Mechanism of spreading of prion and polyglutamine aggregates and role of the cellular prion protein in Huntington's disease

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"Fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza"

(Dante Alighieri, *Divina Commedia*, Inferno, Canto XXVI, 119 – 120)

Acknowledgements

I am left with the acknowledgments…and it turns out it is the most difficult part. I thought I was going to do it with a long, exhaustive list until I realized it is almost impossible to include four years of life in one or two pages.
So, I will be brief and I am certain that each of you will recognize himself in these few words.

A PhD is a unique life experience. My PhD was that!!

When I first arrived in Paris in June 2008, I was a young graduate student and it was my first experience far from family and the friends of a life. I have to admit it… it was terribly hard and I even heard somebody saying, "She will not make it". Four years later, here I am and I made it!!!

In the last four years I grew as a person at the same time that I did as a scientist. If I have to picture myself, I will say I was a kid….experiencing science and life.

I learnt a lot… from each experiment, from the successful ones and especially from the ones that failed. I learnt from the people that accompanied my growth in the lab, the ones that I have known from the very beginning and the ones that just arrived. Thank you for being there with your knowledge, help, support, encouragement and smiles.

I learnt from life and friends here in Paris… Some have a special place but all contributed to the person that I am today (good job, indeed!! ☺). Thank you for being my playmates!!

But all this would not have been possible without my rocks: my parents, my sister and my love. Their unconditional love is my *conditio sine qua non* and I know they will be with me always, taking me through life.

The end of a PhD is the end of a cycle… I am now ready for a new adventure with my backpack full!

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ABSTRACT

Abstract

The pathogenesis of most neurodegenerative diseases, including transmissible diseases like prion encephalopathies, inherited disorders like Huntington's disease, and sporadic diseases like Alzheimer's and Parkinson's diseases, appear to be directly linked to the formation of fibrillar protein aggregates. For many years, the concept of aggregate spreading and infectivity has been confined to prion diseases. However, recent evidence indicate that both extracellular (e.g. amyloid-β) and intracellular (α-synuclein, tau, huntingtin) amyloidogenic protein are able to move (and possibly replicate) within the brains of affected individuals, thereby contributing to the spread of pathology in a prion-like manner (Brundin et al., 2010; Jucker and Walker, 2011; Aguzzi and Rajendran, 2009). Recently another intriguing connection has been made between prions and other aggregation proteinopathies, as it was suggested that the cellular prion protein, PrPC, whose pathological counterpart is responsible for prion diseases, possibly mediates the toxicity of Aβ, the pathogenic protein in Alzheimer's disease, and of other β-conformers independently of the propagation of infectious prions (reviewed in Biasini et al., 2012). However, despite the intense research, many questions in prion and non-prion neurodegenerative diseases are still open regarding both the mechanism of protein aggregate spreading and the mechanism of toxicity.

In the first part of my thesis, I contributed to investigate the role of DCs (dendritic cells) in the spreading of prion infection to neuronal cells. I demonstrated that the transfer of PrPSc from DCs (loaded with prion infected brain homogenate) to primary neurons was triggered by direct cell–cell contact and resulted in transmission of infectivity to the co-cultured neurons. These data confirm the possible role of DCs in prion spreading from the periphery to the nervous system. I also provided a plausible transfer mechanism of PrPSc through tunneling nanotubes (TNTs) shown to connect DCs to primary neurons and excluded the involvement of PrPSc secretion in our system.

In the second part of my thesis, I investigated the mechanisms of the spreading and toxicity of Htt aggregates and the possible role of PrPC in these events. I demonstrated that Htt aggregates transfer between neuronal cells and primary neurons and that cell-cell contact is required. I also showed the involvement of

TNTs in the transfer and reported the aggregation of endogenous wild-type Htt in primary neurons, possibly following the transfer of Htt aggregates. Finally, the last part of my results provides evidences that PrPC is involved in the spreading of the toxicity mediated by mutant Htt in primary neuronal cultures.

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List of Abbreviations

Aβ: Amyloid-β
AD: Alzheimer disease
BDNF: Brain derived neurotrofic factor
BMDC: Bone Marrow Dendritic cell
BSE: Bovine spongiform encephalopathy
CJD: Creutzfeldt-Jakob disease
CNS: Central nervous system
CWD: Chronic Wasting Disease
DC: Dendritic cell DNA: deoxyribonucleic acid
fCJD: familial Creutzfeldt-Jakob disease
FDC: follicular dendritic cell
FFI: Fatal familial insomnia
FSE: feline spongiform encephalopathy
GALT: gut associated lymphatic system
Gnd-HCl: Guanidine hydrochloride
GSS: Gerstmann-Sträussler-Scheinker syndrome
GPI: glycosylphophatidylinositol
GTP: guanosine triphosphate
HIV-1: human immunodeficiency virus 1
HD: Huntington disease
Htt: Huntingtin
IBs: inclusion bodies
iCJD: iatrogenic Creutzfeldt-Jakob disease
IHC: immunohistochemistry
kDa: kilo Dalton
LDL: low-density lipoprotein
M: molar
mRNA: messenger RNA µ: micron
NaOH: sodium hydroxide
NH4Cl: ammonium chloride
nm: nanometer
nvCJD: see vCJD
PD: Parkinson disease
PIPLC: phosphatidylinositol-specific phospholipase C
PNC: peripheral nervous system
PK: proteinase K
PMCA: protein misfolding cyclic amplification
polyQ: polyglutamine
Prion: proteinaceous infectious particles

