1 Laboratories and clinics

All laboratory work included in this thesis has been done in the Neuropathology Laboratory of "Policlinico G.B. Rossi" until May 2019, then moved to the Research Centre LURM (Laboratorio Universitario di Ricerca Medica) of the University of Verona. Some other experiments have been carried out in the laboratory directed by Prof. Mario Buffelli at the Physiology Section of the University of Verona. The microscopy confocal acquisition has been run at CPT (Centro Piattaforme Tecnologiche) of the University of Verona.

Following informed consent, the olfactory mucosa samples were executed by Dr. Luca Sacchetto, otolaryngologist at the Otorhinolaryngology Clinic of "Policlinico G.B. Rossi" and at "Ospedale Civile Maggiore" in Verona.

Part of the OM samples from patients have been collected at the Department of Neurology, Innsbruck Medical University. Other OM samples came from "Clinica Neurologica e di Neuroriabilitazione" of "Azienda Sanitaria Universitaria Integrata di Udine", and from "Unità Operativa Clinica Neurologica" of "Azienda Ospedaliera Universitaria San Martino" in Genova.

All the other OM samples came from "UOC di Neurologia" of "Azienda Ospedaliera Universitaria Integrata Verona".

## 3.2 Healthy subjects recruitment

Thirty healthy volunteers underwent nasal swabbing. These included 15 males (mean age: 50,4 years; range: 22-78 years) and 15 females (mean age: 54,2 years; age range: 19-79 years). Exclusion criteria were determined by the presence of pathology affecting the olfactory function (e.g., recent head trauma, rhinitis or chronic sinus infection, diabetes, stroke, history of smoking/alcohol). Olfactory swabbing was performed following the approval of the Ethical Committee of University Hospital of Verona (Prot.n.28917 June, 15$^{th}$, 2012). Olfactory mucosa sampling was performed after written informed consent.

## 3.3 Patients recruitment

The synuceleinopathies patients have been recruited by Prof. Gianluigi Zanusso, neurologist ("UOC di Neurologia" of "Azienda Ospedaliera Universitaria Integrata Verona"), by Dr. Ambra Stefani (Department of Neurology, of the Innsbruck Medical University), by Francesco Janes ("Clinica Neurologica e di Neuroriabilitazione" of "Azienda Sanitaria Universitaria Integrata di Udine"), and by Prof. Flavio Nobili ("Unità Operativa Clinica Neurologica" of "Azienda Ospedaliera Universitaria San Martino" in Genova). Each patient gave informed consent after the explanation of the purpose of the research and the methodology of the study and before the swabbing procedure.

## 3.4 Olfactory brushing procedure

Olfactory mucosa (OM) samples were obtained by the olfactory brushing (OBg) procedure. It consists of nasal brushing sampling using a flocked swab (Copan Flock Technologies, Italy), which is oriented through the optical guide of a rigid endoscope (Fiberscope Storz 0°) into the nasal vault towards the lateral wall of the olfactory cleft in order to reach the olfactory epithelium. The use of the endoscope ensures that the collection occurs in the neuroepithelium area at the level of the medial face of the middle turbinate (Leopold, Hummel et al. 2000). If necessary and requested by the subject, the ENT specialist performing the brushing could administer a vasoconstrictor (NTR, nasal drops 15 ml: Phenylephrine, Thenyldiamine, Benzalkonium chloride; Teofarma Srl, Italy) to make the nostrils more accessible and the swabbing even more comfortable. The sampling, where possible, was taken in both nostrils. This simple procedure, correctly performed, is rapid, non-invasive, inexpensive and can be repeated several times producing minimal pain or discomfort to the subject. For each swab provided, we collected 1 million living cells on average.

## 3.5 Olfactory mucosa samples processing

The first swab performed from each nostril was directly immersed after the procedure in a 15 ml Falcon tube containing the fixative Diacyte (Diapath S.p.a., Italy). The second swab instead was immediately conserved in a Falcon tube half-filled with Saline.

This protocol, observed for each subject, allowed us to allocate the first samples for the immunocytochemistry analysis because the cellular components contained in it possess a better-preserved morphology and lower chance of blood traces. The following samples collected were commited to the biochemical and molecular biology assays.

The swabs immediately immersed in the Diacyte tube after the sampling procedure were left in the fixative solution at least 30 minutes at room temperature. After the fixation, a brief treatment (10-15 minutes at room

temperature) with the mucolytic CytoRich Red (Diapath S.p.a., Italy) ensured the removal of nasal and other debris and so the cells were recovered vortexing the swab. Then, the samples were centrifuged at 1800 rpm and the resulting pellet was washed with 1X PBS buffer. The cells, from the pellet, were distributed homogeneously in the 1X PBS solution and counted with the Automated Cell Counter (Countess™ II FL; AMQAF1000; Invitrogen). Thus, the appropriate amount of cells was loaded in dedicated CYTOFUNNELs (Thermo Fisher Scientific, Italy) and then placed on cytological slides by the proper cytocentrifuge (CYTOSPIN IV, AHSI, Italy).
As a result, the cytological slides prepared by following the passages described above were immediately ready for the immunocytochemistry analysis, or alternatively, they could be conserved in a specific box at -20°C.

## 3.6 Immunocytochemistry

In this thesis, the indirect immunofluorescence staining technique has been used for the revelation of the primary antibodies binding. This technique exploits a fluorochrome-labeled secondary antibody which recognizes the IgG domain of the bound primary antibody.
The first step of the immunofluorescence protocol was the incubation of the cytological slides for 1 hour at room temperature with a blocking solution containing a mild permeabilization agent (5% of normal serum from the same animal species in which the secondary antibodies are raised; 0,3% of Triton™ X-100; in 1X PBS). Afterwards, the primary antibodies (abs used for the phenotypic characterization are listed in **Table 1**, instead the abs used as neurodegeneration markers in **Table 2**) diluted in a buffer containing 1% of bovine serum albumin and the same permeabilization agent (1% of BSA, 0.3% Triton™ X-100; in 1X PBS), were incubated over the spots of the cytological slides inside a humid chamber overnight at 4°C. After three washings with 1X PBS, which lasted ten minutes each, the secondary antibodies Alexa Fluor-conjugated (Life Technologies, Carlsbad, CA, USA) diluted (1:1000) in the same solution of the

primary abs, were incubated on the microscope slides in the humid chamber, for one hour at room temperature.

Nuclear counterstaining (1:2000) was supplied using 4′,6-diamidino-2-phenylindole (DAPI) diluted 1:2000 in 1X PBS for 5 minutes after three washings in 1X PBS for ten minutes each.

Lastly, glass coverslips were mounted on the microscope slides with ProLong Antifade Mountants for Fixed Cells (Thermo Fisher Scientific Inc., Italy).

**Table 1** ***Primary antibodies used to characterize the epithelial cellular samples***

| Antibody | Host | Antigen | Code (clone) | Company | Working dilution |
|---|---|---|---|---|---|
| **Anti-OMP** | rabbit, polyclonal | Olfactory marker protein | sc-67219 (FL-163) | Santa Cruz | 1:400 |
| **Anti-OMP** | mouse, monoclonal | Olfactory marker protein | sc-365818 (B-6) | Santa Cruz | 1:400 |
| **Anti-OMP** | goat, polyclonal | Olfactory marker protein | 544-10001-WAKO | Wako | 1:400 |
| **Anti-β-tubulin III** | rabbit, polyclonal | aa 441-450 of β-tubulin class III | T2200 (TUJ-1) | Sigma Aldrich | 1:400 |
| **Anti-β-tubulin III** | mouse, monoclonal | aa 443-450 of β-tubulin class III | TU-20 | Sigma Aldrich | 1:300 |
| **Pan-Cytokeratin** | mouse, monoclonal | All isoforms of Cyotkeratin protein family | MA5-15507 | Thermo Fisher | 1:300 |
| **PGP 9.5** | rabbit, polyclonal | Ubiquitin carboxy-terminal hydrolase L1 | Z5116 | DAKO | 1:300 |

**Table 2** ***Primary antibodies used to identify the expression patterns of the major misfolded proteins involved in the main neurodegenerative diseases***

| Antibody | Host | Antigen | Code (clone) | Company | Working dilution |
|---|---|---|---|---|---|
| **Anti- α-synuclein** | mouse, monoclonal | Full length human α -synuclein | ab1903 (4D6) | Abcam | 1:750 |
| **Anti-α-synuclein (phospho S129)** | rabbit, polyclonal | α-synuclein phosporylated in Ser129 ((M-P-SP-E-E) | ab59264 (Phospho S129) | Abcam | 1:2000 |
| **Anti-APP (6E10)** | mouse, monoclonal | aa residues 1-16 of β-amyloid precursor protein | SIG-39320 (6E10) | Covance | 1:200 |
| **Anti-tau 5** | mouse, monoclonal | Total microtubule associated protein | AHB0042 (tau-5) | Thermo Fisher | 1:500 |
| **Anti-4R tau** | rabbit, monoclonal | aa 250-350 of 4R isoform of human tau | ab218314 (EPR21725) | Abcam | 1:500 |
| **Anti-3R tau** | mouse, monoclonal | aa 209-224 residues of 3R isoform of human tau | 05-803 (8E6/C11) | Merck Millipore | 1:500 |
| **Anti-TDP 43** | rabbit, polyclonal | aa residues 1 to 260 of TAR DNA binding protein 43 | 10782-2-AP | ProteinTech | 1:200 |

## 3.7 Imaging

Images of nasal olfactory mucosa preparations were acquired by a confocal inverted Leica TCS SP5 AOBS microscope (Zeiss, Milan, Italy), using a 40x (1.25 NA) and a 63x (1.25 NA) oil objectives. Images were saved as .tiff files; brightness and contrast were adjusted using the Leica Application Suite Advanced Fluorescence (LAS AF) Software (Leica-Mycrosystems, Wetzlar, Germany), ImageJ (NIH, Bethesda, MD, USA) or ImarisX64 7.2.1(Bitplane AG, Zurich Switzerland).

## 3.8 Alpha-synuclein RT-QuIC on olfactory mucosa samples

### 3.8.1 Olfactory brushing samples processing for α-syn RT-QuIC

Following nasal swabbing, one or two swabs were immersed in a 5 ml tube containing 0.9% saline and finally sealed. Tubes from each patient were labeled with a code, stored at 4°C for α-synuclein real-time quacking-induced conversion (α-syn RT-QuIC) assay. Cellular material was dissociated from the brush by vortexing the tubes for one minute at room temperature. After removing the brush from the tube, the cell suspension was centrifuged at 2000×*g* at 4°C for twenty minutes. The supernatant was removed, and the pellet was frozen at −80°C until assayed.

### 3.8.2 Expression and purification of recombinant human α-synuclein

Recombinant α-synuclein was expressed and purified from the periplasmic fraction as reported (Bongianni, Ladogana et al. 2019). Briefly, wild-type human α-synuclein cDNA was cloned in the pET-28a plasmid (Novagen) and transformed into *Escherichia coli* BL21 (DE3). Cell cultures (1 L) were grown at 37 °C to an OD 600nm of 0.3–0.4 and the expression was induced with 0.1 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) for 5 h. Cells were collected by centrifugation, re-suspended in 100 ml of osmotic shock buffer (30 mM Tris–HCl pH 7.2, 40% sucrose, 2 mM EDTA) and incubated for 10 minutes at room temperature. The cells were centrifuged at 12000 rpm, resuspended in 90 ml of cold water with 37.5 ul of saturated $MgCl_2$ solution and, after five minutes incubation on ice, centrifuged again. The supernatant containing the periplasm proteins was boiled for fifteen minutes and cleared by centrifugation. The soluble fraction, enriched in α-synuclein, was subjected to ammonium sulfate precipitation followed by extensively dialysis against 20 mM Tris–HCl, pH 8.0. Further purification of α-synuclein was performed by anion exchange chromatography loading the sample on a Q-Sepharose column (GE Healthcare) equilibrated with the same buffer and eluted with a 0–500 mM linear gradient of NaCl. The purity of α-synuclein was checked by SDS-PAGE. The protein was then dialyzed against 10 mM Sodium Phosphate buffer pH 7.4 and stored at -80 °C until use.

### 3.8.3 Alpha-synuclein RT-QuIC assay

α-synuclein real-time quacking-induced conversion (α-syn RT-QuIC) test has been set up using definite cases of patients with PD, Lewy body dementia (LBD), and MSA as reported (Bongianni, Ladogana et al. 2019).

OM samples were thawed and a disposable inoculating loop (Fisherbrand) was dipped into the pellet to transfer approximately 1 to 2 µl of the pellet into a tube containing 120 µl PBS. The latter tube was sonicated until the pellet was dispersed. For each test, two microliters of diluted OM sample were plated in 98 µl of Reaction Buffer composed of 100 mmol/L phosphate buffer (pH 8.2), 10 $\mu$mol/L thioflavin T (ThT), and 0.05 mg/mL human recombinant full- length (1–140 aa) α- synuclein. The plate was sealed with a plate sealer film (Nalgene Nunc International) and then incubated at 30°C in a BMG FLUOstar Omega plate reader with cycles of 1 min shaking (200 rpm double orbital) and 14 min rest. ThT fluorescence measurements (450 +/- 10 nm excitation and 480 +/- 10 nm emission; bottom read) were taken every 45 min. Four replicate reactions were tested for each sample. The test was considered positive when the reaction occurred in 2 out of 4 wells. For the normalization of the curves, a baseline value was calculated for each sample, when the lag phase reaches the lowest relative fluorescence units (rfu) values, i.e. between 15 and 17 hours. A positive response was defined as a value of > 3 SD above the baseline average of all samples, which is 100000 rfu. This value was calculated as > 3 SD above the average of all the lowest rfu values observed in the lag phase for each sample (i.e. the average between 15 and 17 hours). For sensitivity determinations cut-off time was assessed at 80 hours, based on the results obtained in the definite cases, in order to obtain the best specificity and sensitivity.

# 4. RESULTS

## 4.1 Phenotypic characterization of the epithelial cellular samples

The samples have been primarily analyzed looking at the detection of the phenotypic markers directed towards neuronal olfactory cells. This step was important to ensure that the cellular collection had been made in the olfactory region. The following markers were used: olfactory marker protein (OMP) and neuron-specific class III β-tubulin (TUJ-1) and the protein gene product 9.5 (PGP 9.5). We found that OMP immunoreactivity is mainly intracytoplasmic with a homogenous distribution array, while TUJ-1 shows the main filament pattern tracking the extension of the neural protrusions coming out from the axonal hillock (**Fig. 5**). It has to be pointed out that some non-neuronal shaped cells (probably supporting cells) showed positivity to the OMP antibody. Moreover, we examined the expression of Pan-Cytokeratin (PCK), a typical epithelial marker in combination with PGP 9.5 (**Fig. 6**), a cytoplasmic protein in neurons and neuroendocrine cells, and then with OMP (**Fig. 7**).

PGP 9.5 positivity revealed a coin-shaped figure in the cytosolic apical pole of mature and immature olfactory neurons and in some non-neuronal-shaped cells, likely supporting cells (**Fig. 6**). The supporting cells are large and columnar cells, different from olfactory neurons whose apical part clearly arrange the dendritic knob in the mature cell. The cells we considered immature olfactory neurons displayed a weaker dotted PGP 9.5 positivity inside the apical region (**Fig. 6**, asterisk).

PCK mAb binding all the isoforms of the protein showed a positive signal in all epithelial cells, neuronal and non-neuronal (**Fig. 6** and **Fig. 7**). In the neuronal components, the staining seemed to be confined to the apical intracytoplasmic compartment. In particular, there was a main filamentous cytosolic positivity on the superior dendritic projection (**Fig. 6**) while the non-neuronal cells showed the positive signal located on the plasma membrane. Lastly, the sample characterization was achieved by the combination of PCK and OMP (**Fig. 7**).

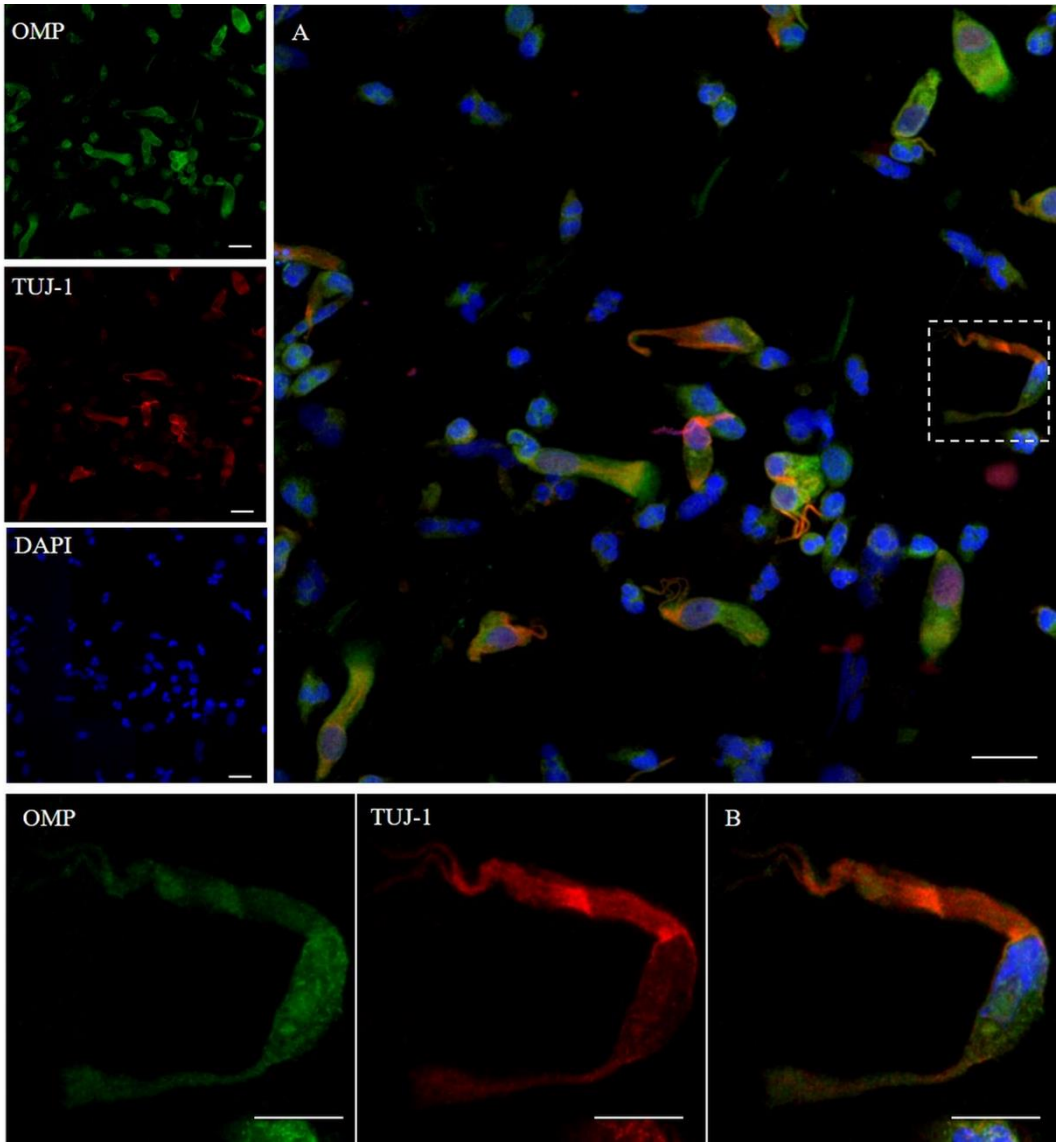


**Fig. 5 OMP and β-tubulin III (TUJ-1) distribution pattern.** OMP expression (green) is evident in the cytoplasm of olfactory neurons with a granular pattern inside the cell body. The TUJ-1 positivity (red) is marked along the neural projections, in particular in the axonal hillock, showing the distinctive β-tubulin III filament pattern. DAPI nuclear staining (blue). Scale bar overview: 20µm. Scale bar detail: 10 µm.