PrPC: cellular (i.e. wild type) prion protein
PrPSc: scrapie (i.e. infectious) prion protein
ROS: reactive oxygen species
SDS: sodium dodecyl sulfate
sCJD: sporadic Creutzfeldt-Jakob disease
TGN: trans-Golgi-network
TME: transmissible mink encephalopathy
TSE: transmissible spongiform encephalopathy
TNT: tunneling nanotubes
UK: United Kingdom
USA: United States of America
vCJD: variant Creutzfeldt-Jakob disease
WB: Western Blot

INTRODUCTION

1. Neurodegenerative diseases: protein conformational disorders

In 1997 Carrel and Lomas put forward the concept that many disorders, which appeared to be unlinked, arose from the same general mechanism that involved the abnormal folding and aggregation of various proteins (Carrell and Lomas, 1997). Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and prion disorders are characterized by progressive accumulation of protein aggregates in selected brain regions. In each disorder, aggregates results from the misfolding of a specific protein or proteins: amyloid-β and tau in Alzheimer's disease, α-synuclein in Parkinson's disease, huntingtin in Huntington's disease and the prion protein (PrP) in prion disorders (reviewed in Ross and Poirier, 2004). Although the disease-associated proteins are very diverse in their primary sequence, they all share a common property: they are soluble and benign for decades but misfold and assemble in amyloid-like deposits in aged neurons.

1.2 Commonalities in amyloid structure

Depending on the disease, amyloid assemblies can be intranuclear, cytoplasmic or extracellular, but they all have a similar tertiary structure, known as cross-β spine or amyloid, that consists of an ordered arrangements of β-sheets (Lührs et al., 2005; Sunde et al., 1997) . Indeed, amyloid deposits are typically composed of 6-10 nm cross-β fibrils in which the polypeptide chain arranged in β-sheets is perpendicular to the long axis of the fibrils and hydrogen bonding is parallel (Figure 1A and B). Originally, the term amyloid was used exclusively to describe extracellular amyloid deposits (i.e., amyloid-β) that could be stained with histological dyes such as Congo red (Figure 1C). Now it is recognized that many cytoplasmic (i.e., synuclein) (Vilar et al., 2008) and even intranuclear inclusions (i.e., polyglutamine huntingtin) (Thakur and Wetzel, 2002; Ross et al., 2003), which do not necessarily stain with these dyes, are composed of ordered fibrillar structures enriched in β-sheets, similar to those of "classical" amyloids. Thus, there seem to be considerable

similarities among the structures of different kinds of disease-related amyloids.

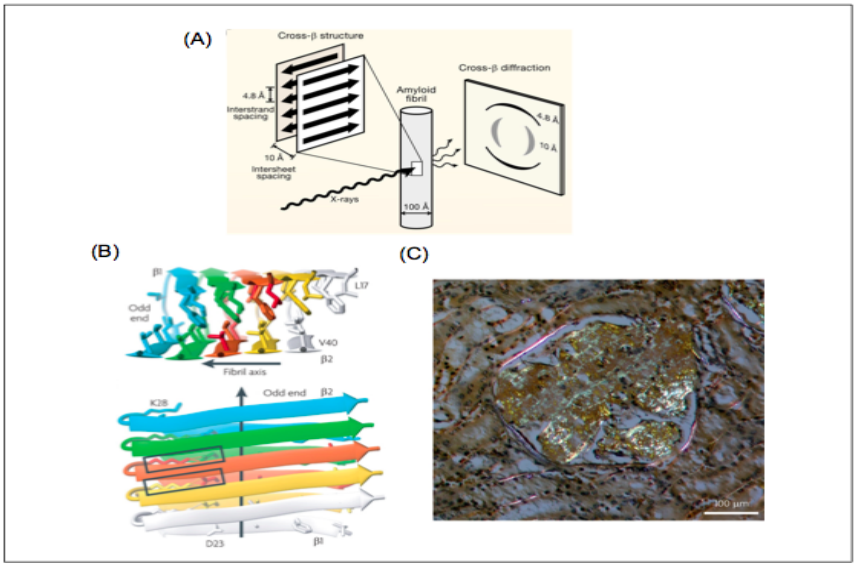

Figure 1 Properties of amyloid fibers. Amyloid-forming proteins are all thought to form similar tertiary structures when aggregated, known as a cross-β spine. (A) The characteristic cross-β diffraction pattern observed when X-rays are directed on amyloid fibers reveals an intersheet spacing of 10 Ä (horizontal) and an interstrand spacing of 4,8 Ä (vertical).(B) Ribbon diagrams of the three-dimensional structure of amyloid-β (Aβ42) (residues 17–40). The cross-β spine consists of an ordered arrangement of β-sheets (thick coloured arrows). (C) Congo red-stained sections of human kidney affected by amyloidosis. The cross-β spine structure is able to intercalate with molecules of the azo-dye Congo red and cause them to emit a characteristic apple-green birefringence upon exposure to polarized light163. This unique feature of amyloids has historically been used to identify them histologically. *Modified from Eisenberg and Jucker, 2012 and Aguzzi and O'Connor, 2010*

1.3 The mechanism and intermediates of protein misfolding

Fibril formation requires considerable structural rearrangements need and implies misfolding of the related native proteins. Protein misfolding can occur because of several reasons (reviewed in Moreno-Gonzalez and Soto, 2011) (i) somatic mutations in the gene sequence leading to the production of a protein unable to adopt the native folding; (ii) errors in the processes of transcription or translation leading to the production of modified proteins unable to properly fold; (iii) failure of the folding and chaperone machinery; (iv) mistakes in the post-translational modifications or trafficking of proteins; (v) structural modification produced by environmental changes and finally (vi) induction of protein misfolding by seeding and cross-seeding mechanisms. The most frequent destiny for misfolded proteins is self-aggregation because the exposure to the solvent of

fragments that are normally buried inside the protein leads to a high degree of stickiness. The β-sheet structural motif offers the most favorable organization for these intermolecular aggregates (Nelson et al., 2005). As result misfolded proteins exist as a large and heterogeneous continuum of polymeric sizes, from soluble oligomers to disordered aggregates to fibrils (ordered aggregated species) (reviewed in Caughey and Lansbury, 2003). Soluble oligomers are small assemblies of misfolded proteins that are present in the soluble fraction of tissue extracts and usually include structures ranging in size from dimers to 24-mers (reviewed in Glabe, 2006). The mechanism of protein misfolding and aggregation follows the so-called "seeding-nucleation" model (reviewed in Soto et al., 2006). In this process, the initial steps of misfolding are thermodynamically unfavorable and progress slowly, until the minimum stable oligomeric unit is formed, and then grows exponentially at a fast speed. There are two kinetic phases in the seeding-nucleation model of polymerization (Figure 2).

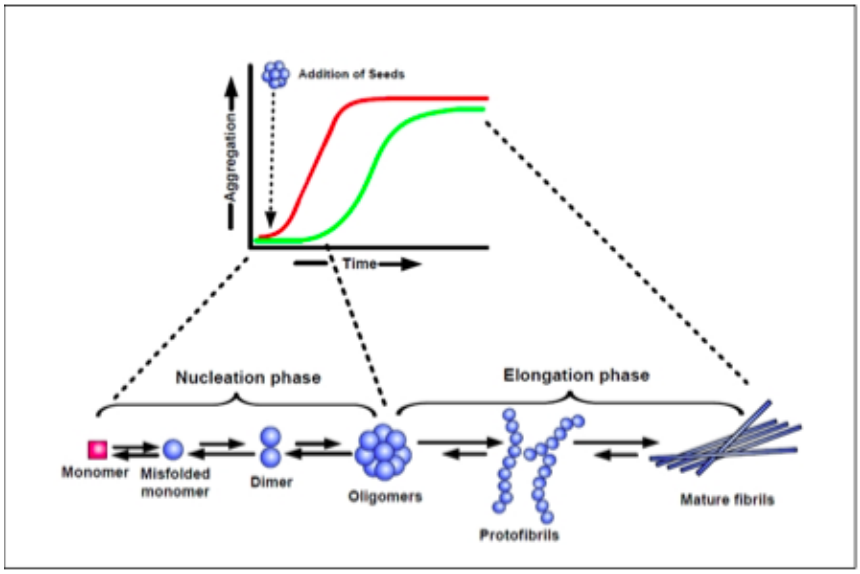

Figure 2 Nucleation-dependent polymerization model of amyloid aggregation. Amyloid formation consists of two phases: (i) a nucleation phase/lag phase, in which monomers undergo conformational change/misfolding and associate to form oligomeric nuclei, and (ii) a elongation phase/growth phase, in which the nuclei rapidly grow by further addition of monomers and form larger polymers/fibrils until saturation. The 'nucleation phase', is thermodynamically unfavourable and occurs gradually, whereas 'elongation phase', is much more favourable process and proceeds quickly. Thus, kinetics of amyloid formation is well represented by a sigmoidal curve with a lag phase followed by rapid growth phase (green curve). The rate limiting step in the process is the formation of nuclei/seeds to promote aggregation. Thus, amyloid formation can be substantially speedup by the addition of preformed seeds (nuclei). The addition of seeds reduces the lag time and induces faster aggregate formation (red curve). *From Kumar and Walker, 2011*

Firstly, during the lag phase, a low amount of misfolded and oligomeric structures are produced in a slow process, generating seeds for the next step. Once nuclei are formed, the elongation phase takes place and results in fast growing of the polymers. The addition of pre-formed seeds can reduce the length of the lag phase, accelerating the exponential phase. Oligomers are perhaps the best seeds to propagate the misfolding process in an exponential manner. However, larger structures like fibrils could be as important to propagate this event *in vivo*, due to their higher resistance to biological clearance than smaller aggregates (reviewed in Moreno-Gonzalez and Soto, 2011).

1.4 A brief overview of some neurodegenerative diseases

In this paragraph, some neurodegenerative diseases are briefly described.
Huntington's disease and prion disorders, also mentioned here, will be described and discussed more extensively in chapter 2 and 3 (prion diseases) and in chapter 5 (Huntington's disease) as they are related to the subject of my PhD work.

1.4.1 Alzheimer's disease

Alzheimer's disease (AD) is a late-onset dementing illness, with progressive loss of memory, task performance, speech, and recognition of people and objects (Thies and Bleiler, 2011). There is degeneration of neurons particularly in the basal forebrain and hippocampus. AD involves two major kinds of protein aggregates. Extracellular aggregates known as neuritic plaques contain as their major constituent the amyloid-β peptide (Aβ), which is derived from proteolytic processing of the amyloid precursor protein (APP) (Figure 3). The Aβ-containing aggregates have β-sheet structure and Congo red reactivity characteristic of amyloid (Thies and Bleiler, 2011). There are also intracellular aggregates of the microtubule-associated protein tau, called neurofibrillary tangles (Selkoe, 2001). The pathogenesis of AD has been greatly clarified by the identification of genetic mutations responsible for rare familial forms of the disease. These mutations occur in APP gene itself and also in the

presenilin genes, which are involved with the cleavage of APP (Goate, 1997).