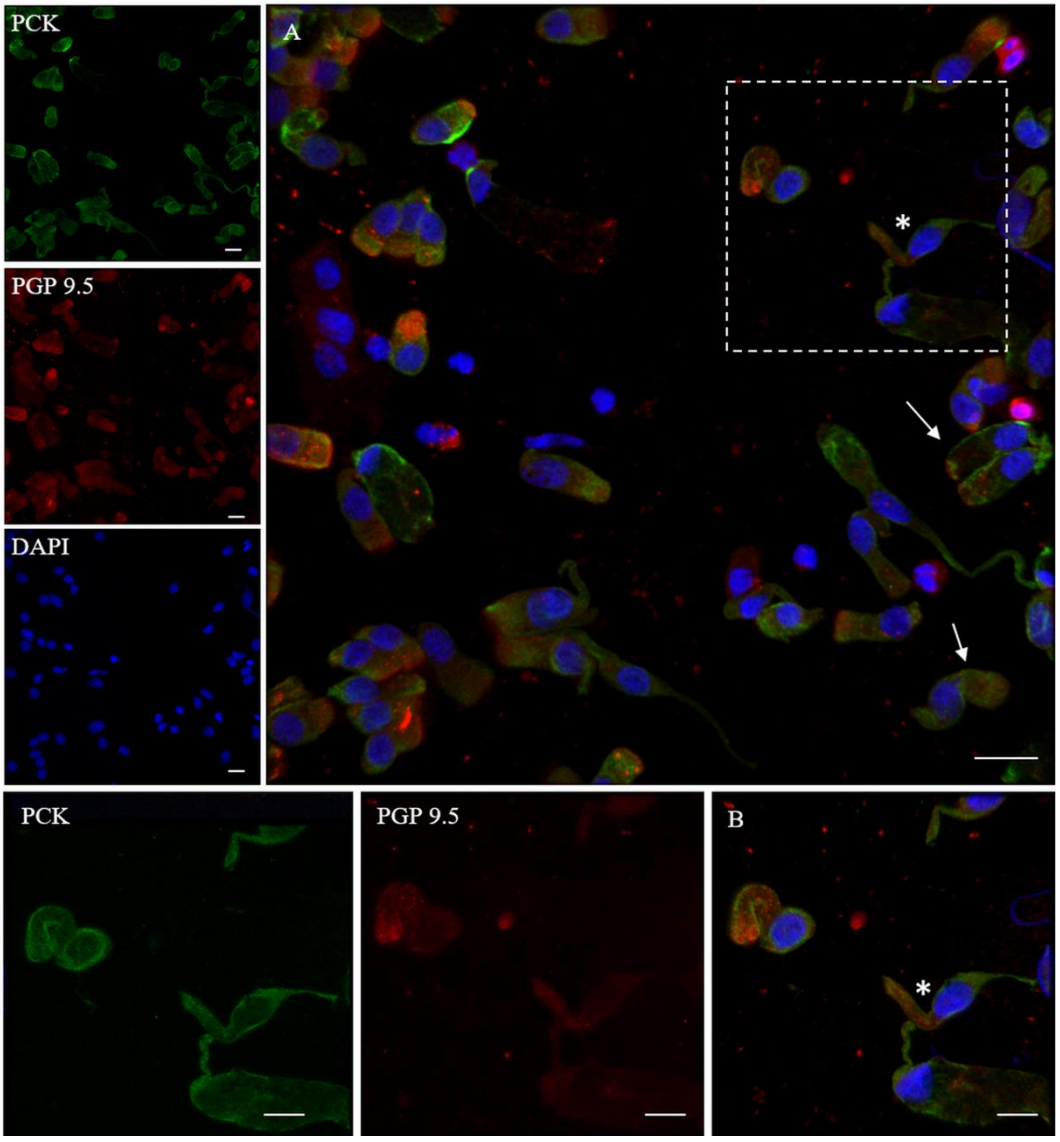


**Fig. 6 Distribution pattern of PGP 9.5 and PCK.** PGP 9.5 (red) shows a dotted positivity in the upper cytosolic region of the neurons (*, square detail). The PCK (green) is identified in the cytosolic compartment of the neurons and in the plasmatic membrane of the other epithelial cells (arrows). DAPI nuclear staining (blue). Scale bar overview: 20 μm. Scale bar detail: 10 μm.

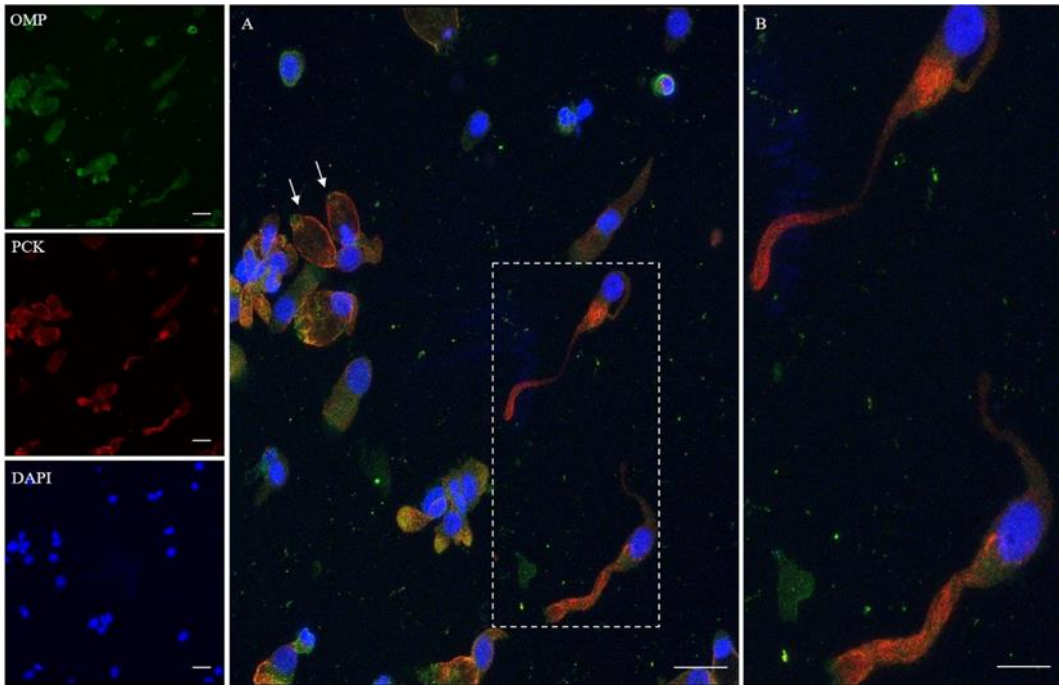


**Fig. 7 Two different patterns of PCK immunostaining.** The distribution for PCK (red) depends on the cell: in the neurons (outlined square, panel **A**), there is a predominant filamentous positivity on the superior dendritic projection, from the center level up to the cilia boundary (detail in panel **B**). In the other cells, the expression is distributed on the boundary of the cell body, all along the plasma membrane (arrows). DAPI nuclear staining (blue). Scale bar overview: 20 μm. Scale bar detail: 10μm.

## 4.2 Expression patterns of ND-associated proteins in healthy subjects

### 4.2.1 α-synuclein

The α-synuclein is an unfolded 140 amino acid protein encoded by the SNCA gene and its function is still not completely known. The distribution of the protein is ubiquitous but it is mainly expressed at the tips of neurons as a pre-synaptic protein. To detect the total expression of α-synuclein in the olfactory samples we selected the referenced 4D6 mAb (Lothian, Lago et al. 2019), which is designed on the non-modified full-length protein as the immunogen. Indeed, the 4D6 mAb binds all the isoforms of the protein, whatever post-translational modification may occur. As shown in **Fig. 8**, the expression pattern of the protein in cells OMP positive with non-neuronal shape is around the cellular membrane, while in the OMP negative and neuronal shaped ones there is mainly a granular positivity inside the cytosol. In particular, the cytosolic α-synuclein deposition is visualized on the dendritic knob.

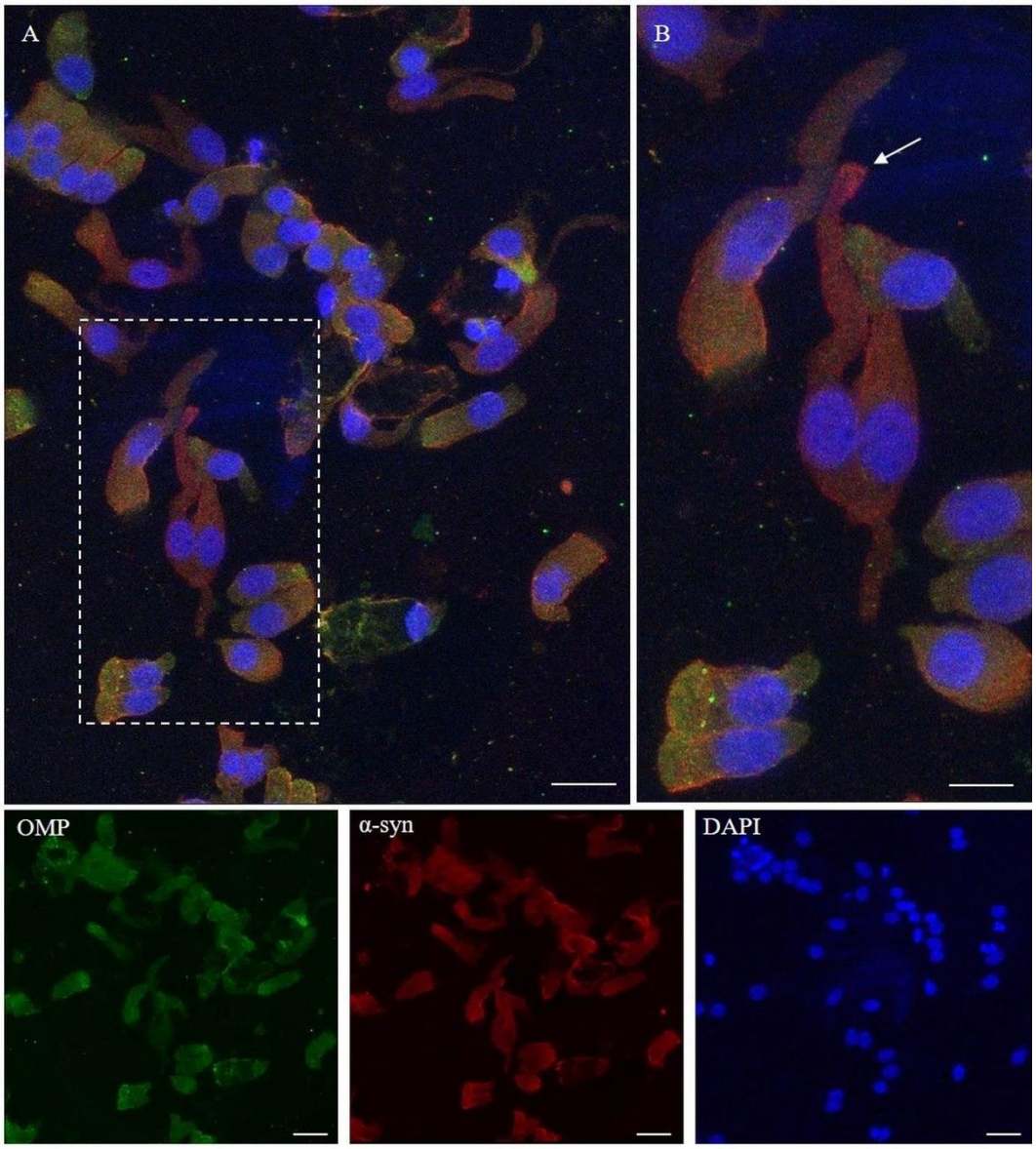


**Fig. 8 Distribution pattern of α-synuclein.** In the neurons, α-synuclein (4D6, red) shows a predominant diffused cytosolic thinly granular labeling particularly localized at the dendritic knob (outlined square in panel **A**, arrow on detail in panel **B**). In the other cells OMP positive (green), α-synuclein shows a positivity around the plasmatic membrane. DAPI nuclear staining (blue). Scale bar overview: 20 μm. Scale bar detail: 10 μm.

### 4.2.2 APP/β-Amyloid

APP gene encodes for the amyloid precursor protein, a transmembrane glycoprotein processed through sequential cleavages performed by different secretases. Amyloid beta (Aβ) is derived from APP by sequential cleavages of β- and γ-secretase. APP is widely expressed in human tissues with great expression in the central nervous system (Wang, Gu et al. 2017).

The 6E10 mAb reacts to residues 1-16 of Aβ and the epitope lies within amino acids 3-8 protein. We found that immunoreactivity to the 6E10 mAb is mainly localized in the neuronal-shaped cells, TUJ1 positive. It shows a dot-like distribution especially in the paranuclear level and at the tips of the cell (**Fig. 9**). Other non-neuronal shaped cells show a faint positivity (**Fig. 9**, arrows).

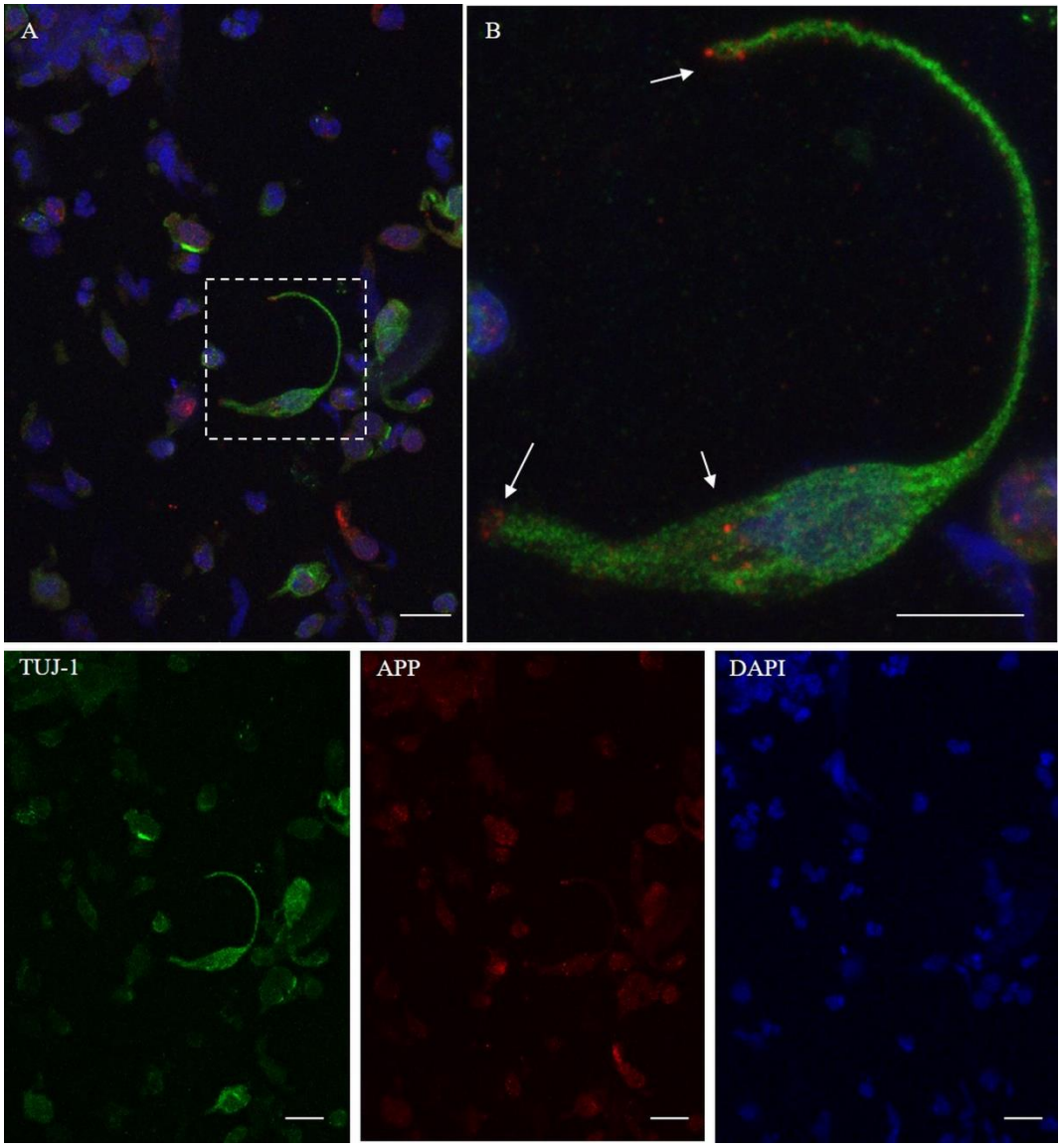


**Fig. 9 Distribution pattern of APP.** In the neurons (TUJ-1 positive, green), APP (red) has a focal expression (dot-like) mainly around the nucleus and at the level of the cellular tips (outlined square in panel **A**, details in panel **B**, arrows). DAPI nuclear staining (blue). Scale bar overview: 20 µm. Scale bar detail: 10 µm.

### 4.2.3 τ-protein

Tau-protein exists in six distinctive isoforms of different lengths in the human nervous system. This protein is a microtubules associated protein (MAP) and its function is to interact with tubulin to stabilize the microtubules framework. The six tau isoforms are generated from MAPT, the tau gene, through alternative messenger RNA (mRNA) splicing. Alternative splicing of exon 10 gives rise to three isoforms with three microtubule-binding repeats (3R) each and three isoforms with four microtubule-binding repeats (4R) each (Goedert, Spillantini et al. 1989).

Immunostaining with total tau antibody (tau-5 clone) (Puvenna, Engeler et al. 2016) (Borin, Saraceno et al. 2018) was detected in the neuronal shaped cells with a cytosolic localization (**Fig. 10**, arrows). The expression pattern is intracellular with a patch framework along the cell body, probably because the protein is associated with the microtubule cytoskeleton in specific cellular districts. Interestingly, when we decided to look at the typing of tau isoforms expressed in the mucosal samples, we found a positivity for the 4R class in the mature neuronal shaped cells. This is remarkably visible within the area underlying the olfactory knob (**Fig. 11, A, B**). On the other hand, for the 3R tau class, we found a very limited detection and the positivity displayed an uneven clustered pattern only in some rare rounded cells, likely globous stem cells (**Fig. 11, C, D**).

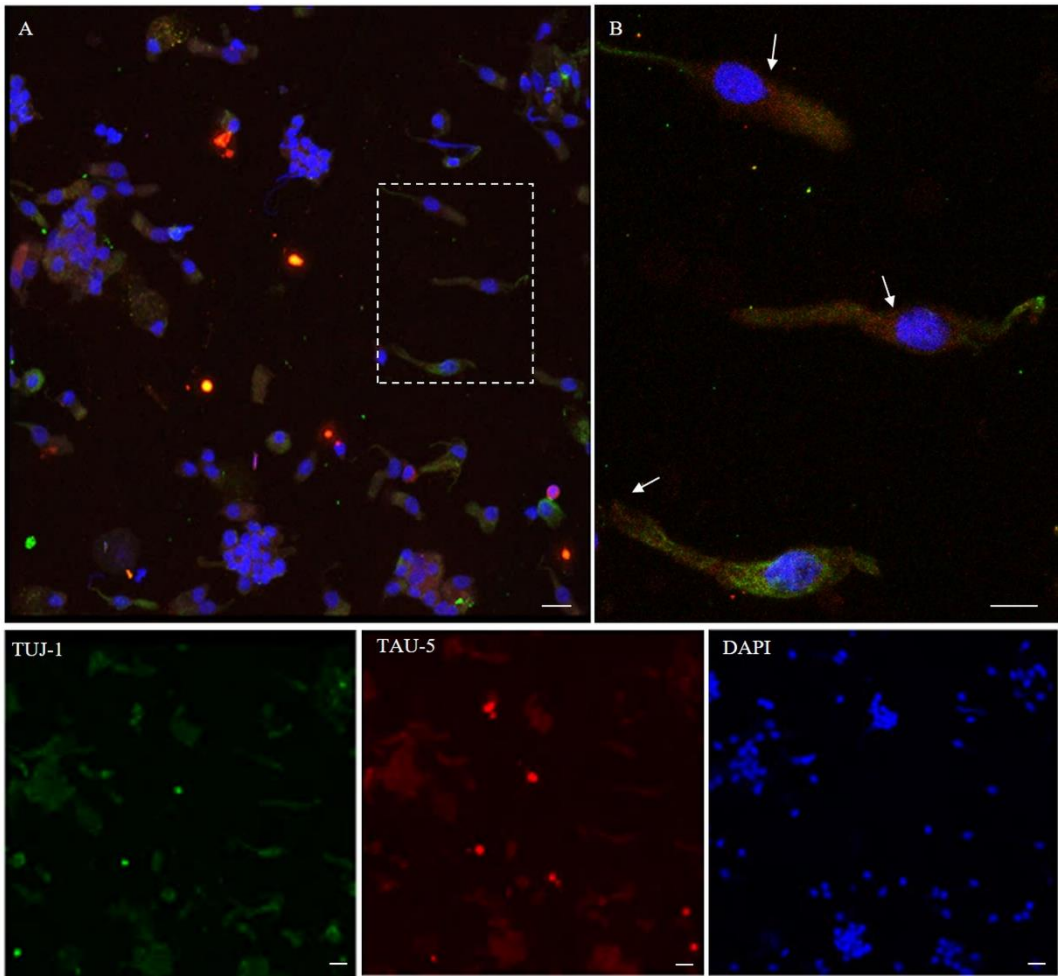


**Fig. 10 Distribution pattern of tau-5.** The total tau protein (tau-5) expression is found all along the cell body with particular intensity in the perinuclear region of the cytosol's neurons (outlined square in panel **A**; detail in panel **B**, arrows). DAPI nuclear staining (blue). Scale bar overview: 20 μm. Scale bar detail: 10 μm.

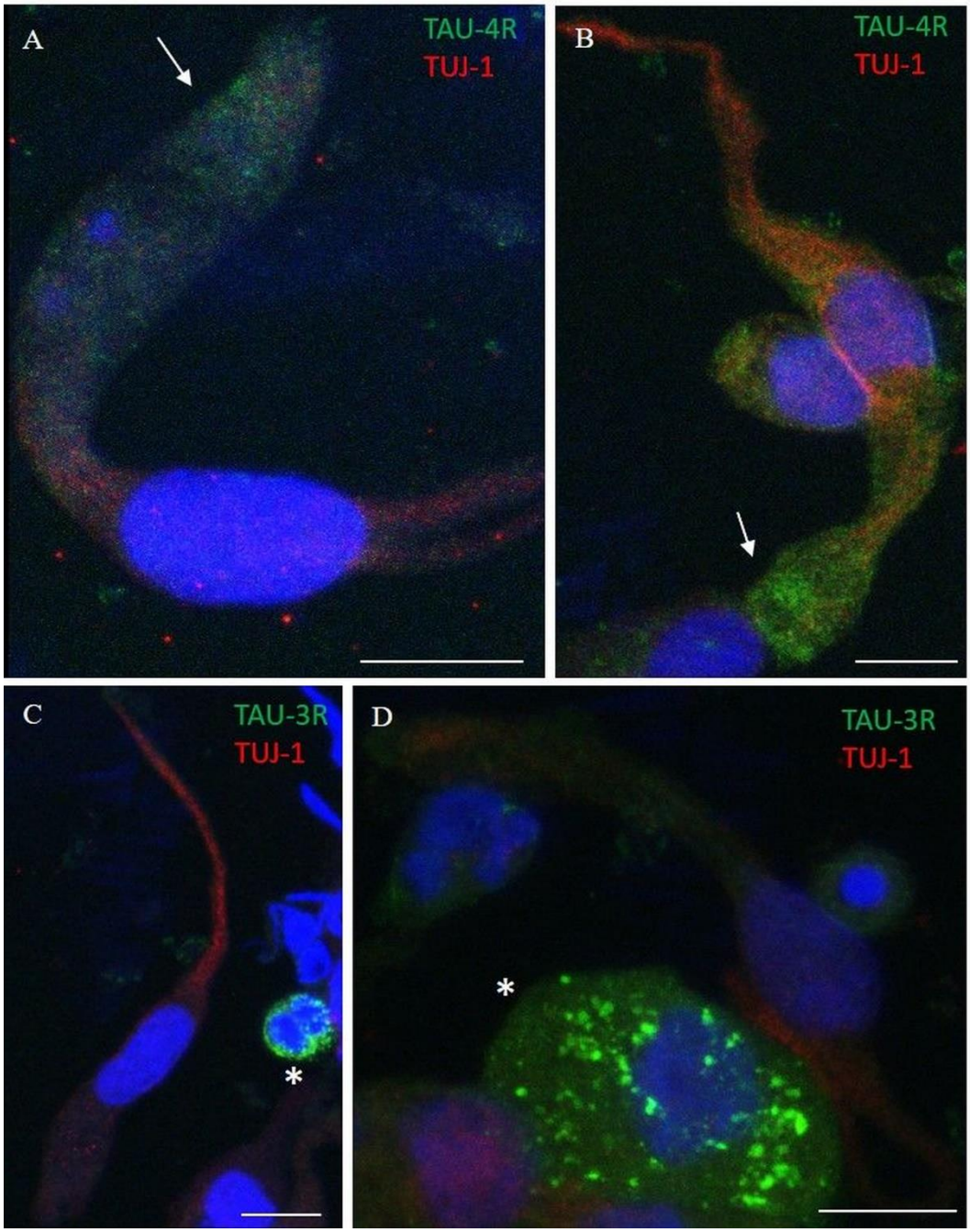


**Fig. 11 Distribution pattern of tau isoforms expression. A** and **B** panels show that the tau 4R isoform (green) is mainly localized in the cytosolic upper part of the neuronal TUJ-1 positive cells (red), close to the dendritic knob (arrows). Concerning the 3R isoform (green), we only found some relatively uneven distributed positive spots in rounded cells, likely globous stem cells, negative for the TUJ-1 antibody (**C** and **D** panels, *). All scale bars: 10 μm.

### 4.2.4 TDP-43

Transactive response DNA binding protein 43 (TDP-43) is a DNA binding protein, originally discovered in a wide-screen investigation for protein factors, which is able to bind the long terminal repeat transactive response element of HIV-1(Ou, Wu et al. 1995). This protein plays a role in transcription, alternative splicing, and messenger RNA (mRNA) stability. Also, it is involved in various cellular processes, including apoptosis, cell division, and axonal transport. Under physiological conditions, TDP-43 travels between the nucleus and cytoplasm, and after a nuclear localization signal, it is imported into the nucleus (Winton, Igaz et al. 2008).

It is reported that TDP-43 beyond neurons, is abundantly expressed in glia, as well as in many other cell types (Kawakami, Arai et al. 2019).

Here immunostaining was detected in the nuclear area and the expression of the protein is wide and homogeneous in all the samples analyzed. The resulting pattern reveals an intense intranuclear distribution with several positive grains around the nucleus in the cytoplasm (**Fig. 12**).

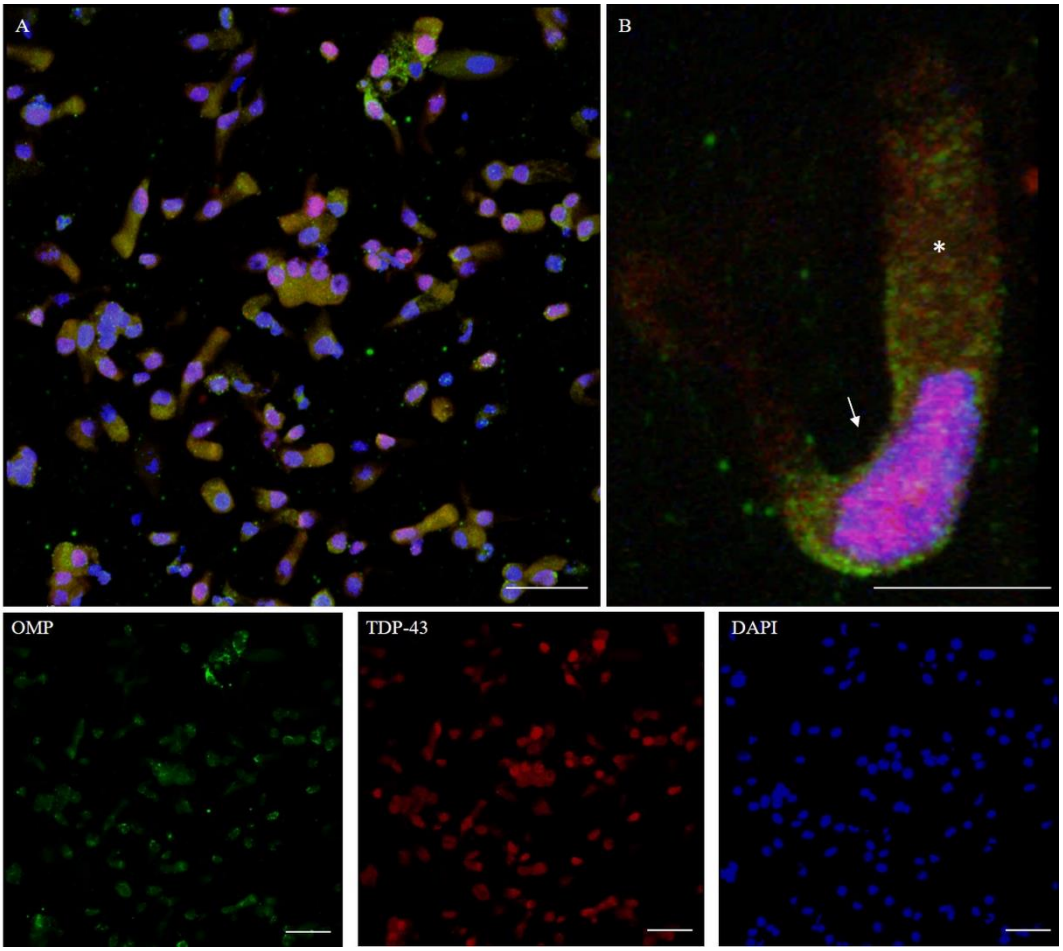


**Fig. 12 Distribution pattern of TDP-43.** The expression pattern of TDP-43 (overview in panel **A**, detail in panel **B**) is clearly nuclear (arrows) beyond weak staining of the cytosol (*). Almost all cells show nuclear positivity. DAPI nuclear staining (blue). Scale bar overview: 40 µm. Scale bar detail: 10 µm.

## 4.3 Evaluation of the α-synuclein expression patterns in synucleinopathies patients

We explored the potential differences in the expression pattern of the α-synuclein between healthy controls and patients affected by synucleinopathies (**Table 3**): Parkinson's disease (PD), Rapid-eye-movement behavioral disorder (RBD), Lewy body dementia (LBD), Multi System Atrophy (MSA). In some cases, age and gender were not available (n.a.) because they derived from a double-blind study. The RT-QuIC results are reported in terms of the ratio between the positive wells and the total 4 wells tested. Grading values of the immunocytochemistry (ICC) analysis are: "negative" (−), "normal" (-/+), "weak" (+), "moderate" (++), "strong" (+++).

**Table 3** ***Demographic profile of the patients with α-synucleinopathies***

| CASE | | AGE | GENDER | C. D. | OE ICC a-syn | OE ICC P-syn | OM a-syn RT-QUIC |
|---|---|---|---|---|---|---|---|
| **1.** | **2017_1** | 59 | M | PD | -/+ | + | 4/4 |
| **2.** | **2017_2** | 45 | M | PD | + | + | 2/4 |
| **3.** | **2017_3** | 58 | F | PD | + | + | 2/4 |
| **4.** | **2017_4** | n.a. | n.a. | RBD | + | ++ | 0/4 |
| **5.** | **2017_5** | n.a. | n.a. | RBD | +++ | +++ | 0/4 |
| **6.** | **2017_6** | n.a. | n.a. | RBD | + | -/+ | 0/4 |
| **7.** | **2017_7** | n.a. | n.a. | RBD | + | ++ | 0/4 |
| **8.** | **2017_8** | n.a. | n.a. | RBD | -/+ | ++ | 0/4 |
| **9.** | **2017_9** | n.a. | n.a. | RBD | + | -/+ | 0/4 |
| **10.** | **2018_1** | 73 | M | LBD | +++ | + | 4/4 |
| **11.** | **2018_2** | 75 | F | MSA | + | ++ | 4/4 |
| **12.** | **2018_3** | n.a. | n.a. | LBD | +++ | + | 4/4 |
| **13.** | **2018_4** | 71 | F | PD | + | -/+ | 2/4 |
| **14.** | **2018_5** | n.a. | M | RBD | + | ++ | 4/4 |
| **15.** | **2018_6** | 71 | M | PD | + | + | 4/4 |
| **16.** | **2018_7** | 75 | M | LBD | +++ | + | 4/4 |
| **17.** | **2018_8** | 68 | M | PD | + | ++ | 4/4 |
| **18.** | **2018_9** | 64 | M | RBD | + | ++ | 4/4 |
| **19.** | **2018_10** | 75 | M | RBD | + | ++ | 3/4 |
| **20.** | **2018_11** | 71 | M | RBD | + | ++ | 1/4 |
| **21.** | **2018_12** | 64 | F | RBD | + | +++ | 4/4 |
| **22.** | **2018_13** | 76 | F | RBD | + | ++ | 4/4 |
| **23.** | **2018_14** | 75 | M | PD | ++ | ++ | 4/4 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **24.** | **2018_15** | 76 | M | LBD | -/+ | -/+ | 0/4 |
| **25.** | **2018_16** | 67 | M | LBD | ++ | +++ | 3/4 |
| **26.** | **2018_17** | 75 | M | PD | ++ | + | 4/4 |
| **27.** | **2018_18** | 70 | M | LBD | ++ | ++ | 4/4 |
| **28.** | **2018_19** | n.a. | F | MSA | -/+ | ++ | 0/4 |
| **29.** | **2019_1** | n.a. | F | LBD | + | ++ | 4/4 |
| **30.** | **2019_2** | n.a. | n.a. | LBD | + | ++ | 4/4 |
| **31.** | **2019_3** | n.a. | n.a. | LBD | + | + | 4/4 |
| **32.** | **2019_4** | n.a. | n.a. | LBD | + | ++ | 2/4 |
| **33.** | **2019_5** | n.a. | n.a. | PD | ++ | +++ | 0/4 |
| **34.** | **2019_6** | 69 | M | PD | + | ++ | 0/4 |
| **35.** | **2019_7** | n.a. | M | PD | ++ | ++ | 2/4 |
| **36.** | **2019_8** | n.a. | n.a. | LBD | + | ++ | 0/4 |
| **37.** | **2019_9** | n.a. | n.a. | LBD | ++ | + | 0/4 |
| **38.** | **2019_10** | n.a. | n.a. | PD | ++ | ++ | 2/4 |
| **39.** | **2019_11** | n.a. | n.a. | LBD | + | ++ | 2/4 |
| **40.** | **2019_12** | n.a. | n.a. | LBD | + | ++ | 3/4 |
| **41.** | **2019_13** | n.a. | n.a. | PD | ++ | ++ | 0/4 |

Immunocytochemistry analysis results revealed a differential expression and distribution both for the total α-synuclein and the phosphorylated isoform, which is more closely involved in the pathology mechanism.

In particular, **Table 4** summarizes the main observed levels of expression compared to the healthy controls for each diagnostic group.

**Table 4** ***Summary of the most recurrent α-synuclein expression pattern in the relative synucleinopathies' group***

| | **PD (n=13)** | **LBD 1 (n=10)** | **LBD 2 (n=4)** | **MSA (n=2)** | **RBD (n=12)** |
|---|---|---|---|---|---|
| **Total α-syn (4D6)** | +/++ | +/++ | +++ | -/+ | + |
| **Phospho α-syn (P-Ser 129)** | +/++ | ++ | +/++ | +++ | ++ |

**PD**: Parkinson's Disease, **LBD 1**: Lewy Body Dementia with pattern 1, **LBD 2**: Lewy Body Dementia with pattern 2, **MSA**: Multi-System Atrophy, **RBD**: Rapid-eye-movement Behavioral Disorder. Grading values of expression: "negative" (−), "normal" (-/+), "weak" (+), "moderate" (++), "strong" (+++).

We also observed distinctive α-synuclein distribution patterns in each pathological group in detail, as described below.

### 4.3.1 Alpha-synuclein expression patterns in PD group

The immunocytochemistry analysis of the total and phosphorylated expression of α-synuclein in the olfactory mucosa brushing samples of patients diagnosed as PD revealed that both antibodies give quite higher signaling compared to normal levels of the controls. In addition, this increased positivity, appeared to be proportional in both isoforms. Moreover, even the distribution patterns were different from the controls. In fact, the pattern related to the PD group showed the presence of the two markers both in the cytosol, particularly inside vesicles at the sub-apical level for the phosphorylated form, and in the nucleus.

In particular, the 4D6 mAb, which recognizes the total α-synuclein, was found throughout the cellular body in a homogeneous manner, especially in the olfactory neurons where showed also denser grains in the terminal knob.

In addition, it seems that in some defined cases the nuclear fraction, lacked the P Ser 129 positivity, while the 4D6 showed a punctuate positivity (**Fig. 13**).

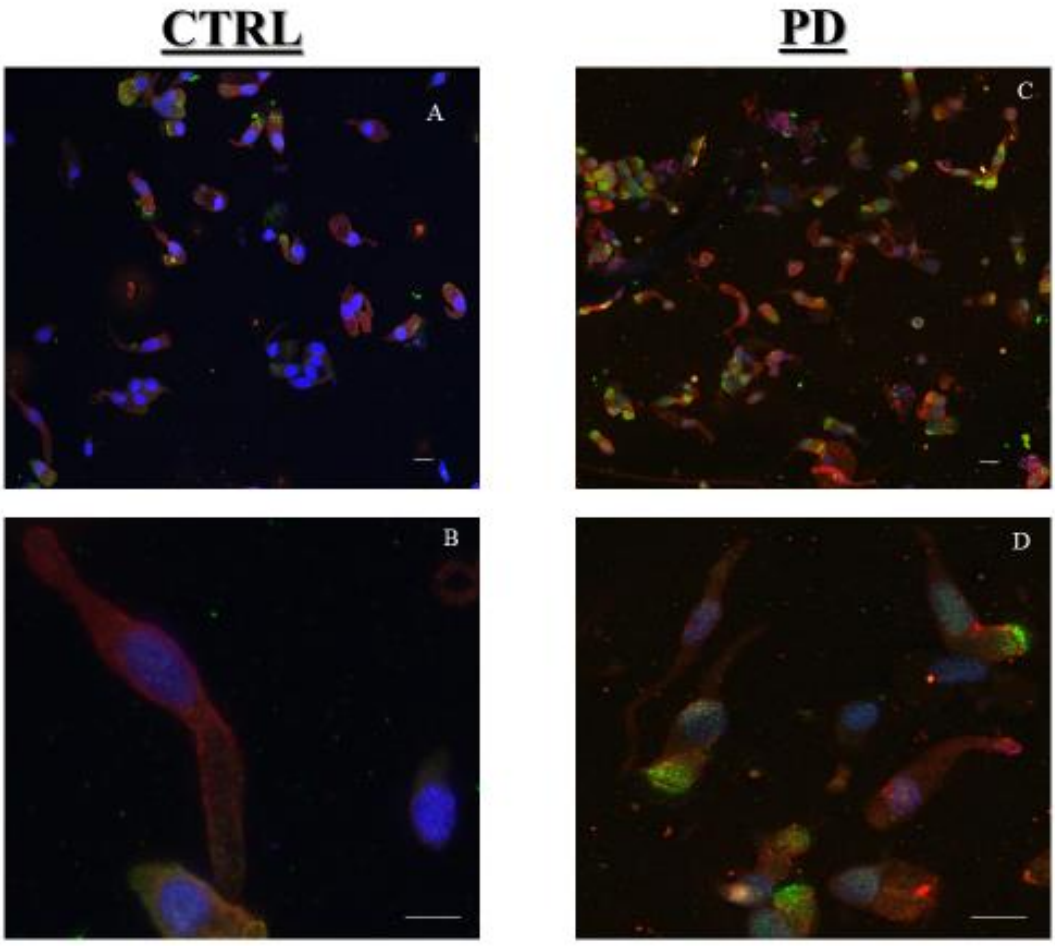


**Fig. 13** Representative immunofluorescence (merge) illustrating the characteristic expression patterns of the total α-synuclein (red) and the phosphorylated isoform (green) in the control group (**A**, **B**); and PD group (**C**, **D**). DAPI nuclear staining (blue). All the superior panels (**A**, **C**) are at 40x, while the panels below (**B**, **D**) at 60X. Scale bars: 10 μm.

### 4.3.2 Alpha-synuclein expression patterns in LBD group

Regarding the expression of α-synuclein in the LBD group, we observed two different expression patterns that may be summarized in two sub-groups: the first one (LBD 1) is characterized by enhanced detection of phosphorylated α-synuclein both in the nucleus and in the cytosol within intracytoplasmic vesicles, while the total form was increased with a minor degree.

In some cases, the intra-nuclear staining showed positivity to both markers with a few tiny dots related to 4D6 mAb (**Fig. 14**).

The second pattern is characterized by abundant granular deposition of the total isoform, which is notably hyper-expressed in the cytosolic compartments. The phosphorylated isoform in this sub-group of LBD patients (LBD 2) appears less augmented than the other LBD sub-group and well-confined to the apical border of the neuroepithelial cells and in the neuronal projections of olfactory neurons (**Fig. 15**).

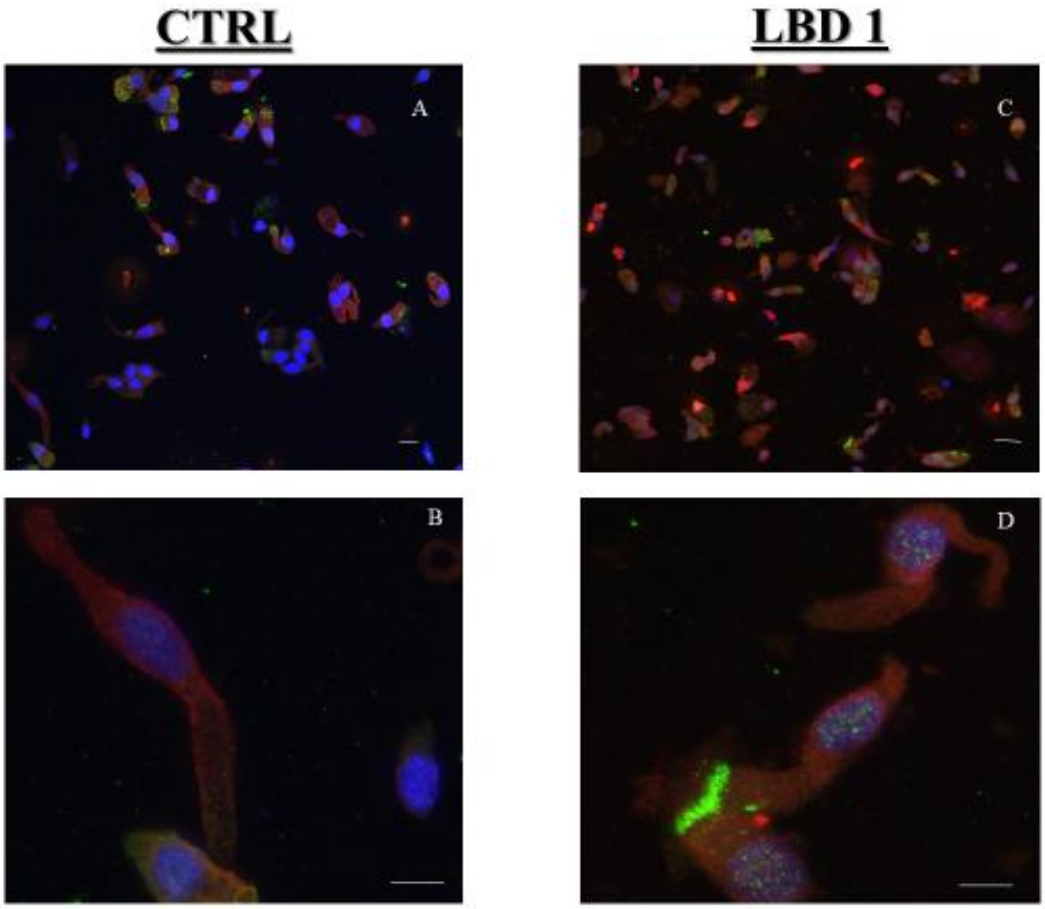


**Fig. 14** Representative immunofluorescence (merge) illustrating the characteristic expression patterns of the total α-synuclein (red) and the phosphorylated isoform (green) in the control group (**A**, **B**), and LBD 1 group (**C**, **D**). DAPI nuclear staining (blue). All the superior panels (**A**, **C**) are at 40x, while the panels below (**B**, **D**) at 60X. Scale bars: 10 µm.