1.4.2 Parkinson's disease

Parkinson's disease (PD) is characterized by resting tremor, rigidity, slow movements and other features such as postural and autonomic instability. It is caused by degeneration of dopaminergic neurons in the substantia nigra of the midbrain and other monoaminergic neurons in the brain stem (Forno, 1996). The discovery of several genes in which mutations cause early-onset forms of PD has greatly accelerated research progress (Dawson and Dawson, 2003). Point mutations or increased gene dosage of the α-synuclein gene cause autosomal dominant PD via a gain-of-function mechanism. Recessive early-onset PD can be caused by mutations in the genes encoding parkin, DJ-1 or PINK1 (Valente et al., 2004), presumably by a loss-of-function mechanism. The pathological hallmark of adult-onset PD is the Lewy body, an inclusion body found in the cytoplasm of neurons, often near the nucleus (Figure 3). Lewy bodies are densest in the substantial nigra but can also be present in monoaminergic, cerebral cortical and other neurons. Aggregates can be found also in neurites, which are referred to as Lewy neurites. A major constituent of Lewy bodies is aggregated alpha-synuclein protein. Lewy bodies can also be labeled for ubiquitin, a synuclein interactor termed synphilin-1, proteasome proteins, and cytoskeletal and other proteins (Ross and Poirier, 2004).

1.4.3 Huntington's disease

Huntington's disease (HD) is a progressive neurodegenerative disorder caused by expansion of a CAG repeat coding for polyglutamine, in the N-terminus of the huntingtin protein (Htt) (HD collaborative group, 1993). Because it is caused by a mutation in a single gene, HD has emerged as a model for studying neurodegenerative disease pathogenesis (Ross and Poirier, 2004). There is a remarkable threshold effect, in the length of the CAG expansion able to cause the disease with polyglutamine stretches of 36 in huntingtin causing disease, whereas 35 do not. In addition, within the expanded range, longer repeats cause earlier onset. Furthermore, the threshold for aggregation in vitro correlates well with the threshold for disease

in humans, consistent with the idea that Htt aggregation is related to pathogenesis (Davies et al., 1997; Scherzinger et al., 1999). Indeed, inclusions containing huntingtin are present in regions of the brain that degenerate. However, the neurons with inclusions do not correspond exactly to the neurons that degenerate. For instance, inclusions are present in the striatum, which is most affected (Vonsattel et al., 1985), but they are more enriched in populations of large interneurons, which are spared, than in medium spiny projection neurons, which are selectively lost (Kuemmerle et al., 1999). Nevertheless, there is a good correlation between the length of the CAG repeat and the density of inclusions (Vonsattel et al., 1985; Kuemmerle et al., 1999; Myers et al., 1988; Becher et al., 1998; Gutekunst et al., 1999). Huntingtin aggregates can be labeled with antibodies against the N-terminus of huntingtin or antibodies anti ubiquitin, a marker for misfolded proteins, and a signal for proteasomal degradation (Figure 3). Defect in the proteasome functions might lead to their accumulation (Venkatraman et al., 2004). The huntingtin aggregates contain fibers and appear to have β-sheet structure characteristic of amyloid (Davies et al., 1997).

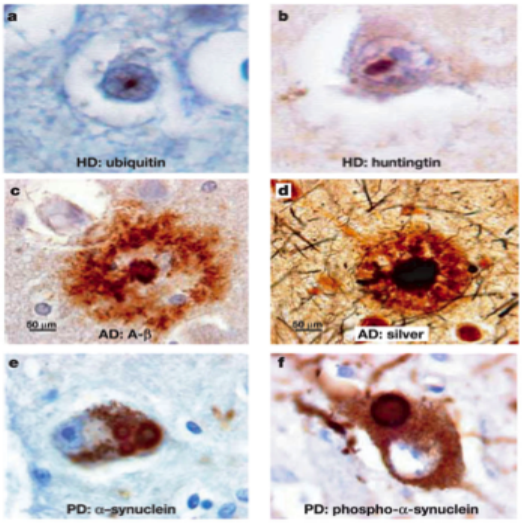

Figure 3 Characteristic neuropathological lesions involve deposition of abnormal proteins, which can be intranuclear, cytoplasmic or extracellular. (a) HD, intranuclear inclusion labeled for ubiquitin (cerebral cortex) (b) HD, intranuclear inclusion labeled for huntingtin (cerebral cortex). (c) AD, neuritic plaque labeled for Abeta (cerebral cortex). (d) AD, neuritic plaque, silver stained. (e) PD, Lewy bodies labeled for alpha-synuclein (fine granular brown label in this and the next panel represent neuromelanin) (substantial nigra). (f) PD, Lewy body labeled for phosphorylated alpha-synuclein (substantia nigra). *Modified from Ross and Poirier, 2004*

1.4.4 Prion disease

Neurodegenerative diseases caused by prions can be sporadic or acquired either by environmental transmission or via genetic mutations (Prusiner, 1998). Environmental pathways include eating prion particles derived from infected brain tissue or surgical implantation by contaminated instruments. Prion disease can also be caused by point mutations in the gene of PrPC, the cellular prion protein, leading to its alterations and misfolding. Pathology can include amyloid plaques that appear similar to those of AD and that can be labeled with PrP antibodies. Prion disease is a prototypical protein conformation disease, in that highly sophisticated studies have shown that it is caused by abnormal protein structure and not by an infective viral agent. Prion aggregation can take place both extracellularly and intracellularly (Ma et al., 2002; Ma and Lindquist, 2002)

2. Infectious amyloids: the prion diseases

Transmissible spongiform encephalopathies (TSE) also known as Prion diseases, are fatal neurodegenerative disorders present both in human and animals which can occur genetically, spontaneously or by infection (Prusiner 1998). Among neurodegenerative diseases, prion disorders are unique because they are infectious, meaning that transmission of the pathology occurs among individuals and across species (reviewed in Aguzzi and Calella, 2009). It is now widely accepted that the infectious agent consists of proteinacious aggregates, called 'prions', derive from a conformational change of a native protein, the cellular protein (PrPC), into its pathological counterpart, PrPSc (Scrapie prion protein) (Prusiner 1998). The nature of the agent has been highly debated for many years (Collinge, 2001). Alper and Griffith developed the theory that some transmissible spongiform encephalopathies were caused by an infectious agent consisting solely of proteins (Alper and Griffith 1967). This hypothesis was formulated to explain the fact that the mysterious infectious agent causing scrapie in sheep and goats and Creutzfeldt-Jacob (CJD) disease in humans resisted to ionizing radiation and nucleases treatments, thus excluding the possibility that the it had a viral origin (Alper and Griffith 1967)(Alper et al., 1967). As a follow up, in 1982 Stanley B. Prusiner and its team at the University of California in San Francisco purified the infectious agent claiming that it consisted only of proteins. They named it "prions" from "proteinaceous infectious particles" that are "resistant to inactivation by most procedures that modify nucleic acid' (Prusiner 1998; Nobel Price in Medicine in 1997). Over the years, compelling evidence has accumulated to support the "protein-only" hypothesis (Prusiner, 1998) that states that the infectious agent associated with TSE is a self-propagating protein in an aberrant or 'misfolded' conformation (Diaz-Espinoza and Soto, 2012). An important proof for this theory was provided by the study of Weissmann and co-workers, which showed that PrP knockout mice are completely resistant to prion infection (Büeler et al., 1992). Consistently, all inherited cases of prion diseases are linked with mutations in the prion protein gene (*PRNP*) and usually have an earlier onset and more severe phenotype than the sporadic forms (Aguzzi and Calella, 2009). Other supporting evidence came from experiments showing that transgenic mice expressing human PrP mutations develop spontaneous diseases

that can be transmissible to wild-type animals with some clinical and neuropathological features of the associated human disease (Jackson et al., 2009; Sigurdson et al., 2009). Furthermore, PrPSc is able to induce misfolding of PrPC in vitro by cyclic amplification of protein misfolding (PMCA), resulting in generation of prion infectious material in the test tube in the absence of living cells (Castilla et al., 2005). One argument often used against the protein only hypothesis is the existence of many phenotypic TSE variants, termed prion strains, with typical features, such as incubation period, clinical signs, characteristic pattern of neuropathological lesions, and specific PrPSc biochemical features, a phenomenon difficult to reconcile with an exclusively proteinaceous infectious agent (Soto and Castilla, 2004). However, recent findings have shown that strain properties can be reproduced during in vitro replication by PMCA, suggesting that all elements enciphering prion strains are encoded in the PrPSc structure (Castilla et al., 2008).

2.1 Human and animal prion diseases: a brief overview

As already mentioned above, transmissible spongiform encephalopathies (TSE) affect both human and animals. They can occur genetically, spontaneously or by infection (Prusiner 1998) (Table1).

Disease	Host	Mechanism of pathogenesis
Kuru	Humans (Fore people)	infection through ritualistic cannibalism
Iatrogenic CJD	Humans	infection from prion-contaminated HGH
Variant CJD	Humans	infection from bovine prions
Familiar CJD	Humans	germline mutations in the PRNP gene
GSS	Humans	germline mutations in the PRNP gene
FFI	Humans	germline mutations in the PRNP gene
Sporadic CJD	Humans	somatic mutation or spontaneous conversion of PrPC into PrPSc
sFI	Humans	matic mutation or spontaneous conversion of PrPC into PrPSc
Scrapie	Sheep	Infection
BSE	Cattle	Infection or sporadic
TME	Mink	Infection with prions from sheep or cattle
CWD	Deer and elk	Infection
FSE	Cats	infection with prion-contaminated bovine tissues or MBM
Exotic ungulate encephalopathy	greater kudu, nyala, oryx	infection with prion-contaminated MBM

Table 1 Human and animals prion diseases

Both animal and human conditions share common histopathological features that include spongiform vacuolation of the gray matter, astrogliosis and neuronal loss with deposition of amyloid plaques (Beck et al., 1982) (Figure 4).

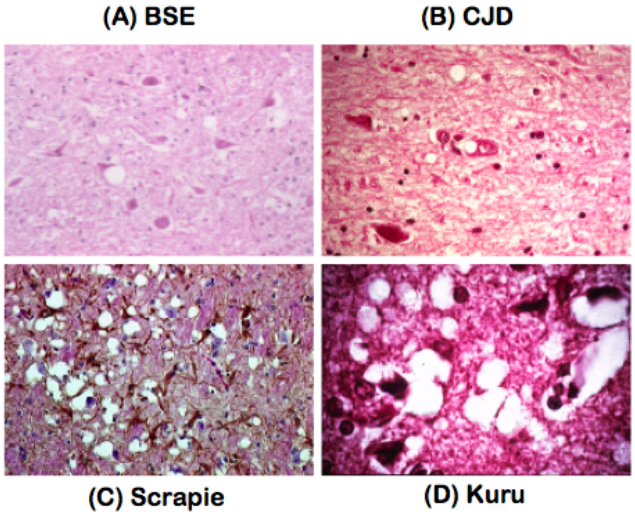

Figure 4 Histopathological features associated with TSEs showing spongiform degeneration and astrocytic gliosis . Analysis of grey matter from brain sections of (A) a BSE-infected cow, (B) an individual affected from CJD, (C) sheep and (D) kuru-affected individual. Modified from http://www.biophys.uni-duesseldorf.de/research/prions/index.html

Also, no infiltration of lymphocytes and macrophages has been detected due to the absence of the immune response (Collinge 2001). TSE are characterized by a long pre-symptomatic period followed, after the appearance of the first symptoms, by a rapid progression that leads inevitably to death. Specific clinical signs are associated with each type of TSE but all include perturbations of the locomotor and sensory system, lack of coordination and progressive dementia (Collinge 2001).