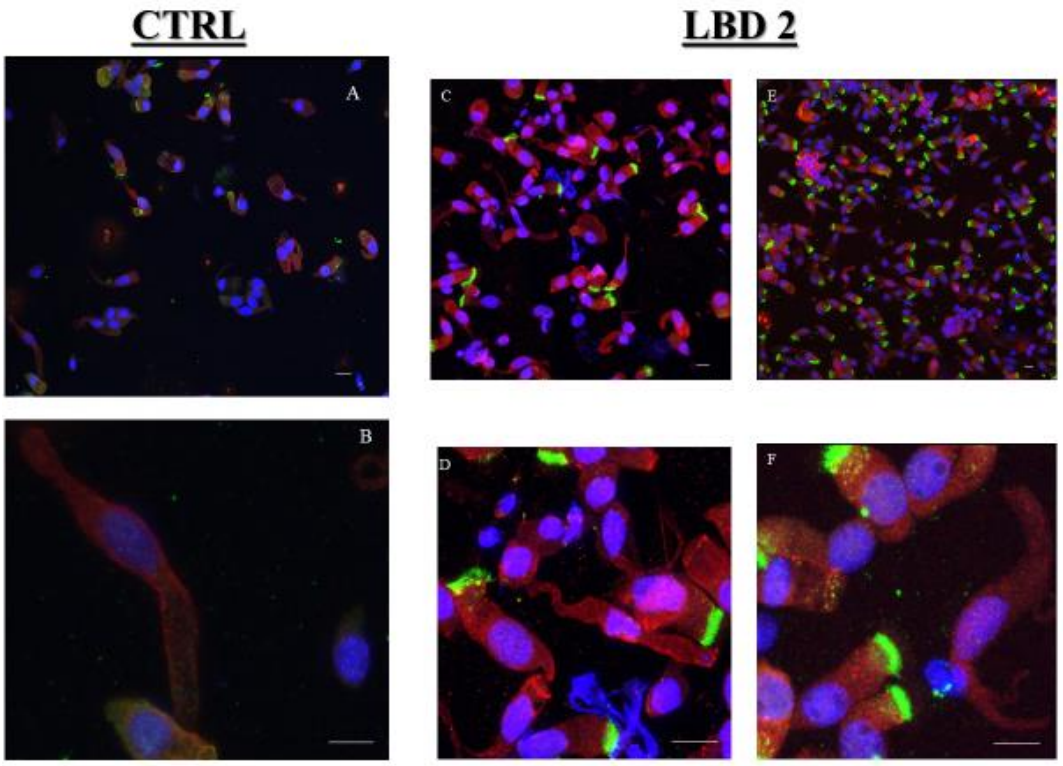


**Fig. 15** Representative immunofluorescence (merge) illustrating the characteristic expression patterns of the total α-synuclein (red) and the phosphorylated isoform (green) in the control group (**A**, **B**), and LBD 2 group (**C**, **D**, **E**, **F**). DAPI nuclear staining (blue). All the superior panels (**A**, **C**, **E**) are at 40x, while the panels below (**B**, **D**, **F**) at 60X. Scale bars: 10 µm.

### 4.3.3 Alpha-synuclein expression patterns in MSA group

With regard to the two cases of MSA, the immunocytochemical analysis revealed a clear increase of the phosphorylated form throughout the sample, unlike the form recognized by the 4D6 mAb, which is not very different from the control levels.

Specifically, the phosphorylated isoform was found particularly concentrated in the apex of the neuroepithelial cells with some cytosolic vesicles present in the sub-apical region.

The total isoform, albeit less expressed than the other marker, showed a granular cytosolic distribution, which had not been observed in the control group.

In one case, some neuroepithelial cells showed weak P Ser 129 signal also in the nuclear fraction (**Fig. 16,** panel **C**).

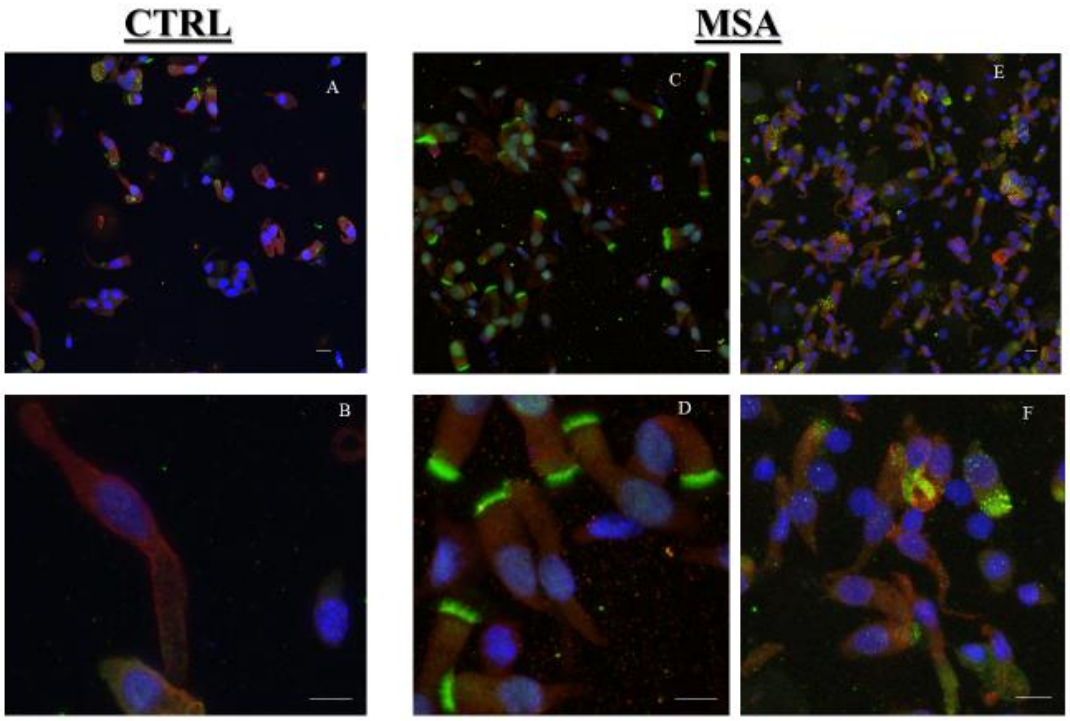


**Fig. 16** Representative immunofluorescence (merge) illustrating the characteristic expression patterns of the total α-synuclein (red) and the phosphorylated isoform (green) in the control group (**A**, **B**); and MSA group (**C**, **D**). DAPI nuclear staining (blue). All the superior panels (**A, C**, **E**) are at 40x, while the panels below (**B**, **D**, **F**) at 60X. Scale bars: 10 μm.

### 4.3.4 Alpha-synuclein expression patterns in RBD group

The immunocytochemical analysis of α-synuclein expression in the RBD group showed a homogeneous distribution of the grading values: altogether, the phosphorylated isoform increased much more than the total form of the pre-synaptic protein.

Only one case owned strong hyper-expression for both the markers in an equal manner.

In general, the P Ser 129 revealed an enhanced expression in the cytosol, especially in apical regions, and it was sometimes observed deposited in the nucleus.

As for the 4D6 mAb detection, we observed concomitantly a smaller increase characterized by particular distribution featuring a tiny cytosolic pattern lacking the intra-nuclear signal.

It has to be pointed out that the olfactory neurons showed greater cytoplasmic expression of the total isoform, compared to the other neuroepithelial cells of the same sample (**Fig. 17**).

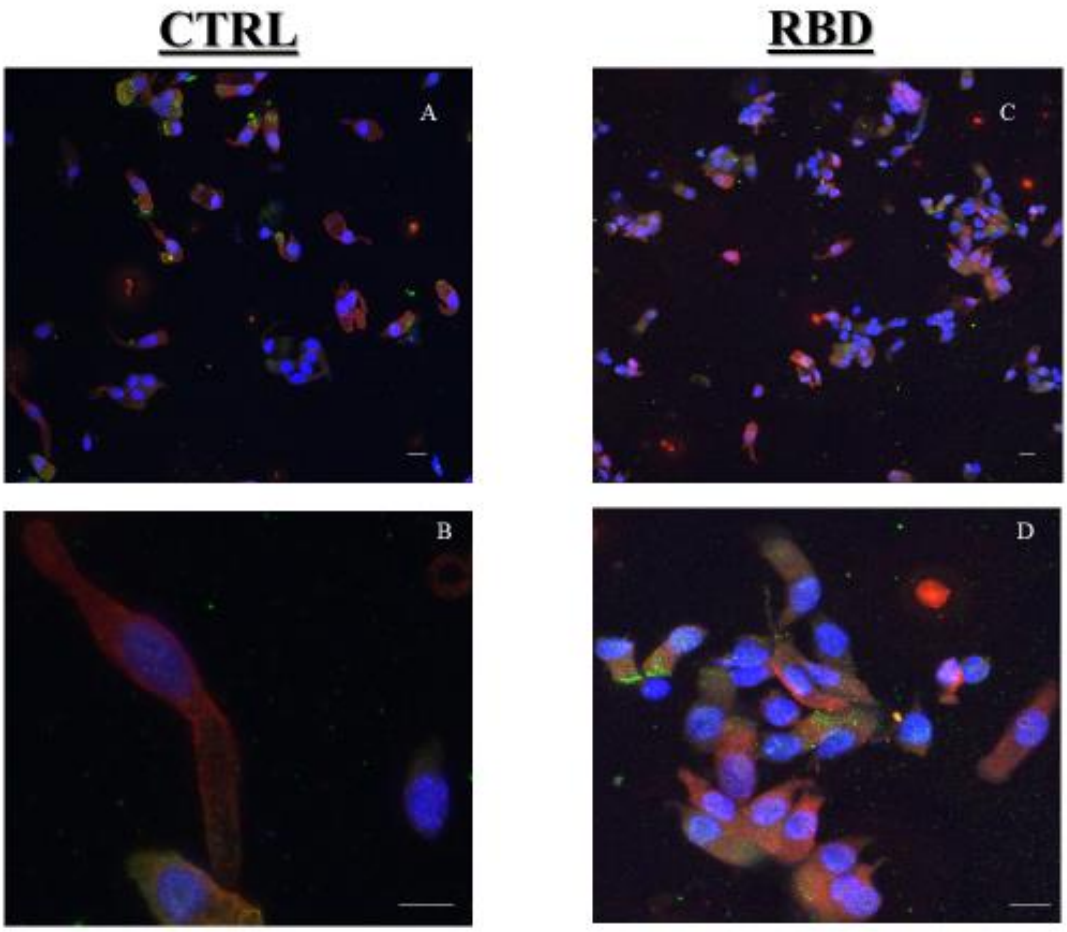


**Fig. 17** Representative immunofluorescence (merge) illustrating the characteristic expression patterns of the total α-synuclein (red) and the phosphorylated isoform (green) in the control group (**A**, **B**), and RBD group (**C**, **D**). DAPI nuclear staining (blue). All the superior panels (**A**, **C**) are at 40x, while the panels below (**B**, **D**) at 60X. Scale bars: 10 µm.

## 4.4 Alpha-synuclein expression patterns in relation to the RT-QuIC seeding activity in synucleinopathies patients

Lastly, we compared the α-synuclein expression patterns between RT-QuIC negative and RT-QuIC positive pathological OM samples. Indeed, the α-syn RT-QuIC assay has proven to be a specific test and sensitive to reveal the seeding activity of the pre-synaptic protein in OE samples.

We primarily found the phosphorylated a-synuclein isoform in the nucleus of the positive samples for the seeding activity (see arrows in **Fig**. **18**). In particular, the phosphorylated isoform showed a variable degree of the nuclear detection signal within 56,1% (23 out 41) of the total analyzed samples, with a percentage of 88,4% inside the group of positive cases (23 out 26).

Differently, the nuclear deposition of the total isoform was observed in the 30,7% of the α-syn RT-QuIC positive cases (8 out 26). Moreover, 75% (6 out 8) of these last positive samples, showed P-ser-129 in the nucleus with a lower intensity compared to the simultaneous nuclear detection of the total α-synuclein.

On the other hand, we found a nuclear positivity of one of the two markers also in 20% of RT-QuIC negative cases.

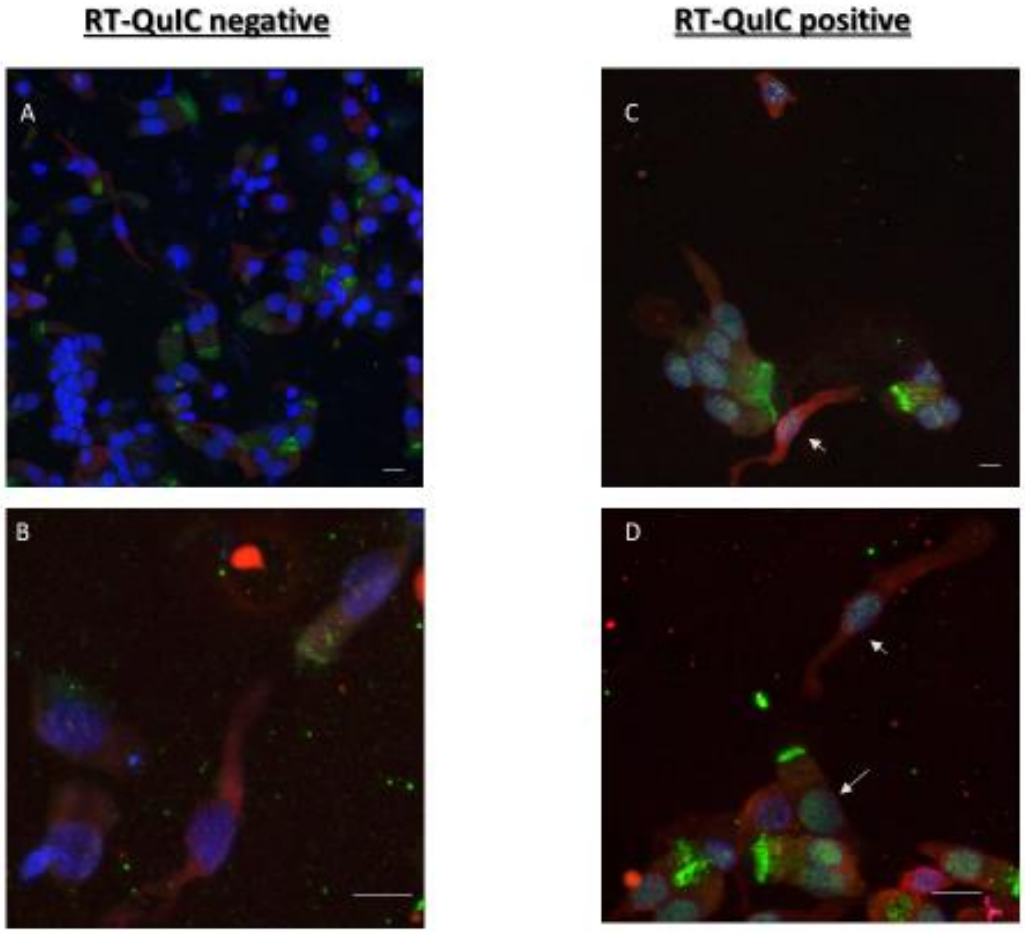


**Fig. 18** Representative immunofluorescence (merge) illustrating the characteristic expression patterns of the total α-synuclein (red) and the phosphorylated isoform (green) in the RT-QuIC negative group (**A**, **B**), and RT-QuIC positive group (**C**, **D**). The arrows point to the nuclear localization of the P Ser 129. DAPI nuclear staining (blue). All the superior panels (**A**, **C**) are at 40x, while the panels below (**B**, **D**) at 60X. Scale bars: 10 µm.

# 5. DISCUSSION

In this thesis work, we investigated:

1) first of all, the phenotype of the olfactory cells, especially olfactory neurons collected by the olfactory brushing technique in humans;

2) secondly, the presence and the related expression patterns of the main NDs-associated proteins in healthy controls;

3) thirdly, the α-synuclein expression pattern variations in patients with synucleopathies;

4) lastly, the α-synuclein expression patterns in relation to the α-syn RT-QuIC seeding activity results in the same patients.

Olfactory impairment is recognized as a prodromal symptom in patients with neurodegenerative diseases suggesting that the olfactory system and particularly the olfactory mucosa represent an intriguing neural tissue for the exploration of the mechanisms underlying the neurodegenerative process.
In this thesis, we showed that the human olfactory neuroepithelium can be easily sampled by the olfactory brushing technique, collecting neurons and cells from neuronal lineage for morphological and immunocytochemical investigations. Olfactory brushing allows gentle collection of olfactory mucosa, it can be easily performed, it is non-invasive, non–traumatic and produces minimal pain or discomfort. The collected cells show a well-preserved native morphology, enabling the recognition of olfactory neurons, characterized by the typical bipolar slender shape, and their discrimination from other sample cellular components.

## 5.1 Phenotypic characterization of the olfactory neuroepithelium cells

Since the variety of cells retrieved from each sampling was highly unpredictable and the cell typing was unsuitable to be addressed only by the morphology analysis, our study firstly aimed at the phenotypic characterization with ensuing identification of specific neuronal markers for the ONs.

OMP was the first discovered marker for ONs (Monti-Graziadei, Margolis et al. 1978), associated with the olfactory transduction (Albeanu, Provost et al. 2018) towards the olfactory bulb. The OMP staining showed the characteristic intracytoplasmic localization of the protein (Kream and Margolis 1984).

However, the positive signal was also detected in some non-neuronal shaped cells (probably supporting cells and olfactory neurons not yet mature). This unexpected pattern of detection of the OMP was repeatedly observed, using all the different antibody clones for the marker, some of which had been also removed from the market.

OMP is widely accepted as a marker of mature ONs, even though its physiological function is not fully understood (Nakashima, Nakashima et al. 2019). In addition, OMP was also detected in some non-olfactory tissues, which are not classified as classical chemosensory tissues (Kang, Kim et al. 2015).

We found that around 30% of the total cells were OMP positive, and they showed both neuronal and non-neuronal morphology. Taking into account that, we analyzed the OM samples obtained from the middle turbinate, we cannot exclude that OMP expression in apparently non-neuronal cells might indicate a modulatory paracrine role of these cells on the olfactory function of the ONs. In middle turbinate, ONs are unevenly distributed, compared to the ones of the mucosal surface covering the *lamina cribra*. Moreover, the olfactory epithelium in humans is not clear-cut from the non-olfactory tissue, whereas in rodents there is a marked boundary. In addition, in the airway system, it was showed that ciliated cells have chemosensory features (Shah, Ben-Shahar et al. 2009); (Merigo, Benati et al. 2012) and, as reported recently in the rat trachea, it is possible that the various epithelial cell populations cooperate within an intricated cellular network,

each of them with different chemosensory properties (Lasconi, Pifferi et al. 2019). Furthermore, because of the extremely dynamic properties of the olfactory epithelium, OMP positivity in apparently non-neuronal shaped cells might indicate that these olfactory cells did not ultimate their maturation process. Indeed, cytological preparation from a heterogeneous epithelium like the olfactory mucosa disrupts the architectural integrity of the epithelial surface. In that way, the topographical information providing cues regarding the neuronal maturation path is lost.

Furthermore, β-tubulin III (specifically TUJ-1 marker), as already reported in previous studies on olfactory biopsies (Holbrook, Wu et al. 2011, Tanos, Saibene et al. 2017) (Durante, Kurtenbach et al. 2020), could be considered an efficient neuronal marker.

The marking obtained with the TUJ-1 clone of the β-tubulin III is located mainly within the axonal projection, and it is particularly evident on the axonal hillock showing a filament pattern. The combination of OMP and TUJ-1, provided effective ONs identification is effective and both markers proved useful to focus the study of the neurodegeneration-associated proteins on this cell type.

In addition, we showed that the PGP 9.5, a neural marker, which is commonly adopted as a neuronal-specific marker, labeled ONs showing punctuate staining in the cytoplasm while other ciliated and tubular cells different from neurons, showed rounded staining in the cytoplasmic apical pole. This labeling pattern confirms previous comparable results described in adults and developing humans (Johnson, Eller et al. 1997) (Witt, Bormann et al. 2009) (Holbrook, Wu et al. 2011), showing that the respiratory epithelium marker β-tubulin type IV co-labeled with the PGP 9.5 positive non-neuronal shaped cells. Therefore, we cannot exclude that the non-neuronal shaped PGP 9.5 positive cells we found might be not yet mature neurons or isolated metaplastic olfactory neurons possibly related to subclinical inflammatory *status*. Also the sustentacular-like cells positive to the PGP 9.5 that we found could be cells derived from common progenitors to the olfactory neurons, and consequently, they express general neural marker as the protein gene product 9.5 (Guo, Packard et al. 2010).

In addition, we have characterized the other epithelial cells of the olfactory epithelium using a monoclonal antibody (mAb) against all the isoforms of the cytokeratin (PCK), which have been proved to be expressed in the olfactory epithelium (Witt, Bormann et al. 2009) (Chen, Kachramanoglou et al. 2014) (Hahn, Han et al. 2005). We assessed this general epithelial marker in combination with marker PGP 9.5 and then with OMP.

PCK, although it marked nearly all the epithelial cells, showed a different distribution pattern depending on the cell lineage, with an intracytoplasmic localization in ONs and a plasmatic membrane marking in the supporting cells. In fact, the two different cellular classes, derive from the same progenitor originating from the horizontal stem cells, then committed in the neuronal or glial direction depending on the specific growth factors and differentiation stimuli present (Goldstein, Fang et al. 1998, Sicard, Feron et al. 1998).

Therefore, after this first part of the study, we decided to set and optimize the sampling and immunocytochemistry techniques. The achievement of these improvements was a prerequisite that allowed us to characterize the phenotype of the OM cells recovered by the nasal brushing procedure. In particular we focused on ONs, due to the fact that with had lost the the structural overview of the tissue and its cytoarchitecture.

Finally, through the lens of our methodological approach, we could assess the health state and the integrity of the OM cellular constituents as if we were taking a snapshot of the single cells in the sampling's instant and thus investigate whether potential alterations occur under disease conditions.

## 5.2 Expression patterns of ND-associated proteins in olfactory cells of healthy subjects

In the second phase of our study, we evaluated the expression pattern of some neurodegeneration-associated proteins, which had been previously investigated only in rodents or in a few *postmortem* or bioptic human studies (Rey, Wesson et al. 2018). The physiological production of those proteins which are prone to self-aggregate represents the condition "*sine qua non*" for triggering the aggregation cascade and the relative pathological pathway. According to Braak's "dual hit theory" and Doty's "olfactory vector hypothesis", the basal expression of protein with prion-like features in the olfactory epithelium could supply the brain of aberrant species via anterograde axonal route (Hawkes, Del Tredici et al. 2007) (Doty 2008). Indeed, in some neurodegenerative disorders such as Alzheimer's disease or Parkinson's disease different studies have shown the presence of Lewy neurites, neurofibrillary tangles or β-amyloid deposits in ONs (Crino, Greenberg et al. 1995); (Talamo, Rudel et al. 1989); (Trojanowski, Newman et al. 1991); (Lee, Goedert et al. 1993); (Funabe, Takao et al. 2013); (Saito, Shioya et al. 2016). These findings indicate that misfolded species with seeding activity may be transported to the olfactory bulb glomeruli where they accumulate and aggregate. According to this perspective, it might be difficult to observe the typical pathological changes in the ONs, since they undergo a complete recycle every three months and this time-course might be not sufficient to carry out the aggregation process.

For these reasons, we decided to investigate the normal expression of neurodegeneration-associated proteins in olfactory neurons from healthy subjects. This investigation revealed distinctive expressions for each selected protein, which are described in the following sections.

### 5.2.1 α-synuclein

At present, no comprehensive mapping studies of the normal α-synuclein expression have been performed in human whole-brain. The partial investigations in the peripheral regions, included in Braak's theory, had been either conducted in the enteric nervous system (Böttner, Zorenkov et al. 2012) or explored the pathological forms of the protein (Bloch, Probst et al. 2006). However, in elderly healthy subjects, consistent levels of α-synuclein were seen in olfactory brain areas (Freer, Sormanni et al. 2016) and only one study evaluated the α-synuclein expression in the human olfactory mucosa, showing a positive signal not only in basal cells and ONs but also in supporting cells and Bowman's gland component (Duda, Shah et al. 1999). Our results are in line with these previous findings and reveal the constitutive expression of α-synuclein in the olfactory epithelium cells. In particular, we showed the non-exclusive neuronal expression of α-synuclein in the OE, which agrees with the notion that such protein is ubiquitous (Baltic, Perovic et al. 2004). The relative distribution patterns described a membrane localization in neuronal and non-neuronal cells, while the intracytoplasmic deposition was only found in ONs.

### 5.2.2 APP/β-Amyloid

APP is a long integral membrane glycoprotein that forms Aβ-peptide after the proteolytic secretase cleavage, whose amyloid fibrillar form is the primary component of amyloid plaques found in the brains of AD patients. Although the native biological role of APP is of obvious diagnostic and therapeutic interest, its thorough understanding remains elusive.

APP has been considered a non-exclusive neuronal protein for more than twenty years, but the recent notion regarding its localization claims that it is predominantly expressed in the forebrain, in which the olfactory system is contained (Corbett and Buss 2014). There are no data available showing the APP expression in olfactory structures in humans, whereas it was found in the olfactory

bulb of rats beyond various cortical olfactory regions. Unfortunately, the AON and the OE were not investigated (Struble, Dhanraj et al. 1998)(Rey, Wesson et al. 2018).

According to the findings in olfactory epithelium biopsies (Arnold, Lee et al. 2010), we showed the physiological expression of the APP in ONs, displaying a dotted cytosolic pattern both at the perinuclear level and within the axonal and dendritic terminations. Similar results have been previously reported specifically for β-Amyloid in AD patients (LaFerla, Green et al. 2007).

### 5.2.3 τ-protein

Tau is a microtubule-associated protein (MAP) originally purified from brain and recognized for its ability to promote microtubule assembly *in vitro* (Weingarten, Lockwood et al. 1975). A pioneering study on the OE in AD patients conducted in 1993 showed the expression of tau in the axons of the olfactory neurons during their dystrophic transformation (Lee, Goedert et al. 1993).

The physiological Tau expression has not been evaluated in human olfactory structures (Rey, Wesson et al. 2018). Olfactory regions were only explored in rats, showing that tau is greatly expressed in ONs and in their axons in the olfactory bulb, beyond mitral cells and interneurons (Viereck, Tucker et al. 1989) (Schoenfeld and Obar 1994).

In this thesis, we showed that total tau protein was mainly localized around the nuclear compartment and in the apical region of human ONs. Some dotted positivity appeared in clustered cells, and such form of the protein may be released from the dying neurons or secreted by a physiological mechanism of defense to prevent an elevated intracellular concentration of the protein (Perea, Llorens-Martín et al. 2018) (Pooler, Phillips et al. 2013). We observed sporadically a clear well-compartmentalized concentration of tau in a cytosolic organelle, probably Golgi apparatus during the processing preceding the secretion. (Mohamed, Desjardins et al. 2017).

The analysis of tau isoforms typing revealed no detection of 3R isoform, apart from some rare positive globous cells, likely stem cells, in line with the notion

that such isoform is expressed during developmental stages (Goedert, Spillantini et al. 1989). 4R isoforms were found under the knob of TUJ-1 positive mature olfactory neurons.

### 5.2.4 TDP-43

Originally TDP-43 was discovered during a wide-screen investigation into protein factors having the ability to bind the long terminal repeat transactive response element of HIV-1(Ou, Wu et al. 1995). Under physiological conditions, TDP-43 travels between the nucleus and cytoplasm, and after a nuclear localization signal, it is imported into the nucleus (Winton, Igaz et al. 2008). Even if TDP-43 is mainly located in the nucleus, in healthy state, a significant 30% of the normal protein can be found in the cytoplasm (Kawakami, Arai et al. 2019) and its export is largely driven by passive diffusion (Ou, Wu et al. 1995).

TDP-43 was identified as the major brain component associated with amyotrophic lateral sclerosis (ALS), as well as with frontotemporal lobar degeneration (FTLD) (Neumann, Sampathu et al. 2006). Under pathological conditions, TDP-43 is discharged from the nucleus and gathers in the cytoplasm (Arai, Hasegawa et al. 2006). The translocation into the cytoplasm appears to be the result of compromised nuclear transport and solubility of the protein. This idea is consistent with the evidence that TDP-43 seeds in the cytoplasm promote its mislocalization by affecting nuclear transport and with the presence of proteins involved in nucleo-cytoplasm transport in pathological TDP-43 aggregates (Chou, Zhang et al. 2018).

At any rate, the neurotoxicity of aberrant TDP-43 has not been completely clarified yet. Although the localization of TDP-43 in the cytoplasm is widely thought to be the cause of the TDP-43 nuclear fraction loss, some studies indicate that there is no correlation between the nuclear depletion and the neuronal toxicity, while the presence of cytoplasmic mutant of TDP-43 induces the neurodegeneration process (Kawakami, Arai et al. 2019).

Interestingly, as it happens with the other ND-associated protein, the accumulation of TDP-43 is related to many post-translational modifications, such

as hyperphosphorylation, ubiquitination and abnormally cleavage generating C-terminal fragments (Neumann, Sampathu et al. 2006). Moreover, TDP-43 inclusions are thioflavin-S positive (Bigio, Wu et al. 2013), suggesting that the aggregation occurs in a way that is analogous to amyloid-like fibrils.

Our analysis of TDP-43 physiological expression in the OE brushing samples reveals a widespread production of the protein in the majority of the cells collected. Furthermore, the expression pattern of the protein we found is consistent with the general typing of TDP-43, showing a preponderant nuclear localization of the protein, with weak positive signals in the cytosolic region.

### 5.2.5 Overall Discussion on the Expression of ND-associated-proteins in healthy subjects

We have shown for the first time that ONs, express all the marker proteins investigated: α-synuclein, APP/β-Amyloid, TDP-43, and tau protein. In addition, for some of these proteins, we found a non-exclusive neuronal expression, as for the TDP-43 and α-synuclein.

These findings suggest that, directly or indirectly, the potential contribution of ONs and the other olfactory epithelium cells cannot be excluded, from the triggering of neurodegeneration processes (Rey, Wesson et al. 2018). In this regard, glial and supporting cells of the olfactory tissue and OB could play a crucial role in the onset mechanisms of the pathology. The key role of the sustentacular cells in the neurodegenerative pathways should be associated with the loss of their essential functions in protecting brain structures against external insults and in sustaining neurons for proper transduction into the brain. Moreover, further pieces of evidence in line with this concept came from several animal studies in which the intranasal administration of toxicants produced analogous alterations to the pathological signs (Prediger, Aguiar et al. 2012).

The study of the constitutive expression of the main misfolding-prone proteins in healthy human olfactory mucosa represents a very promising research matter. The fact that the olfactory neuroepithelium, including not only ONs but also the other neighboring cells, is highly exposed to viral and/or toxic/environmental insults,

might trigger the protein misfolding in these cells and disrupt the physiological interaction of the different cell types, resulting in olfactory signal impairment and dysfunction. Misfolded proteins may form early oligomers which might be transported through the axonal anterograde pathway to the OB, where they might assembly into fibrillary structures and amyloid deposits. In support to this hypothesis are the occurrence of smell loss, the pathological involvement of the olfactory pathways in the initial stages of the ND, and the evidence that xenobiotics epidemiologically linked to these diseases can enter the brain via the OM.

## 5.3 Alpha-synuclein expression patterns in synucleinopathies patients

In the further phase of our study, we focused on the exploration of variations in α-synuclein expression patterns under pathological conditions.

Thus, we compared the expression levels and the distribution patterns of the α-synuclein between healthy controls and patients affected by synucleinopathies such as Parkinson's disease (PD), Rapid-eye-movement behavioral disorder (RBD), Lewy body dementia (LBD), and Multiple System Atrophy (MSA).

The shreds of the experimental evidence introductory to this investigation derived from previous studies in autopsic olfactory epithelium specimens of patients with the same disorders as the aforementioned synucleinopathies (Funabe, Takao et al. 2013) (Saito, Shioya et al. 2016).

On the other hand, we were aware that in another investigation on OE biopsies from patients affected by Parkinson's disease, Witt et al. have reported no histochemical detection of α-synuclein immunolabeling (Witt, Bormann et al. 2009). Another work preceding the aforementioned studies investigated synuclein family proteins in biopsies of patients with various synucleinopathies. The results of this *antemortem* investigation revealed abnormal dystrophic neurites in the OE of patients with PD, LBD, AD, and MSA, even though similar detection had been observed also in the OE of normal controls (Duda, Shah et al. 1999).

Our immunocytochemistry analysis showed changes of the expression patterns between normal controls and synucleinopathies' patients, both for the total α-synuclein detected by the 4D6 mAb and for the isoform phosphorylated in Serine 129 (Ser 129). In this last isoform, the native protein underwent the most relevant post-translational modification involved in the pathogenesis mechanisms (Fujiwara, Hasegawa et al. 2002). Specifically, we revealed a different distribution array for both markers in each synucleinopathies group. Particularly, we observed the principal patterns associated with each clinical diagnosis: in the PD group, we frequently recognized a parallel increase of expression, from weak to moderate, for both α-synuclein isoforms. Besides, we found positive signals of the two markers in the nuclear and cytosolic compartments. The variability of the

α-synuclein expression inside the PD group could reflect the different stages of maturity of the disease in the brain (Covell, Robinson et al. 2017).

In the OM samples from patients with LBD, we identified two different expression patterns. The first one is characterized by a moderate rise of detection of the phosphorylated α-synuclein in the nucleus and inside apical vesicles. The second pattern is characterized by abundant granular deposition of the total isoform in the cytosol, especially in the soma of the ONs. The phosphorylated isoform in this subset of LBD patients appears not to have increased and to be well-confined in the brush border of the epithelial cells and in the knob of olfactory neurons.

The finding of these two different patterns in the LBD group is in line with the previously reported morphological description of the α-synuclein deposition in the CNS neurons and may correspond to former phases of Lewy intracytoplasmic inclusions staging in the brain (Gómez-Tortosa, Newell et al. 2000). In particular, the previous study found two different patterns of Lewy bodies distribution in paralimbic cortices, hence our results may represent a starting point from which to trace the differential pathological pathways.

In the few OM samples of MSA patients, we have seen a remarkable intensification of the phosphorylated isoform signal, concomitantly with the normal expression of the total α-synuclein. Even in this case, it is possible that the lower detection of the total α-synuclein in the cytosolic compartment of ONs is correlated with the more-extraneuronal glial implication of the pathology in the MSA phenotype (Tu, Galvin et al. 1998).

In the RBD group, the increase of expression in the total isoform was less marked than that in the phosphorylated isoform. A strong increase of the P-Ser 129 with a web-shape deposition within the nucleus has been often found.

Our results support the mechanism of general α-synuclein overexpression just reported in genetic forms of PD (Singleton, Farrer et al. 2003).

The novelty emerging from our study is that, beside a quantitative increase of the general protein expression, in the olfactory epithelium, we also observed a qualitative alteration in the protein distribution, which was well mirrored in the variations of the α-synuclein expression pattern. In particular, each specific form

of synucleinopathy analyzed owned a distinctive pattern of expression for both markers, and above all, the phosphorylated isoform showed the major quantitative and qualitative variance between groups.

We think that stochastic events could evoke a transient α-synuclein over-expression, so that it makes the native protein an available substrate to the misfolding mechanisms, which in turn, might trigger the pathology in specific districts prone to neurodegeneration. Nevertheless, the α-synuclein expression increase could be also, a physiological attempt to preserve the correct synaptic function in response to an injury. It seems that the local increase of the synaptic protein might serve as a defense reaction against viral infection, especially after demonstrating its viral RNA restriction activity in the brain (Beatman, Massey et al. 2016). Other quantitative studies on the mRNA and the extent of the related protein showed that the levels of soluble α-synuclein found in LBD and PD were lower in comparison with the control, although there was no evidence of a corresponding decrease in α-synuclein mRNA levels (Quinn, Coulson et al. 2012). This discrepancy might reflect an altered protein synthesis or maturation, or the removal of the soluble detectable state of the protein, because of the normal turnover or the misregulated conversion to an insoluble form. Consequently, the protein depletion may decrease the physiologic accomplishment for any cellular homeostatic role it may play, contributing to a loss-of-function and neurotoxicity.

On the basis of these considerations, it is plausible that the α-synuclein increased levels we found in the diverse synucleinopathy groups are not the consequence of increased gene synthesis, but rather they result from a dysregulation of its maturation, or of its turn-over and degradation (Engelender 2008).

The aforementioned studies analyzed the α-synuclein levels from different CNS areas of patients with severe disease in an advanced stage. Therefore, the results might not correspond to the regulation mechanisms of expression in the early phases of the disease. For instance, the total α-synuclein rate we found in a long-term established disease like PD was on average lower compared to the RBD group, which is considered a pro-dromal condition of α-synucleinopathy. This discrepancy might be due to either a constant protein translocation from the olfactory neurons to the olfactory bulb via anterograde transport, or to the

continuous misfolding process that renders the normal target protein undetectable by the antibodies. Another explanation could be that the levels of the native protein expression at the onset of the disease (as RBD condition) increased because of the physiological response to restore the cellular homeostasis.

We hypothesize that protein misfolding events might occur on the ground of the continuous injuries that the olfactory neuroepithelium has to face. The ensuing α-synuclein expression increase becomes considerable at the very early stages of the disease. As a result, the dysregulated activation of defense mechanisms accelerates the aberrant protein processing leading to aggregation and pathology spread within the olfactory bulbs. According to this concept, the variations of the α-synuclein patterns we found in each synucleinopathy group, might derive from the impairment of distinctive expression pathways and different post-translational modifications promoted by the specific defense mechanisms activated.

Importantly, a larger study must confirm the very promising results obtained so far, given their limits because of the small sampling size.

## 5.4 Alpha-synuclein expression patterns in relation to the RT-QuIC α-synuclein seeding activity in synucleinopathies patients

With regard to the last part of the study, we analyzed the immunocytochemistry expression patterns of the α-synuclein in relation to the α-syn RT-QuIC results in synucleinopathies' patients. Specifically, we evaluated the variability in the expression levels and the distribution patterns of the two α-synuclein markers, among RT-QuIC positive and RT-QuIC negative samples. In particular, we explored distinctive differences in the expression patterns able to provide insights about the seeding activity detected by the α-syn RT-QuIC assay.

After that, we developed a specific α-synuclein RT-QuIC assay in our laboratories that has proven to be specific and sensitive to reveal the aggregation seeding activity of α-synuclein (Bongianni, Ladogana et al. 2019).

Through the immunocytochemistry examination of the α-synuclein expression in the same samples tested by α-syn RT-QuIC, we found that the main difference between the RT-QuIC positive and RT-QuIC negative specimens lied on the distribution of the phosphorylated isoform.

The P Ser 129 marker owned a nuclear localization in the majority of the cases analyzed (23 out 41, 56,11%) mirroring the positivity to the RT-QuIC tests (26 out 41, 63,4%). In particular, we observed that 88,4% of the RT-QuIC positive samples revealed a nuclear detection of the phosphorylated isoform, in a web-shape or a punctate form.

The total α-synuclein instead, showed nuclear localization in 30.7% (8 out 26) of RT-QuIC positive cases. 75% (6 out 8) of these last positive samples showed detection of the total α-synuclein together with P ser 129 in the nucleus.

On the other hand, we found nuclear positivity of one of the two markers even in 20% of RT-QuIC negative cases.

We argue that despite the high sensibility of the nuclear P Ser 129 as a biomarker of the α-synuclein seeding activity detected by RT-QuIC, the lower specificity might be related to several factors. Firstly, though still controversial, the finding of the nuclear localization of α-synuclein and its post-translational isoforms had been reported since the original discovery of the protein (Maroteaux, Campanelli

et al. 1988) (Wales, Pinho et al. 2013) (Schell, Hasegawa et al. 2009) (Surguchov 2015). Secondly, the presence of the pre-synaptic protein in the nucleus could be also promoted by the physiological DNA damage response pathway (Schaser, Osterberg et al. 2019), even in absence of or long before the seeding activity induction. The ultimate aspect concerns the issue of the detection of the truncated isoforms, which are translocated in the nucleus (Zhou, Xu et al. 2013). Indeed, they are largely undetectable by the antibodies, similarly to the misfolded species that continuously gobble the proteins and decrease the soluble fractions available for the retrieval (Jucker and Walker 2018) (Osterberg, Spinelli et al. 2015).