2.1.1 Animal

Prion diseases occur in many animals and more frequently as infectious disorders (Table 1). The most known are scrapie in sheep and goat, bovine spongiform encephalopathy (BSE) in cattle, transmissible mink encephalopathy (TME) (Marsh et al., 1991), chronic wasting disease (CWD) of mule deer and elk (Williams & Young 1980) and the more recently described feline spongiform encephalopathy (Wyatt et al., 1991).

- Scrapie

Scrapie is the prototype of prion diseases. Its name originates from the main clinical symptom, an itching sensation caused by the disease that induces the animal to scrape its fleece off (Figure 2). The work of Cuillé and Chelle in 1936 provided the demonstration that scrapie can be transmitted to goats upon injection with scrapie infected brain homogenate. (La maladie dite tremblante du mouton est-elle inocuable? C. R. Acad. Sci. 203, 1552-1554.). Since then, scrapie has effectively been transmitted experimentally into sheep (Gordon, 1946) and other species including laboratory mice (CHANDLER, 1961), demonstrating that it can cross the 'species barrier' and it is currently used as model in prion research. To date, scrapie has never been shown to pose a threat to human health (Brown and Bradley, 1998).

- Bovine Spongiform encephalopathy

BSE, also known as 'Mad Cow disease' ('la vache folle'), has raised the attention of the public for the first time in 1986 in Great Britain where it appeared like an epidemic disease in which nearly one million cows were infected with prions (Anderson et al., 1996). Clinical symptoms include changes in temperament and movement disorders. Since the incubation time for BSE is around 5 years, infected cattle slaughtered at 2 or 3 years of age were in a pre-symptomatic phase and therefore not recognized as afflicted by BSE (Stekel et al., 1996). The disease is caused by meat and bone meal (fed primarily to dairy cows) derived from offal of sheep, cattle (probably affected by a rare sporadic BSE), pigs and chickens as they represent high sources of nutrients (Wilesmith et al., 1991; Nathanson et al., 1997). Changes in the feeding system eradicated the epidemic that reached its peak in 1992, but sporadic cases can still arise occasionally (Colby and Prusiner, 2011).

Brain extracts derived from prion-infected cows can transmit the disease to mice, cattle, sheep and pigs after intracerebral inoculation (Dawson et al 1990a; Dawson et al 1990b; Fraser et al., 1988 Aguzzi and Calella 2009). More importantly, and differently from scrapie, BSE can be transmitted to humans resulting in a new variant of the disease, vCJD, by ingestion of contaminated food (see below, section 2.1.2). In 1994, the first cases of vCJD in teenagers and young adults occurred in Britain (Will et al., 1996) and in 1996 one case presenting unusual neuropathological features that did not match with CJD cases (Prusiner 1998), was recognized in France (Chazot et al., 1996).

2.1.2 Human

Human prion diseases, traditionally classified into Creutzfeldt-Jakob disease (CJD), Gerstmann-Straüssler-Scheinker disease (GSS) and Kuru, have been subsequently divided into three etiological categories: sporadic, acquired, and inherited (Table 1).

- Sporadic prion diseases

The sporadic forms (sCJD) were the first to be described by Creutzfeldt-Jakob in 1920. They are the most frequent among CJD forms, accounting for 80 to 90% of the cases, and present worldwide without sexual preference with an annual incidence of one per million (de Pedro-Cuesta et al 2006). The causes of sCJD are not known and probably derive from a spontaneous misfolding of PrPC into PrPSc (Hsiao et al., 1989; Prusiner, 1989). Alternatively, it has been proposed that the disease could be due to a somatic mutation of the prion protein gene, *PRNP*, or infrequent amplification of low levels of PrPSc that are part of "normal" protein homeostasis (Colby and Prusiner 2011). Susceptibility to sCJD disease is influenced by a polymorphism at residue 129 of *PRNP* (Meade, 2006) and homozygosity predisposes not only to sporadic but also the acquired forms of CJD.

The typical onset of the disease is at ~ 60 years old which quickly progresses in 4-5 months leading to death (Johnson and Gibbs, 1998). The pathology is limited to the central nervous system, where neuronal loss occurs with progressive vacuolization in the absence of amyloid plaques (Spero and Lazibat, 2010). Infected sCJD brains can transmit the disease to experimental animals by intracerebral injection (Brown et al., 1994).

- Inherited prion diseases

Around 15% of human prion disease is inherited and in all cases to date over 40 different mutations in *PRNP* are associated with genetic forms of prion disease (Maeda et al 2006). According to the clinical symptoms, they have been classified as Gerstmann-Straüssler-Scheinker syndrome (GSS) (MASTERS et al., 1981), familiar (f) CJD and fatal familiar insomnia (FFI) (Lugaresi et al., 1986).

The first reports of *PRNP* mutations described insertion and missense mutations in families with dominantly inherited neurodegenerative diseases (Owen et al., 1989; Hsiao et al., 1989). Over 40 different types of *PRNP* mutations have been found and *PRNP* analysis allows for pre-symptomatic diagnosis of

inherited prion disease (Collinge, 2005). They include point mutations leading to amino acid substitutions or premature stop codons mostly affecting the region between the second and the third helix of the carboxy-terminus and octapeptide repeat insertions (OPRI) (Figure 5).

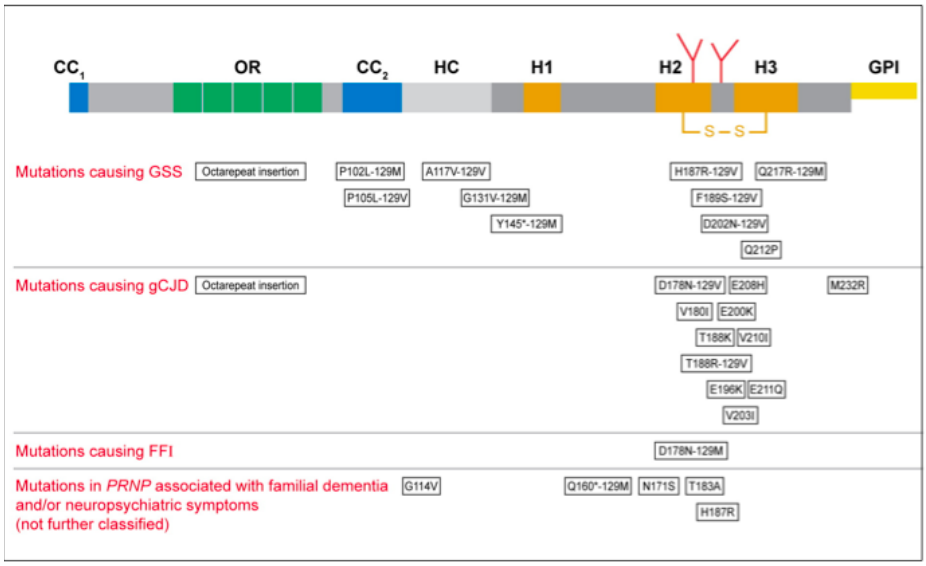

Figure 5 The human PrPC protein and its mutants. The mature human PrPC protein contains 208 amino acid residues. It features two positively charged amino acid clusters denoted CC1 and CC2 (blue boxes), an octapeptide repeat region (OR) (green boxes), a hydrophobic core (HC) (gray box), three α-helixes (H1-H3) (red boxes), one disulphide bond (S–S) between cysteine residues 179 and 214, and two potential sites for N-linked glycosylation (red forks) at residues 181 and 197. A glycosylphosphatidylinositol anchor (GPI) (yellow box) is attached to the C-terminus of PrP. This figure indicates in black framed boxes point mutations and insertions found in the human *PRNP* gene in patients with prion disease. The associated polymorphisms of codon 129 (methionine M or valine V) are indicated. Amino acids are given in single-letter code. The asterisk indicates a stop codon; therefore, this mutation results in a truncated protein. *From Aguzzi et al 2008*

The pathology of this group of prion diseases can vary depending on the actual mutation, as well as on the polymorphisms at codon 129, that represent a key determinant of genetic susceptibility to acquired and sporadic prion diseases (Collinge 2001). Also, given the heterogeneity in clinical signs, the effects of unidentified cellular modifiers and environmental factors should be taken into account (Kovacs and Budka, 2008).

- Acquired prion diseases

Infectious forms of prion diseases include kuru, iatrogenic CJD (iCJD) and variant CJD (vCJD).

Kuru was firstly described in the '60s by Gajdusek and Zigas (Gajdusek and Zigas, 1957) as an endemic disease among some aborigines tribes of New Guinea, particularly in the Fore Tribe and

neighboring tribes. The route of transmission was attributed to the cannibalistic rituals through ingestion of the brains of their dead relatives in an attempt to immortalize them. The typical progression for kuru is progressive cerebellar ataxia, evolving in few months with a very broad incubation period of 4 to 40 years. With the end of cannibalism in Papua New Guinea, kuru is now eliminated (Aguzzi et al., 2008).

Iatrogenic CJD is a rare form of prion disease deriving from accidental transmission during the course of medical or surgical procedures. In 1974, the first case of iCJD caused by corneal transplantation of a graft derived from a patient suffering from sCJD was reported (Duffy et al., 1974). Later on, other routes of transmission derived from prion-tainted human growth hormones and gonadotropin, dura mater grafts and blood transfusion were also reported. The incubation period ranges between 1 and 15 years and death occurs around 15 months from the onset of the symptoms (Colby and Prusiner, 2011; Prusiner, 1998).

Among the infectious forms of prion diseases, the variant form (vCJD) is the one that has caught the attention of the public the most. Indeed, in 1996 a major epidemic of vCJD appeared in different countries, particularly in the UK, where the number of reported cases had the highest incidence (about 150) (Will et al., 1996). Patients are generally young at the onset of the disease (average onset is at 29 years), have a significant longer disease course, present florid plaque deposits (vacuolization) in the brain and are homozygous for methionine at position 129 in the *PRNP* gene that suggests a genetic susceptibility for vCJD. Interestingly, in experimentally infected mice, prions from patients with vCJD and prions from BSE-cattle gave similar pathological and biochemical characteristics (i.e., incubation period and localization in brain), leading researchers to conclude that the most likely cause for vCJD in humans is the consumption of BSE-contaminated food (Bruce et al., 1997; Hill et al., 1997) . A single case of vCJD in a patient heterozygous at codon 129 has also been reported, raising the possibility of a second wave (with late onset) of “mad cow”–related deaths (Kashi et al 2009).