However, in rodents, a previous study ascribed the pathological implications of the human α-synuclein to their nuclear localization (Wakamatsu, Ishii et al. 2007). Moreover, the nuclear localization of α-synuclein has been reported to enhance its oligomerization and aggregation capacity (Goers, Manning-Bog et al. 2003) (Lázaro, Rodrigues et al. 2014) and its neurotoxicity activity (Kontopoulos, Parvin et al. 2006) (Fares, Ait-Bouziad et al. 2014).

In conclusion, we showed that most of the OM samples positive for the α-syn RT-QuIC in a cohort of patients suffering from synucleinopathies revealed a nuclear localization of the P Ser 129 biomarker, unlike the negative cases, providing a feasible prediction of the seeding activity assay and a related pathogenetic explanation.

# 6. CONCLUSION

For the first time, we provided in this study the phenotypic characterization of the human olfactory cells collected by OBg. In particular, we identified the TUJ-1 neuronal marker for ONs identification.

We demonstrated that ONs, and in some cases also the neighboring cells, constitutively express all the selected NDs-associated proteins under physiological conditions, including α-synuclein, APP/β-Amyloid, TDP-43, and tau protein.

Subsequently, we explored the possibility to distinguish the several synucleinopathies based on the α-synuclein expression patterns analysis in the OE and we showed a different expression pattern of P-Ser 129 in each distinct synucleinopathy. This might be a novel complementary analysis to the existing techniques for a more accurate diagnosis of α-synucleinopathy with a clinical impact on therapy and prognosis.

Consequently, we investigated the significance of α-synuclein expression patterns variations at the light of RT-QuIC results. Specifically, RT-QuIC positive and negative samples revealed distinct qualitative alterations in the distribution patterns of P a-syn. In particular, we observed the nuclear deposition of P Ser 129 in the large majority of the RT-QuIC positive samples.

The pathological implications of this nuclear localization need to be deeply investigated in a larger group of patients. However, the simultaneous analyses of immunocytochemistry and RT-QuIC on the same OM sample might be a valuable approach to unravel the pathogenetic processing of α-synuclein in its early phase of aggregation.

Of course, these findings, if validated, might be applied in therapeutic trials aimed to inhibit α-synuclein aggregates formation.

# 7. BIBLIOGRAPHY


Agur, A. M. R., J. C. B. Grant, A. F. Dalley and A. F. Dalley (2005). Grant's Atlas of Anatomy, Lippincott Williams & Wilkins.

Albeanu, D. F., A. C. Provost, P. Agarwal, E. R. Soucy, J. D. Zak and V. N. Murthy (2018). "Olfactory marker protein (OMP) regulates formation and refinement of the olfactory glomerular map." Nature Communications **9**(1): 5073.

Alizadeh, R., S. K. Kamrava, Z. Bagher, M. Farhadi, M. Falah, F. Moradi, M. E. Boroujeni, M. Soleimani, A. Kamyab and A. J. N. l. Komeili (2019). "Human olfactory stem cells: As a promising source of dopaminergic neuron-like cells for treatment of Parkinson's disease." **696**: 52-59.

Arai, T., M. Hasegawa, H. Akiyama, K. Ikeda, T. Nonaka, H. Mori, D. Mann, K. Tsuchiya, M. Yoshida and Y. Hashizume (2006). "TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis." Biochemical and biophysical research communications **351**(3): 602-611.

Arnold, S. E., E. B. Lee, P. J. Moberg, L. Stutzbach, H. Kazi, L.-Y. Han, V. M. Y. Lee and J. Q. Trojanowski (2010). "Olfactory epithelium amyloid-beta and paired helical filament-tau pathology in Alzheimer disease." Annals of neurology **67**(4): 462-469.

Asai, H., S. Ikezu, S. Tsunoda, M. Medalla, J. Luebke, T. Haydar, B. Wolozin, O. Butovsky, S. Kügler and T. Ikezu (2015). "Depletion of microglia and inhibition of exosome synthesis halt tau propagation." Nature neuroscience **18**(11): 1584-1593.

Atarashi, R., K. Sano, K. Satoh and N. Nishida (2011). "Real-time quaking-induced conversion: a highly sensitive assay for prion detection." Prion **5**(3): 150-153.

Atarashi, R., K. Satoh, K. Sano, T. Fuse, N. Yamaguchi, D. Ishibashi, T. Matsubara, T. Nakagaki, H. Yamanaka and S. Shirabe (2011). "Ultrasensitive human prion detection in cerebrospinal fluid by real-time quaking-induced conversion." Nature medicine **17**(2): 175.

Baltic, S., M. Perovic, A. Mladenovic, N. Raicevic, S. Ruzdijic, L. Rakic and S. Kanazir (2004). "α-Synuclein is expressed in different tissues during human fetal development." Journal of Molecular Neuroscience **22**(3): 199-203.

Bartels, T., J. G. Choi and D. J. Selkoe (2011). "α-Synuclein occurs physiologically as a helically folded tetramer that resists aggregation." Nature **477**(7362): 107-110.

Beach, T. G., C. H. Adler, L. Lue, L. I. Sue, J. Bachalakuri, J. Henry-Watson, J. Sasse, S. Boyer, S. Shirohi, R. Brooks, J. Eschbacher, C. L. White, 3rd, H. Akiyama, J. Caviness, H. A. Shill, D. J. Connor, M. N. Sabbagh, D. G. Walker and C. Arizona Parkinson's Disease (2009). "Unified staging system for Lewy body disorders: correlation with nigrostriatal degeneration, cognitive impairment and motor dysfunction." Acta neuropathologica **117**(6): 613-634.

Beach, T. G., C. L. White, 3rd, C. L. Hladik, M. N. Sabbagh, D. J. Connor, H. A. Shill, L. I. Sue, J. Sasse, J. Bachalakuri, J. Henry-Watson, H. Akiyama, C. H. Adler and C. Arizona Parkinson's Disease (2009). "Olfactory bulb alpha-synucleinopathy has high specificity and sensitivity for Lewy body disorders." Acta neuropathologica **117**(2): 169-174.

Beatman, E. L., A. Massey, K. D. Shives, K. S. Burrack, M. Chamanian, T. E. Morrison and J. D. Beckham (2016). "Alpha-synuclein expression restricts RNA viral infections in the brain." Journal of virology **90**(6): 2767-2782.

Berghard, A. and L. B. Buck (1996). "Sensory transduction in vomeronasal neurons: evidence for G alpha o, G alpha i2, and adenylyl cyclase II as major components of a pheromone signaling cascade." Journal of Neuroscience **16**(3): 909-918.

Bero, A. W., P. Yan, J. H. Roh, J. R. Cirrito, F. R. Stewart, M. E. Raichle, J.-M. Lee and D. M. Holtzman (2011). "Neuronal activity regulates the regional vulnerability to amyloid-β deposition." Nature Neuroscience **14**(6): 750-756.

Biedlingmaier, J. F. and P. J. Whelan (1996). "Analysis for Olfactory Epithelium using Olfactory Marker Protein on Endoscopically Harvested Middle Turbinates." American Journal of Rhinology **10**(4): 221-224.

Bigio, E. H., J. Y. Wu, H.-X. Deng, E. N. Bit-Ivan, Q. Mao, R. Ganti, M. Peterson, N. Siddique, C. Geula and T. Siddique (2013). "Inclusions in

frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP) and amyotrophic lateral sclerosis (ALS), but not FTLD with FUS proteinopathy (FTLD-FUS), have properties of amyloid." Acta neuropathologica **125**(3): 463-465.

Bloch, A., A. Probst, H. Bissig, H. Adams and M. Tolnay (2006). "α-Synuclein pathology of the spinal and peripheral autonomic nervous system in neurologically unimpaired elderly subjects." Neuropathology and Applied Neurobiology **32**(3): 284-295.

Bloom, G. and H. Engström (1952). "The structure of the epithelial surface in the olfactory region." Experimental Cell Research **3**(4): 699-701.

Boesveldt, S., D. Verbaan, D. L. Knol, M. Visser, S. M. van Rooden, J. J. van Hilten and H. W. Berendse (2008). "A comparative study of odor identification and odor discrimination deficits in Parkinson's disease." Movement Disorders **23**(14): 1984-1990.

Bongianni, M., A. Ladogana, S. Capaldi, S. Klotz, S. Baiardi, A. Cagnin, D. Perra, M. Fiorini, A. Poleggi, G. Legname, T. Cattaruzza, F. Janes, M. Tabaton, B. Ghetti, S. Monaco, G. G. Kovacs, P. Parchi, M. Pocchiari and G. Zanusso (2019). "α-Synuclein RT-QuIC assay in cerebrospinal fluid of patients with dementia with Lewy bodies." Annals of clinical and translational neurology **6**(10): 2120-2126.

Bongianni, M., C. Orrù, B. R. Groveman, L. Sacchetto, M. Fiorini, G. Tonoli, G. Triva, S. Capaldi, S. Testi and S. Ferrari (2017). "Diagnosis of human prion disease using real-time quaking-induced conversion testing of olfactory mucosa and cerebrospinal fluid samples." JAMA neurology **74**(2): 155-162.

Borgmann-Winter, K., N. Rawson, H.-Y. Wang, H. Wang, M. Macdonald, M. Ozdener, K. Yee, G. Gomez, J. Xu and B. J. N. Bryant (2009). "Human olfactory epithelial cells generated in vitro express diverse neuronal characteristics." **158**(2): 642-653.

Borin, M., C. Saraceno, M. Catania, E. Lorenzetto, V. Pontelli, A. Paterlini, S. Fostinelli, A. Avesani, G. Di Fede, G. Zanusso, L. Benussi, G. Binetti, S. Zorzan, R. Ghidoni, M. Buffelli and S. Bolognin (2018). "Rac1 activation links

tau hyperphosphorylation and Aβ dysmetabolism in Alzheimer's disease." Acta Neuropathologica Communications **6**(1): 61.

Böttner, M., D. Zorenkov, I. Hellwig, M. Barrenschee, J. Harde, T. Fricke, G. Deuschl, J.-H. Egberts, T. Becker, A. Fritscher-Ravens, A. Arlt and T. Wedel (2012). "Expression pattern and localization of alpha-synuclein in the human enteric nervous system." Neurobiology of Disease **48**(3): 474-480.

Bower, J. H., D. M. Maraganore, S. K. McDonnell and W. A. Rocca (1997). "Incidence of progressive supranuclear palsy and multiple system atrophy in Olmsted County, Minnesota, 1976 to 1990." Neurology **49**(5): 1284-1288.

Braak, H. and E. Braak (1991). "Neuropathological stageing of Alzheimer-related changes." Acta neuropathologica **82**(4): 239-259.

Braak, H., R. A. I. de Vos, J. Bohl and K. Del Tredici (2006). "Gastric α-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for Parkinson's disease-related brain pathology." Neuroscience Letters **396**(1): 67-72.

Braak, H., E. Ghebremedhin, U. Rüb, H. Bratzke and K. Del Tredici (2004). "Stages in the development of Parkinson's disease-related pathology." Cell and Tissue Research **318**(1): 121-134.

Braak, H., K. D. Tredici, U. Rüb, R. A. I. de Vos, E. N. H. Jansen Steur and E. Braak (2003). "Staging of brain pathology related to sporadic Parkinson's disease." Neurobiology of Aging **24**(2): 197-211.

Brahic, M., L. Bousset, G. Bieri, R. Melki and A. D. Gitler (2016). "Axonal transport and secretion of fibrillar forms of α-synuclein, Aβ42 peptide and HTTExon 1." Acta neuropathologica **131**(4): 539-548.

Brann, J. H. and S. J. J. F. i. n. Firestein (2014). "A lifetime of neurogenesis in the olfactory system." **8**: 182.

Brettschneider, J., K. Del Tredici, V. M.-Y. Lee and J. Q. Trojanowski (2015). "Spreading of pathology in neurodegenerative diseases: a focus on human studies." Nature Reviews Neuroscience **16**(2): 109-120.

Breydo, L. and V. N. Uversky (2015). "Structural, morphological, and functional diversity of amyloid oligomers." FEBS letters **589**(19): 2640-2648.

Buck, L. and R. Axel (1991). "A novel multigene family may encode odorant receptors: a molecular basis for odor recognition." Cell **65**(1): 175-187.

Buiakova, O., H. Baker, J. Scott, A. Farbman, R. Kream, M. Grillo, L. Franzen, M. Richman, L. Davis and S. J. P. o. t. N. A. o. S. Abbondanzo (1996). "Olfactory marker protein (OMP) gene deletion causes altered physiological activity of olfactory sensory neurons." **93**(18): 9858-9863.

Burke, R. E., W. T. Dauer and J. P. G. Vonsattel (2008). "A critical evaluation of the Braak staging scheme for Parkinson's disease." Annals of neurology **64**(5): 485-491.

Burré, J., M. Sharma and T. C. Südhof (2014). "α-Synuclein assembles into higher-order multimers upon membrane binding to promote SNARE complex formation." Proceedings of the National Academy of Sciences **111**(40): E4274-E4283.

Calof, A. L. and D. M. J. N. Chikaraishi (1989). "Analysis of neurogenesis in a mammalian neuroepithelium: proliferation and differentiation of an olfactory neuron precursor in vitro." **3**(1): 115-127.

Caughey, B. and P. T. Lansbury Jr (2003). "Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders." Annual review of neuroscience **26**(1): 267-298.

Chabrat, A., E. Lacassagne, R. Billiras, S. Landron, A. Pontisso-Mahout, H. Darville, A. Dupront, F. Coge, E. Schenker and D. J. S. c. i. Piwnica (2019). "Pharmacological Transdifferentiation of Human Nasal Olfactory Stem Cells into Dopaminergic Neurons." **2019**.

Chaudhuri, K. R., D. G. Healy and A. H. Schapira (2006). "Non-motor symptoms of Parkinson's disease: diagnosis and management." The Lancet Neurology **5**(3): 235-245.

Chen, C. R., C. Kachramanoglou, D. Li, P. Andrews and D. Choi (2014). "Anatomy and cellular constituents of the human olfactory mucosa: a review." Journal of neurological surgery. Part B, Skull base **75**(5): 293-300.

Chen, H., S. Dadsetan, A. F. Fomina and Q. J. N. d. Gong (2008). "Expressing exogenous functional odorant receptors in cultured olfactory sensory neurons." **3**(1): 22.

Chen, M., S. Tian, X. Yang, A. P. Lane, R. R. Reed and H. J. J. o. N. Liu (2014). "Wnt-responsive Lgr5+ globose basal cells function as multipotent olfactory epithelium progenitor cells." **34**(24): 8268-8276.

Cheng, H.-C., C. M. Ulane and R. E. Burke (2010). "Clinical progression in Parkinson disease and the neurobiology of axons." Annals of Neurology **67**(6): 715-725.

Chou, C.-C., Y. Zhang, M. E. Umoh, S. W. Vaughan, I. Lorenzini, F. Liu, M. Sayegh, P. G. Donlin-Asp, Y. H. Chen and D. M. Duong (2018). "TDP-43 pathology disrupts nuclear pore complexes and nucleocytoplasmic transport in ALS/FTD." Nature neuroscience **21**(2): 228.

Chuah, M. and A. I. J. J. o. N. Farbman (1983). "Olfactory bulb increases marker protein in olfactory receptor cells." **3**(11): 2197-2205.

Colby, D. W., Q. Zhang, S. Wang, D. Groth, G. Legname, D. Riesner and S. B. Prusiner (2007). "Prion detection by an amyloid seeding assay." Proceedings of the National Academy of Sciences of the United States of America **104**(52): 20914-20919.

Collinge, J. (2016). "Mammalian prions and their wider relevance in neurodegenerative diseases." Nature **539**(7628): 217-226.

Connolly, B. S. and A. E. Lang (2014). "Pharmacological treatment of Parkinson disease: a review." Jama **311**(16): 1670-1683.

Corbett, G. T. and E. W. Buss (2014). "Non-neuronal cells exacerbate β-amyloid aggregation in the aged brain." The Journal of neuroscience : the official journal of the Society for Neuroscience **34**(30): 9825-9827.

Covell, D. J., J. L. Robinson, R. S. Akhtar, M. Grossman, D. Weintraub, H. M. Bucklin, R. M. Pitkin, D. Riddle, A. Yousef, J. Q. Trojanowski and V. M. Y. Lee (2017). "Novel conformation-selective alpha-synuclein antibodies raised against different in vitro fibril forms show distinct patterns of Lewy pathology in Parkinson's disease." Neuropathology and applied neurobiology **43**(7): 604-620.

Crino, P. B., B. Greenberg, J. A. Martin, V. M.-Y. Lee, W. D. Hill and J. Q. Trojanowski (1995). "β-Amyloid peptide and amyloid precursor proteins in olfactory mucosa of patients with Alzheimer's disease, Parkinson's disease, and

Down syndrome." Annals of Otology, Rhinology & Laryngology **104**(8): 655-661.

Cunningham, A., P. Manis, R. R. Reed and G. J. N. Ronnett (1999). "Olfactory receptor neurons exist as distinct subclasses of immature and mature cells in primary culture." **93**(4): 1301-1312.

Dalton, P. H., C. J. C. o. i. a. Jaen and c. immunology (2010). "Responses to odors in occupational environments." **10**(2): 127-132.

De Lau, L. M. and M. M. Breteler (2006). "Epidemiology of Parkinson's disease." The Lancet Neurology **5**(6): 525-535.

De Luca, C. M. G., A. E. Elia, S. M. Portaleone, F. A. Cazzaniga, M. Rossi, E. Bistaffa, E. De Cecco, J. Narkiewicz, G. Salzano and O. Carletta (2019). "Efficient RT-QuIC seeding activity for α-synuclein in olfactory mucosa samples of patients with Parkinson's disease and multiple system atrophy." Translational Neurodegeneration **8**(1): 24.

Delay, R. J. and V. E. Dionne (2003). "Coupling between sensory neurons in the olfactory epithelium." Chemical senses **28**(9): 807-815.

DeVos, S. L., B. T. Corjuc, D. H. Oakley, C. K. Nobuhara, R. N. Bannon, A. Chase, C. Commins, J. A. Gonzalez, P. M. Dooley, M. P. Frosch and B. T. Hyman (2018). "Synaptic Tau Seeding Precedes Tau Pathology in Human Alzheimer's Disease Brain." Frontiers in neuroscience **12**: 267-267.

Doty, R. L. (2008). "The olfactory vector hypothesis of neurodegenerative disease: Is it viable?" Annals of Neurology **63**(1): 7-15.

Doty, R. L. (2009). "The Olfactory System and Its Disorders." Semin Neurol **29**(01): 074-081.

Douek, E., L. Bannister and H. C. Dodson (1975). Olfaction and Its Disorders: Recent Advances in the Pathology of Olfaction, SAGE Publications.

Duda, J. E., U. Shah, S. E. Arnold, V. M. Y. Lee and J. Q. Trojanowski (1999). "The Expression of α-, β-, and γ-Synucleins in Olfactory Mucosa from Patients with and without Neurodegenerative Diseases." Experimental Neurology **160**(2): 515-522.

Durante, M. A., S. Kurtenbach, Z. B. Sargi, J. W. Harbour, R. Choi, S. Kurtenbach, G. M. Goss, H. Matsunami and B. J. J. N. n. Goldstein (2020).

"Single-cell analysis of olfactory neurogenesis and differentiation in adult humans." **23**(3): 323-326.

Engelender, S. (2008). "Ubiquitination of α-synuclein and autophagy in Parkinson's disease." Autophagy **4**(3): 372-374.

Escada, P. A., C. Lima and J. M. da Silva (2009). "The human olfactory mucosa." European Archives of Oto-Rhino-Laryngology **266**(11): 1675-1680.

Evgrafov, O. V., B. B. Wrobel, X. Kang, G. Simpson, D. Malaspina and J. A. J. P. g. Knowles (2011). "Olfactory neuroepithelium-derived neural progenitor cells as a model system for investigating the molecular mechanisms of neuropsychiatric disorders." **21**(5): 217-228.

Fairfoul, G., L. I. McGuire, S. Pal, J. W. Ironside, J. Neumann, S. Christie, C. Joachim, M. Esiri, S. G. Evetts and M. Rolinski (2016). "Alpha- synuclein RT- Qu IC in the CSF of patients with alpha- synucleinopathies." Annals of clinical and translational neurology **3**(10): 812-818.

Farbman, A. I. and F. L. J. D. b. Margolis (1980). "Olfactory marker protein during ontogeny: immunohistochemical localization." **74**(1): 205-215.

Fares, M.-B., N. Ait-Bouziad, I. Dikiy, M. K. Mbefo, A. Jovičić, A. Kiely, J. L. Holton, S.-J. Lee, A. D. Gitler and D. Eliezer (2014). "The novel Parkinson's disease linked mutation G51D attenuates in vitro aggregation and membrane binding of α-synuclein, and enhances its secretion and nuclear localization in cells." Human molecular genetics **23**(17): 4491-4509.

Fauvet, B., M. K. Mbefo, M.-B. Fares, C. Desobry, S. Michael, M. T. Ardah, E. Tsika, P. Coune, M. Prudent, N. Lion, D. Eliezer, D. J. Moore, B. Schneider, P. Aebischer, O. M. El-Agnaf, E. Masliah and H. A. Lashuel (2012). "α-Synuclein in Central Nervous System and from Erythrocytes, Mammalian Cells, and Escherichia coli Exists Predominantly as Disordered Monomer." Journal of Biological Chemistry **287**(19): 15345-15364.

Feron, F., C. Perry, J. Cochrane, P. Licina, A. Nowitzke, S. Urquhart, T. Geraghty and A. J. B. Mackay-Sim (2005). "Autologous olfactory ensheathing cell transplantation in human spinal cord injury." **128**(12): 2951-2960.

Féron, F., C. Perry, J. J. McGrath, A. J. A. o. o. h. Mackay-Sim and n. surgery (1998). "New techniques for biopsy and culture of human olfactory epithelial neurons." **124**(8): 861-866.

Ferreon, A. C. M., Y. Gambin, E. A. Lemke and A. A. Deniz (2009). "Interplay of α-synuclein binding and conformational switching probed by single-molecule fluorescence." Proceedings of the National Academy of Sciences **106**(14): 5645-5650.

Fitzpatrick, A. W., G. T. Debelouchina, M. J. Bayro, D. K. Clare, M. A. Caporini, V. S. Bajaj, C. P. Jaroniec, L. Wang, V. Ladizhansky and S. A. Müller (2013). "Atomic structure and hierarchical assembly of a cross-β amyloid fibril." Proceedings of the National Academy of Sciences **110**(14): 5468-5473.

Freer, R., P. Sormanni, G. Vecchi, P. Ciryam, C. M. Dobson and M. Vendruscolo (2016). "A protein homeostasis signature in healthy brains recapitulates tissue vulnerability to Alzheimer's disease." Science advances **2**(8): e1600947.

Freiherr, J., M. Wiesmann, M. Witt, A. Welge-Lüssen and T. Hummel (2013). "Functional anatomy of the olfactory system II: Central relays, pathways, and their function." Management of smell and taste disorders: a practical guide for clinicians. Stuttgart: Thieme: 27-38.

Fujiwara, H., M. Hasegawa, N. Dohmae, A. Kawashima, E. Masliah, M. S. Goldberg, J. Shen, K. Takio and T. Iwatsubo (2002). "α-Synuclein is phosphorylated in synucleinopathy lesions." Nature cell biology **4**(2): 160.

Funabe, S., M. Takao, Y. Saito, H. Hatsuta, M. Sugiyama, S. Ito, K. Kanemaru, M. Sawabe, T. Arai, H. Mochizuki, N. Hattori and S. Murayama (2013). "Neuropathologic analysis of Lewy-related α-synucleinopathy in olfactory mucosa." Neuropathology **33**(1): 47-58.

Ganowiak, K., P. Hultman, U. Engstrom, A. Gustavsson and P. Westermark (1994). "Fibrils from synthetic amyloid-related peptides enhance development of experimental AA-amyloidosis in mice." Biochemical and biophysical research communications **199**(1): 306-312.

Garcia, E. C. D., A. C. Rossaneis, A. S. Pipino, G. V. Gomes, F. de Rezende Pinna, R. L. Voegels, R. L. Doty, W. A. Verri and M. A. J. E. A. o. O.-R.-L. Fornazieri (2020). "Safety and efficacy of superior turbinate biopsies as a

source of olfactory epithelium appropriate for morphological analysis." **277**(2): 483-492.

George, J. M. (2001). "The synucleins." Genome Biology **3**(1): reviews3002.3001.

Georgiou, M., J. N. dos Reis, R. Wood, P. P. Esteban, V. Roberton, C. Mason, D. Li, Y. Li, D. Choi and I. J. S. r. Wall (2018). "Bioprocessing strategies to enhance the challenging isolation of neuro-regenerative cells from olfactory mucosa." **8**(1): 1-12.

Gilman, S., G. Wenning, P. A. Low, D. Brooks, C. Mathias, J. Trojanowski, N. W. Wood, C. Colosimo, A. Dürr and C. Fowler (2008). "Second consensus statement on the diagnosis of multiple system atrophy." Neurology **71**(9): 670-676.

Girard, S. D., A. Devéze, E. Nivet, B. Gepner, F. S. Roman and F. J. J. Féron (2011). "Isolating nasal olfactory stem cells from rodents or humans." (54): e2762.

Godoy, M. D. C. L., R. L. Voegels, F. de Rezende Pinna, R. Imamura and J. M. Farfel (2015). "Olfaction in neurologic and neurodegenerative diseases: a literature review." International archives of otorhinolaryngology **19**(02): 176-179.

Goedert, M. (2015). "Alzheimer's and Parkinson's diseases: The prion concept in relation to assembled Aβ, tau, and α-synuclein." Science **349**(6248): 1255555.

Goedert, M., B. Falcon, F. Clavaguera and M. Tolnay (2014). "Prion-like mechanisms in the pathogenesis of tauopathies and synucleinopathies." Current neurology and neuroscience reports **14**(11): 495.

Goedert, M., R. Jakes and M. G. Spillantini (2017). "The synucleinopathies: twenty years on." Journal of Parkinson's disease **7**(s1): S51-S69.

Goedert, M., M. G. Spillantini, K. Del Tredici and H. Braak (2013). "100 years of Lewy pathology." Nature Reviews Neurology **9**(1): 13-24.

Goedert, M., M. G. Spillantini, R. Jakes, D. Rutherford and R. A. Crowther (1989). "Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease." Neuron **3**(4): 519-526.

Goers, J., A. B. Manning-Bog, A. L. McCormack, I. S. Millett, S. Doniach, D. A. Di Monte, V. N. Uversky and A. L. Fink (2003). "Nuclear localization of α-synuclein and its interaction with histones." Biochemistry **42**(28): 8465-8471.

Goldstein, B. J., H. Fang, S. L. Youngentob and J. E. J. N. Schwob (1998). "Transplantation of multipotent progenitors from the adult olfactory epithelium." **9**(7): 1611-1617.

Gómez-Tortosa, E., K. Newell, M. C. Irizarry, J. L. Sanders and B. T. Hyman (2000). "α-Synuclein immunoreactivity in dementia with Lewy bodies: morphological staging and comparison with ubiquitin immunostaining." Acta neuropathologica **99**(4): 352-357.

Gomez, G., N. E. Rawson, C. G. Hahn, R. Michaels and D. J. J. o. n. r. Restrepo (2000). "Characteristics of odorant elicited calcium changes in cultured human olfactory neurons." **62**(5): 737-749.

Gong, Q. J. C. p. i. n. (2012). "Culture of mouse olfactory sensory neurons." **58**(1): 3.24. 21-23.24. 14.

Gonzales, F., A. I. Farbman and R. C. J. J. o. n. m. Gesteland (1985). "Cell and explant culture of olfactory chemoreceptor cells." **14**(2): 77-90.

Graziadei, G. M. and P. P. C. J. J. o. n. Graziadei (1979). "Neurogenesis and neuron regeneration in the olfactory system of mammals. II. Degeneration and reconstitution of the olfactory sensory neurons after axotomy." **8**(2): 197-213.

Graziadei, P. P. and A. G. M. Graziadei (1983). "Regeneration in the olfactory system of vertebrates." American journal of otolaryngology **4**(4): 228-233.

Grill, R. J. and S. K. J. J. o. N. Pixley (1997). "In vitro generation of adult rat olfactory sensory neurons and regulation of maturation by coculture with CNS tissues." **17**(9): 3120-3127.

Groveman, B. R., C. D. Orrù, A. G. Hughson, L. D. Raymond, G. Zanusso, B. Ghetti, K. J. Campbell, J. Safar, D. Galasko and B. Caughey (2018). "Rapid and ultra-sensitive quantitation of disease-associated α-synuclein seeds in brain and cerebrospinal fluid by αSyn RT-QuIC." Acta neuropathologica communications **6**(1): 7.

Guo, Z., A. Packard, R. C. Krolewski, M. T. Harris, G. L. Manglapus and J. E. J. J. o. C. N. Schwob (2010). "Expression of pax6 and sox2 in adult olfactory epithelium." **518**(21): 4395-4418.

Hahn, C.-G., L.-Y. Han, N. E. Rawson, N. Mirza, K. Borgmann-Winter, R. H. Lenox and S. E. Arnold (2005). "In vivo and in vitro neurogenesis in human olfactory epithelium." Journal of Comparative Neurology **483**(2): 154-163.

Hahn, C. G., L. Y. Han, N. E. Rawson, N. Mirza, K. Borgmann- Winter, R. H. Lenox and S. E. J. J. o. C. N. Arnold (2005). "In vivo and in vitro neurogenesis in human olfactory epithelium." **483**(2): 154-163.

Hamilton, R. L. (2000). "Lewy bodies in Alzheimer's disease: a neuropathological review of 145 cases using α- synuclein immunohistochemistry." Brain pathology **10**(3): 378-384.

Hawkes, C. H., K. Del Tredici and H. Braak (2007). "Parkinson's disease: a dual-hit hypothesis." Neuropathology and Applied Neurobiology **33**(6): 599-614.

Heymann, E. W. (2006). "The neglected sense–olfaction in primate behavior, ecology, and evolution." American Journal of Primatology **68**(6): 519-524.

Holbrook, E. H., K. E. M. Szumowski and J. E. Schwob (1995). "An immunochemical, ultrastructural, and developmental characterization of the horizontal basal cells of rat olfactory epithelium." Journal of Comparative Neurology **363**(1): 129-146.

Holbrook, E. H., E. Wu, W. T. Curry, D. T. Lin and J. E. Schwob (2011). "Immunohistochemical characterization of human olfactory tissue." The Laryngoscope **121**(8): 1687-1701.

Idotta, C., E. Tibaldi, A. M. Brunati, M. A. Pagano, M. Cadamuro, A. Miola, A. Martini, N. Favaretto, D. Cazzador and A. J. E. P. Favaro (2019). "Olfactory neuroepithelium alterations and cognitive correlates in schizophrenia." **61**: 23-32.

Iturria-Medina, Y. and A. C. Evans (2015). "On the central role of brain connectivity in neurodegenerative disease progression." Frontiers in aging neuroscience **7**: 90.

Jafari, F. H. (2006). A Histological Study Of Human Olfactory Mucosa: regional Distribution And Age Related Changes, University Of Health Sciences, Lahore.

Jafek, B. W., B. Murrow, R. Michaels, D. Restrepo and M. Linschoten (2002). "Biopsies of human olfactory epithelium." Chemical senses **27**(7): 623-628.

Jakes, R., M. G. Spillantini and M. Goedert (1994). "Identification of two distinct synucleins from human brain." FEBS Letters **345**(1): 27-32.

Jankovic, J. (2008). "Parkinson's disease: clinical features and diagnosis." Journal of neurology, neurosurgery & psychiatry **79**(4): 368-376.

Jarrett, J. T. and P. T. Lansbury Jr (1993). "Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie?" Cell **73**(6): 1055-1058.

Jellinger, K. A. (2003). "Neuropathological spectrum of synucleinopathies." Movement Disorders **18**(S6): 2-12.

Jenkins, P. M., D. P. McEwen and J. R. Martens (2009). "Olfactory cilia: linking sensory cilia function and human disease." Chemical senses **34**(5): 451-464.

Johnson, E. W., P. M. Eller and B. W. Jafek (1997). "Protein gene product 9.5-like and calbindin- like immunoreactivity in the nasal respiratory mucosa of perinatal humans." The Anatomical Record: An Official Publication of the American Association of Anatomists **247**(1): 38-45.

Joiner, A. M., W. W. Green, J. C. McIntyre, B. L. Allen, J. E. Schwob and J. R. Martens (2015). "Primary Cilia on Horizontal Basal Cells Regulate Regeneration of the Olfactory Epithelium." The Journal of neuroscience : the official journal of the Society for Neuroscience **35**(40): 13761-13772.

Jucker, M. and L. C. Walker (2013). "Self-propagation of pathogenic protein aggregates in neurodegenerative diseases." Nature **501**(7465): 45-51.

Jucker, M. and L. C. Walker (2018). "Propagation and spread of pathogenic protein assemblies in neurodegenerative diseases." Nature neuroscience **21**(10): 1341-1349.

Jürgen M. Peters, M.D. ,, Thomas Hummel, M.D., Ph.D. ,, Tilman Kratzsch, M.D. ,, Jörn Lötsch, M.D., Ph.D. ,, Carsten Skarke, M.D. , and and Lutz Frölich, M.D., Ph.D. (2003). "Olfactory Function in Mild Cognitive Impairment and

Alzheimer's Disease: An Investigation Using Psychophysical and Electrophysiological Techniques." American Journal of Psychiatry **160**(11): 1995-2002.

Kang, N., H. Kim, Y. Jae, N. Lee, C. R. Ku, F. Margolis, E. J. Lee, Y. Y. Bahk, M.-S. Kim and J. Koo (2015). "Olfactory marker protein expression is an indicator of olfactory receptor-associated events in non-olfactory tissues." PLoS One **10**(1): e0116097.

Kawakami, I., T. Arai and M. Hasegawa (2019). "The basis of clinicopathological heterogeneity in TDP-43 proteinopathy." Acta Neuropathologica **138**(5): 751-770.

Keller, A. and F. J. J. o. n. Margolis (1975). "IMMUNOLOGICAL STUDIES OF THE RAT OLFACTORY MARKER PROTEIN 1." **24**(6): 1101-1106.

Kocisko, D. A., J. H. Come, S. A. Priola, B. Chesebro, G. J. Raymond, P. T. Lansbury and B. Caughey (1994). "Cell-free formation of protease-resistant prion protein." Nature **370**(6489): 471.

Kontopoulos, E., J. D. Parvin and M. B. Feany (2006). "α-synuclein acts in the nucleus to inhibit histone acetylation and promote neurotoxicity." Human molecular genetics **15**(20): 3012-3023.

Kream, R. M. and F. L. Margolis (1984). "Olfactory marker protein: turnover and transport in normal and regenerating neurons." The Journal of neuroscience : the official journal of the Society for Neuroscience **4**(3): 868-879.

Krieger, J. and H. Breer (1999). "Olfactory Reception in Invertebrates." Science **286**(5440): 720-723.

Krolewski, R. C., A. Packard and J. E. Schwob (2013). "Global expression profiling of globose basal cells and neurogenic progression within the olfactory epithelium." The Journal of comparative neurology **521**(4): 833-859.

Krstic, R. V. (2013). Human microscopic anatomy: an atlas for students of medicine and biology, Springer Science & Business Media.

Kurtenbach, S., G. M. Goss, S. Goncalves, R. Choi, J. M. Hare, N. Chaudhari and B. J. J. S. c. r. Goldstein (2019). "Cell-Based Therapy Restores Olfactory Function in an Inducible Model of Hyposmia." **12**(6): 1354-1365.

LaFerla, F. M., K. N. Green and S. Oddo (2007). "Intracellular amyloid-β in Alzheimer's disease." Nature Reviews Neuroscience **8**(7): 499.

Lane, A. P., G. Gomez, T. Dankulich, H. Wang, W. E. Bolger and N. E. Rawson (2002). "The superior turbinate as a source of functional human olfactory receptor neurons." The Laryngoscope **112**(7): 1183-1189.

Lanza, D. C., D. A. Deems, R. L. Doty, D. Crawford, J. Carter Rowley III, D. W. Kennedy, A. Sajjadian and D. J. T. L. Moran (1994). "The effect of human olfactory biopsy on olfaction: a preliminary report." **104**(7): 837-840.

Lasconi, C., S. Pifferi, A. Hernandez-Clavijo, F. Merigo, M. P. Cecchini, K. Y. Gonzalez-Velandia, E. Agostinelli, A. Sbarbati and A. Menini (2019). "Bitter tastants and artificial sweeteners activate a subset of epithelial cells in acute tissue slices of the rat trachea." Scientific Reports **9**(1): 8834.

Lázaro, D. F., E. F. Rodrigues, R. Langohr, H. Shahpasandzadeh, T. Ribeiro, P. Guerreiro, E. Gerhardt, K. Kröhnert, J. Klucken and M. D. Pereira (2014). "Systematic comparison of the effects of alpha-synuclein mutations on its oligomerization and aggregation." PLoS genetics **10**(11): e1004741.

Lee, J.-G., S. Takahama, G. Zhang, S. I. Tomarev and Y. Ye (2016). "Unconventional secretion of misfolded proteins promotes adaptation to proteasome dysfunction in mammalian cells." Nature Cell Biology **18**: 765.

Lee, J. H., M. Goedert, W. D. Hill, V. M.-Y. Lee and J. Q. Trojanowski (1993). "Tau proteins are abnormally expressed in olfactory epithelium of Alzheimer patients and developmentally regulated in human fetal spinal cord." Experimental neurology **121**(1): 93-105.

Lema Tomé, C. M., T. Tyson, N. L. Rey, S. Grathwohl, M. Britschgi and P. Brundin (2013). "Inflammation and α-synuclein's prion-like behavior in Parkinson's disease--is there a link?" Molecular neurobiology **47**(2): 561-574.

Leopold, D. A., T. Hummel, J. E. Schwob, S. C. Hong, M. Knecht and G. Kobal (2000). "Anterior Distribution of Human Olfactory Epithelium." The Laryngoscope **110**(3): 417-421.

Leung, C. T., P. A. Coulombe and R. R. Reed (2007). "Contribution of olfactory neural stem cells to tissue maintenance and regeneration." Nature neuroscience **10**(6): 720.

Liu, N., C. Shields and F. J. E. n. Roisen (1998). "Primary culture of adult mouse olfactory receptor neurons." **151**(2): 173-183.

Lothian, A., L. Lago, S. Mukherjee, A. R. Connor, C. Fowler, C. A. McLean, M. Horne, C. L. Masters, R. Cappai and B. R. Roberts (2019). "Characterization of the metal status of natively purified alpha-synuclein from human blood, brain tissue, or recombinant sources using size exclusion ICP-MS reveals no significant binding of Cu, Fe or Zn." Metallomics **11**(1): 128-140.

Lovell, M. A., B. W. Jafek, D. T. Moran and J. C. J. A. o. O. Rowley (1982). "Biopsy of human olfactory mucosa: An instrument and a technique." **108**(4): 247-249.

Luk, K. C., V. Kehm, J. Carroll, B. Zhang, P. O'Brien, J. Q. Trojanowski and V. M. Y. Lee (2012). "Pathological α-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice." Science (New York, N.Y.) **338**(6109): 949-953.

Luk, K. C., V. M. Kehm, B. Zhang, P. O'Brien, J. Q. Trojanowski and V. M. Y. Lee (2012). "Intracerebral inoculation of pathological α-synuclein initiates a rapidly progressive neurodegenerative α-synucleinopathy in mice." The Journal of experimental medicine **209**(5): 975-986.

Maroteaux, L., J. Campanelli and R. Scheller (1988). "Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal." The Journal of Neuroscience **8**(8): 2804-2815.

Masliah, E. (2005). "[alpha]-synuclein immunization in a model of Parkinson's disease." The Neuroscientist **11**(6): 523-524.

Matigian, N., G. Abrahamsen, R. Sutharsan, A. L. Cook, A. M. Vitale, A. Nouwens, B. Bellette, J. An, M. Anderson, A. G. J. D. m. Beckhouse and mechanisms (2010). "Disease-specific, neurosphere-derived cells as models for brain disorders." **3**(11-12): 785-798.

McCurdy, R. D., F. Féron, C. Perry, D. C. Chant, D. McLean, N. Matigian, N. K. Hayward, J. J. McGrath and A. J. S. r. Mackay-Sim (2006). "Cell cycle alterations in biopsied olfactory neuroepithelium in schizophrenia and bipolar I disorder using cell culture and gene expression analyses." **82**(2-3): 163-173.

McKeith, I. (2007). Dementia with Lewy bodies. Handbook of clinical Neurology, Elsevier. **84:** 531-548.

McKeith, I. G., B. F. Boeve, D. W. Dickson, G. Halliday, J.-P. Taylor, D. Weintraub, D. Aarsland, J. Galvin, J. Attems and C. G. Ballard (2017). "Diagnosis and management of dementia with Lewy bodies: Fourth consensus report of the DLB Consortium." Neurology **89**(1): 88-100.

Meade, R. M., D. P. Fairlie and J. M. Mason (2019). "Alpha-synuclein structure and Parkinson's disease – lessons and emerging principles." Molecular Neurodegeneration **14**(1): 29.

Menco, B. P. M. (1984). "Ciliated and microvillous structures of rat olfactory and nasal respiratory epithelia." Cell and tissue research **235**(2): 225-241.

Merigo, F., D. Benati, M. Cristofoletti, F. Amarù, F. Osculati and A. Sbarbati (2012). "Glucose transporter/T1R3- expressing cells in rat tracheal epithelium." Journal of anatomy **221**(2): 138-150.

Miragall, F. and G. M. J. B. r. Graziadei (1982). "Experimental studies on the olfactory marker protein. II. Appearance of the olfactory marker protein during differentiation of the olfactory sensory neurons of mouse: an immunohistochemical and autoradiographic study." **239**(1): 245-250.

Mohamed, N.-V., A. Desjardins and N. Leclerc (2017). "Tau secretion is correlated to an increase of Golgi dynamics." PLOS ONE **12**(5): e0178288.

Monti-Graziadei, G., F. Margolis, J. Harding and P. P. C. Graziadei (1978). "Immunocytochemistry of olfactory marker protein." The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society **25**: 1311-1316.

Moran, D. T., J. C. Rowley III and B. W. Jafek (1982). "Electron microscopy of human olfactory epithelium reveals a new cell type: the microvillar cell." Brain research **253**(1-2): 39-46.

Moran, D. T., J. C. Rowley, B. W. Jafek and M. A. Lovell (1982). "The fine structure of the olfactory mucosa in man." Journal of neurocytology **11**(5): 721-746.

Morrison, E. E. and R. M. Costanzo (1990). "Morphology of the human olfactory epithelium." Journal of Comparative Neurology **297**(1): 1-13.

Moulton, D., G. Celebi and R. Fink (1970). Olfaction in mammals—two aspects: proliferation of cells in the olfactory epithelium and sensitivity to odours. Ciba Foundation Symposium- Internal Secretions of the Pancreas (Colloquia on Endocrinology), Wiley Online Library.

Murrell, W., G. R. Bushell, J. Livesey, J. McGrath, K. MacDonald, P. R. Bates and A. J. N. Mackay-Sim (1996). "Neurogenesis in adult human." **7**(6): 1189-1194.

Murrell, W., F. Féron, A. Wetzig, N. Cameron, K. Splatt, B. Bellette, J. Bianco, C. Perry, G. Lee and A. J. D. d. a. o. p. o. t. A. A. o. A. Mackay- Sim (2005). "Multipotent stem cells from adult olfactory mucosa." **233**(2): 496-515.

Nakashima, N., K. Nakashima, A. Takaku-Nakashima and M. Takano (2019). "Olfactory receptor neurons express olfactory marker protein but not calpain 5 from the same genomic locus." Molecular brain **12**(1): 54.

Nakashima, T., C. P. Kimmelman and J. B. Snow (1984). "Structure of human fetal and adult olfactory neuroepithelium." Archives of otolaryngology **110**(10): 641-646.

Neumann, M., D. M. Sampathu, L. K. Kwong, A. C. Truax, M. C. Micsenyi, T. T. Chou, J. Bruce, T. Schuck, M. Grossman and C. M. Clark (2006). "Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis." Science **314**(5796): 130-133.

Orrú, C. D., M. Bongianni, G. Tonoli, S. Ferrari, A. G. Hughson, B. R. Groveman, M. Fiorini, M. Pocchiari, S. Monaco, B. Caughey and G. Zanusso (2014). "A Test for Creutzfeldt–Jakob Disease Using Nasal Brushings." New England Journal of Medicine **371**(6): 519-529.

Orrú, C. D., B. R. Groveman, A. G. Hughson, G. Zanusso, M. B. Coulthart and B. Caughey (2015). "Rapid and sensitive RT-QuIC detection of human Creutzfeldt-Jakob disease using cerebrospinal fluid." MBio **6**(1): e02451-02414.

Osterberg, V. R., K. J. Spinelli, L. J. Weston, K. C. Luk, R. L. Woltjer and V. K. Unni (2015). "Progressive aggregation of alpha-synuclein and selective degeneration of lewy inclusion-bearing neurons in a mouse model of parkinsonism." Cell reports **10**(8): 1252-1260.

Ou, S., F. Wu, D. Harrich, L. F. García-Martínez and R. B. Gaynor (1995). "Cloning and characterization of a novel cellular protein, TDP-43, that binds to human immunodeficiency virus type 1 TAR DNA sequence motifs." Journal of virology **69**(6): 3584-3596.

Paik, S. I., M. N. Lehman, A. M. Seiden, H. J. Duncan, D. V. J. A. o. O. H. Smith and N. Surgery (1992). "Human olfactory biopsy: the influence of age and receptor distribution." **118**(7): 731-738.

Palmqvist, S., M. Schöll, O. Strandberg, N. Mattsson, E. Stomrud, H. Zetterberg, K. Blennow, S. Landau, W. Jagust and O. Hansson (2017). "Earliest accumulation of β-amyloid occurs within the default-mode network and concurrently affects brain connectivity." Nature communications **8**(1): 1214-1214.

Pearce, R. K. B., C. H. Hawkes and S. E. Daniel (1995). "The anterior olfactory nucleus in Parkinson's disease." Movement Disorders **10**(3): 283-287.

Perea, J. R., M. Llorens-Martín, J. Ávila and M. Bolós (2018). "The Role of Microglia in the Spread of Tau: Relevance for Tauopathies." Frontiers in cellular neuroscience **12**: 172-172.

Pixley, S. K. and R. Y. J. D. B. R. Pun (1990). "Cultured rat olfactory neurons are excitable and respond to odors." **53**(1): 125-130.

Polymeropoulos, M. H., C. Lavedan, E. Leroy, S. E. Ide, A. Dehejia, A. Dutra, B. Pike, H. Root, J. Rubenstein and R. Boyer (1997). "Mutation in the α-synuclein gene identified in families with Parkinson's disease." science **276**(5321): 2045-2047.

Polyzonis, B., P. Kafandaris, P. Gigis and T. Demetriou (1979). "An electron microscopic study of human olfactory mucosa." Journal of anatomy **128**(Pt 1): 77.

Pooler, A. M., E. C. Phillips, D. H. W. Lau, W. Noble and D. P. Hanger (2013). "Physiological release of endogenous tau is stimulated by neuronal activity." EMBO reports **14**(4): 389-394.

Prediger, R. D. S., A. S. Aguiar, F. C. Matheus, R. Walz, L. Antoury, R. Raisman-Vozari and R. L. Doty (2012). "Intranasal Administration of Neurotoxicants in

Animals: Support for the Olfactory Vector Hypothesis of Parkinson's Disease." Neurotoxicity Research **21**(1): 90-116.

Puvenna, V., M. Engeler, M. Banjara, C. Brennan, P. Schreiber, A. Dadas, A. Bahrami, J. Solanki, A. Bandyopadhyay, J. K. Morris, C. Bernick, C. Ghosh, E. Rapp, J. J. Bazarian and D. Janigro (2016). "Is phosphorylated tau unique to chronic traumatic encephalopathy? Phosphorylated tau in epileptic brain and chronic traumatic encephalopathy." Brain Research **1630**: 225-240.

Quinn, J. G., D. T. Coulson, S. Brockbank, N. Beyer, R. Ravid, J. Hellemans, G. B. Irvine and J. A. Johnston (2012). "α-Synuclein mRNA and soluble α-synuclein protein levels in post-mortem brain from patients with Parkinson's disease, dementia with Lewy bodies, and Alzheimer's disease." Brain research **1459**: 71-80.

Rambaran, R. N. and L. C. Serpell (2008). "Amyloid fibrils: abnormal protein assembly." Prion **2**(3): 112-117.

Rey, N. L., J. A. Steiner, N. Maroof, K. C. Luk, Z. Madaj, J. Q. Trojanowski, V. M.-Y. Lee and P. Brundin (2016). "Widespread transneuronal propagation of α-synucleinopathy triggered in olfactory bulb mimics prodromal Parkinson's disease." The Journal of Experimental Medicine **213**(9): 1759-1778.

Rey, N. L., D. W. Wesson and P. Brundin (2018). "The olfactory bulb as the entry site for prion-like propagation in neurodegenerative diseases." Neurobiology of disease **109**(Pt B): 226-248.

Richardson, J. T. E. and G. M. Zucco (1989). "Cognition and olfaction: A review." Psychological Bulletin **105**(3): 352-360.

Ross, C. A. and M. A. Poirier (2004). "Protein aggregation and neurodegenerative disease." Nature Medicine **10**(7): S10-S17.

Saito, Y., A. Shioya, T. Sano, H. Sumikura, M. Murata and S. Murayama (2016). "Lewy body pathology involves the olfactory cells in Parkinson's disease and related disorders." Movement Disorders **31**(1): 135-138.

Sammeta, N. and T. S. J. J. o. C. N. McClintock (2010). "Chemical stress induces the unfolded protein response in olfactory sensory neurons." **518**(10): 1825-1836.

Sanchez-Guajardo, V., A. Annibali, P. H. Jensen and M. Romero-Ramos (2013). "α-Synuclein vaccination prevents the accumulation of parkinson disease-like pathologic inclusions in striatum in association with regulatory T cell recruitment in a rat model." Journal of Neuropathology & Experimental Neurology **72**(7): 624-645.

Sano, K., R. Atarashi, K. Satoh, D. Ishibashi, T. Nakagaki, Y. Iwasaki, M. Yoshida, S. Murayama, K. Mishima and N. Nishida (2018). "Prion-like seeding of misfolded α-synuclein in the brains of dementia with Lewy body patients in RT-QUIC." Molecular neurobiology **55**(5): 3916-3930.

Schaser, A. J., V. R. Osterberg, S. E. Dent, T. L. Stackhouse, C. M. Wakeham, S. W. Boutros, L. J. Weston, N. Owen, T. A. Weissman and E. Luna (2019). "Alpha-synuclein is a DNA binding protein that modulates DNA repair with implications for Lewy body disorders." Scientific reports **9**(1): 1-19.

Schell, H., T. Hasegawa, M. Neumann and P. Kahle (2009). "Nuclear and neuritic distribution of serine-129 phosphorylated α-synuclein in transgenic mice." Neuroscience **160**(4): 796-804.

Schoenfeld, T. A. and R. A. Obar (1994). Diverse distribution and function of fibrous microtubule-associated proteins in the nervous system. International review of cytology, Elsevier. **151:** 67-137.

Selvaraj, S. and S. Piramanayagam (2019). "Impact of gene mutation in the development of Parkinson's disease." Genes & diseases **6**(2): 120-128.

Shah, A. S., Y. Ben-Shahar, T. O. Moninger, J. N. Kline and M. J. Welsh (2009). "Motile cilia of human airway epithelia are chemosensory." Science **325**(5944): 1131-1134.

Shahnawaz, M., T. Tokuda, M. Waragai, N. Mendez, R. Ishii, C. Trenkwalder, B. Mollenhauer and C. Soto (2017). "Development of a biochemical diagnosis of Parkinson disease by detection of α-synuclein misfolded aggregates in cerebrospinal fluid." JAMA neurology **74**(2): 163-172.

Sicard, G., F. Feron, J. Andrieu, A. Holley and A. J. A. o. t. N. Y. A. o. S. Mackaysim (1998). "Generation of neurons from a nonneuronal precursor in adult olfactory epithelium in vitro." **855**(1): 223-225.

Singleton, A. B., M. Farrer, J. Johnson, A. Singleton, S. Hague, J. Kachergus, M. Hulihan, T. Peuralinna, A. Dutra, R. Nussbaum, S. Lincoln, A. Crawley, M. Hanson, D. Maraganore, C. Adler, M. R. Cookson, M. Muenter, M. Baptista, D. Miller, J. Blancato, J. Hardy and K. Gwinn-Hardy (2003). "α-Synuclein Locus Triplication Causes Parkinson's Disease." Science **302**(5646): 841-841.

Sinnatamby, C. S. (2011). Last's Anatomy e-Book: Regional and Applied, Elsevier Health Sciences.

Smart, I. J. J. o. a. (1971). "Location and orientation of mitotic figures in the developing mouse olfactory epithelium." **109**(Pt 2): 243.

Soto, C. (2003). "Unfolding the role of protein misfolding in neurodegenerative diseases." Nature Reviews Neuroscience **4**(1): 49.

Soto, C. (2012). "Transmissible proteins: expanding the prion heresy." Cell **149**(5): 968-977.

Soto, C., L. Estrada and J. Castilla (2006). "Amyloids, prions and the inherent infectious nature of misfolded protein aggregates." Trends in biochemical sciences **31**(3): 150-155.

Soto, C. and S. Pritzkow (2018). "Protein misfolding, aggregation, and conformational strains in neurodegenerative diseases." Nature neuroscience **21**(10): 1332-1340.

Spillantini, M. G., M. L. Schmidt, V. M. Y. Lee, J. Q. Trojanowski, R. Jakes and M. Goedert (1997). "α-Synuclein in Lewy bodies." Nature **388**(6645): 839-840.

Struble, R. G., D. N. Dhanraj, Y. Mei, M. Wilson, R. Wang and V. Ramkumar (1998). "β-Amyloid precursor protein-like immunoreactivity is upregulated during olfactory nerve regeneration in adult rats." Brain research **780**(1): 129-137.

Surguchov, A. (2015). Chapter Four - Intracellular Dynamics of Synucleins: "Here, There and Everywhere". International Review of Cell and Molecular Biology. K. W. Jeon, Academic Press. **320:** 103-169.

Tabaton, M., S. Cammarata, G. Mancardi, G. Cordone, G. Perry and C. Loeb (1991). "Abnormal tau- reactive filaments in olfactory mucosa in biopsy specimens of patients with probable Alzheimer's disease." Neurology **41**(3): 391-391.

Talamo, B. R., R. Rudel, K. S. Kosik, V. M.-Y. Lee, S. Neff, L. Adelman and J. S. Kauer (1989). "Pathological changes in olfactory neurons in patients with Alzheimer's disease." Nature **337**(6209): 736.

Tanos, T., A. M. Saibene, C. Pipolo, P. Battaglia, G. Felisati and A. Rubio (2017). "Isolation of putative stem cells present in human adult olfactory mucosa." PloS one **12**(7): e0181151-e0181151.

Trojanowski, J. Q., P. D. Newman, W. D. Hill and V. M. Y. Lee (1991). "Human olfactory epithelium in normal aging, Alzheimer's disease, and other neurodegenerative disorders." Journal of comparative neurology **310**(3): 365-376.

Tsuboi, Y., Z. K. Wszolek, N. R. Graff-Radford, N. Cookson and D. W. Dickson (2003). "Tau pathology in the olfactory bulb correlates with Braak stage, Lewy body pathology and apolipoprotein ε4." Neuropathology and Applied Neurobiology **29**(5): 503-510.

Tu, P. h., J. E. Galvin, M. Baba, B. Giasson, T. Tomita, S. Leight, S. Nakajo, T. Iwatsubo, J. Q. Trojanowski and V. M. Y. Lee (1998). "Glial cytoplasmic inclusions in white matter oligodendrocytes of multiple system atrophy brains contain insoluble α- synuclein." Annals of neurology **44**(3): 415-422.

Tuttle, M. D., G. Comellas, A. J. Nieuwkoop, D. J. Covell, D. A. Berthold, K. D. Kloepper, J. M. Courtney, J. K. Kim, A. M. Barclay and A. Kendall (2016). "Solid-state NMR structure of a pathogenic fibril of full-length human α-synuclein." Nature structural & molecular biology **23**(5): 409.

Uéda, K., H. Fukushima, E. Masliah, Y. Xia, A. Iwai, M. Yoshimoto, D. A. Otero, J. Kondo, Y. Ihara and T. Saitoh (1993). "Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease." Proceedings of the National Academy of Sciences **90**(23): 11282-11286.

Ulmer, T. S., A. Bax, N. B. Cole and R. L. Nussbaum (2005). "Structure and Dynamics of Micelle-bound Human α-Synuclein." Journal of Biological Chemistry **280**(10): 9595-9603.

van Rumund, A., A. J. Green, G. Fairfoul, R. A. Esselink, B. R. Bloem and M. M. Verbeek (2019). "α- Synuclein real- time quaking- induced conversion in the

cerebrospinal fluid of uncertain cases of parkinsonism." Annals of neurology **85**(5): 777-781.

Vannelli, G. B., F. Ensoli, R. Zonefrati, Y.-h. Kubota, A. Arcangeli, A. Becchetti, G. Camici, T. Barni, C. J. Thiele and G. J. J. o. N. Balboni (1995). "Neuroblast long-term cell cultures from human fetal olfactory epithelium respond to odors." **15**(6): 4382-4394.

Vargas, G. and M. T. J. C. s. Lucero (1999). "A method for maintaining odor-responsive adult rat olfactory receptor neurons in short-term culture." **24**(2): 211-216.

Viereck, C., R. P. Tucker and A. Matus (1989). "The adult rat olfactory system expresses microtubule-associated proteins found in the developing brain." Journal of Neuroscience **9**(10): 3547-3557.

Wakamatsu, M., A. Ishii, Y. Ukai, J. Sakagami, S. Iwata, M. Ono, K. Matsumoto, A. Nakamura, N. Tada and K. Kobayashi (2007). "Accumulation of phosphorylated α- synuclein in dopaminergic neurons of transgenic mice that express human α- synuclein." Journal of neuroscience research **85**(8): 1819-1825.

Wales, P., R. Pinho, D. F. Lázaro and T. F. Outeiro (2013). "Limelight on alpha-synuclein: pathological and mechanistic implications in neurodegeneration." Journal of Parkinson's disease **3**(4): 415-459.

Walker, L. C. and M. Jucker (2015). "Neurodegenerative diseases: expanding the prion concept." Annual review of neuroscience **38**: 87-103.

Wang, J., B. J. Gu, C. L. Masters and Y.-J. Wang (2017). "A systemic view of Alzheimer disease—insights from amyloid-β metabolism beyond the brain." Nature Reviews Neurology **13**(10): 612.

Weiler, E. and A. I. Farbman (1998). "Supporting cell proliferation in the olfactory epithelium decreases postnatally." Glia **22**(4): 315-328.

Weingarten, M. D., A. H. Lockwood, S. Y. Hwo and M. W. Kirschner (1975). "A protein factor essential for microtubule assembly." Proceedings of the National Academy of Sciences of the United States of America **72**(5): 1858-1862.

Welge-Lüssen, A. and T. Hummel (2004). Management of smell and taste disorders, Thieme Verlag, Stuttgart.

Werner, S., E. J. J. o. O. M. Nies and Toxicology (2018). "Olfactory dysfunction revisited: a reappraisal of work-related olfactory dysfunction caused by chemicals." **13**(1): 28.

Westphal, C. H. and S. S. Chandra (2013). "Monomeric synucleins generate membrane curvature." The Journal of biological chemistry **288**(3): 1829-1840.

Whitman, M. C. and C. A. J. P. i. n. Greer (2009). "Adult neurogenesis and the olfactory system." **89**(2): 162-175.

Wilham, J. M., C. D. Orrú, R. A. Bessen, R. Atarashi, K. Sano, B. Race, K. D. Meade-White, L. M. Taubner, A. Timmes and B. Caughey (2010). "Rapid end-point quantitation of prion seeding activity with sensitivity comparable to bioassays." PLoS pathogens **6**(12): e1001217.

Winton, M. J., L. M. Igaz, M. M. Wong, L. K. Kwong, J. Q. Trojanowski and V. M.-Y. Lee (2008). "Disturbance of nuclear and cytoplasmic TAR DNA-binding protein (TDP-43) induces disease-like redistribution, sequestration, and aggregate formation." Journal of Biological Chemistry **283**(19): 13302-13309.

Witt, M., K. Bormann, V. Gudziol, K. Pehlke, K. Barth, A. Minovi, A. Hähner, H. Reichmann and T. Hummel (2009). "Biopsies of olfactory epithelium in patients with Parkinson's disease." Movement Disorders **24**(6): 906-914.

Wolozin, B., T. Sunderland, B.-b. Zheng, J. Resau, B. Dufy, J. Barker, R. Swerdlow and H. J. J. o. M. N. Coon (1992). "Continuous culture of neuronal cells from adult human olfactory epithelium." **3**(3): 137-146.

Wrobel, B. B., J. M. Mazza, O. V. Evgrafov and J. A. Knowles (2013). Assessing the efficacy of endoscopic office olfactory biopsy sites to produce neural progenitor cell cultures for the study of neuropsychiatric disorders. International forum of allergy & rhinology, Wiley Online Library.

Wyatt, T. (2014). Pheromones and animal behavior: Chemical signals and signatures, second edition.

Wysocki, C. J. and G. Preti (2004). "Facts, fallacies, fears, and frustrations with human pheromones." The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology **281A**(1): 1201-1211.

Xing, Y., A. Nakamura, T. Chiba, K. Kogishi, T. Matsushita, F. Li, Z. Guo, M. Hosokawa, M. Mori and K. Higuchi (2001). "Transmission of mouse senile amyloidosis." Laboratory investigation **81**(4): 493.

Yamada, K. and T. Iwatsubo (2018). "Extracellular α-synuclein levels are regulated by neuronal activity." Molecular neurodegeneration **13**(1): 9-9.

Ye, L., J. Rasmussen, S. A. Kaeser, A.-M. Marzesco, U. Obermüller, J. Mahler, J. Schelle, J. Odenthal, C. Krüger, S. K. Fritschi, L. C. Walker, M. Staufenbiel, F. Baumann and M. Jucker (2017). "Aβ seeding potency peaks in the early stages of cerebral β-amyloidosis." EMBO reports **18**(9): 1536-1544.

Zhou, M., S. Xu, J. Mi, K. Uéda and P. Chan (2013). "Nuclear translocation of alpha-synuclein increases susceptibility of MES23. 5 cells to oxidative stress." Brain research **1500**: 19-27.

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