2.2 Prion protein gene

The prion protein gene (*PRNP*) belongs to the *PRN* gene family that consists of *PRND*, encoding the Doppel protein (Moore et al.,

1999), and *SPRN*, encoding Shadoo (Watts and Westaway, 2007). *PRNP* is located in the short arm of the chromosome 20 in humans and in a homologous region in mouse chromosome 2 (Sparkers et al 1986). The open-reading frame (ORF), responsible for the transduction of the PrPC protein, resides in a single exon in all known mammalian prions and avian genes *PRNP* (Basler et al 1986) (Westaway et al., 1987). However, the gene itself comprises two to three exons that contain untranslated sequences including the promoter and termination sequence (Hsiao et al., 1989; Gabriel et al., 1992). The PrP promoter contains multiple copies of GC-rich repeats that represent a well-known binding site for the transcription factor Sp1 site driving expression in many different tissues (McKnight and Tjian, 1986). *PRNP* transcript is constitutively expressed in different tissues and especially within the brain of different animals, but is highly regulated during development (Chesebro et al., 1985; Oesch et al., 1985). In addition, *PRNP* mRNA does not increase during the course of prion disease (Oesch et al 1985).
Furthermore, high levels of similarities in the *PRNP* sequence have been found by aligning more than 40 translated sequences from different species (Colby and Prusiner 2011). This highlights the importance of PrPC protein functions and explains why the gene has been conserved through evolution. However, variations in PrP sequences exist both between species and between individuals within species (Figure 6), thus affecting their susceptibility to prion, as mentioned above in section 2.1.2.

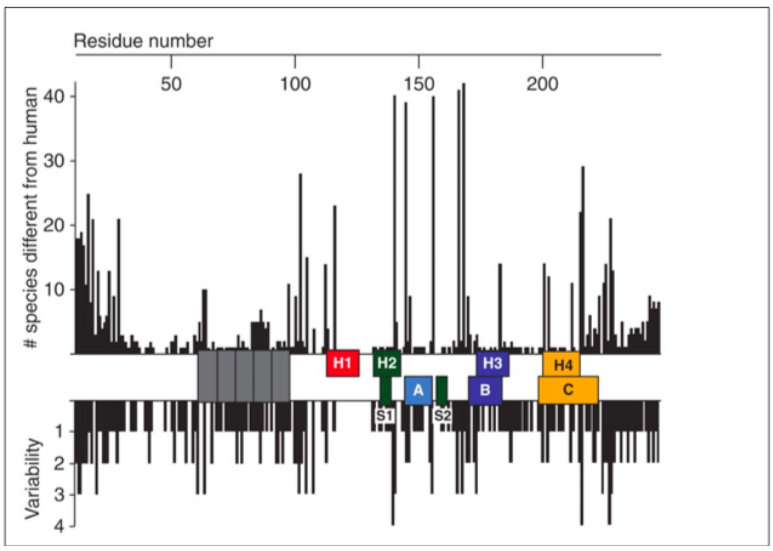

Figure 6 Variation of in the prion protein gene. Species variations of the prion protein gene. The x-axis represents the human PrP sequence, with the five octarepeats and H1–H4 regions of the putative secondary structure shown, as well as the three α-helices A, B, and C and the two β-strands S1 and S2 as determined by

NMR. Vertical bars above the axis indicate the number of species that differ from the human sequence at each position. Below the axis, the length of the bars indicates the number of alternative amino acids at each position in the alignment. *From Colby and Prusiner, 2011*

Knock-out mice for *PRNP* gene (as *Prnp* 0/0, Zürich I and *Prnp* -/-, Edinburgh) have been generated from different laboratories (Bueler et al 1992; Manson et al 1994). These mice are vital, do not show particular signs of alterations and develop normally. In contrast, other mice models ablated of *PRNP* (Ngsk *Prnp* 0/0 and Rcm0 *Prnp* 0/0) did show some dysfunction, afterwards attributed to abnormal expression of Doppel and due to the technique used to engineer these mice (Sakaguchi et al., 1996; Moore et al., 1999). But, in agreement with the 'prion-only' hypothesis all these mice are resistant to prion infection (Aguzzi et al 2008).

2.3 The cellular prion protein: structure and function

PrPC is a ubiquitous glycoprotein expressed early in embryogenesis and present in high levels in the central nervous system in adult, particularly in neurons but also in glial cells (Manson et al 1992; Harris et al 1993; Moser et al 1995; Ford et al 2002). PrPC normally localizes on the extracellular leaflet of the plasma membrane where it associates with cholesterol-enriched lipid rafts. In neurons, PrPC is predominant in axons and dendrites (Mironov et al., 2003). It seems to be excluded from synaptic vesicles but present within the synaptic specialization and perisynaptically, so its role at the level of the synapse is still controversial (Fournier et al., 1995; Laine et al 2002; Vassallo and Herms, 2003). In addition, PrPC is widely expressed in the immune system, in hematopoietic stem cells and mature lymphoid and myeloid compartments (Isaacs et al., 2006). Also, many other tissues and organs like the spleen, intestines, the skin, muscles and the heart have been found positive for PrPC expression. The PrPC precursor is a protein of 254 amino acids (Figure 7A).

After cleavage of a 22 amino acids signal peptide in the endoplasmic reticulum (ER), a glycosylphosphatidilinositol (GPI) anchor, which mediates its anchoring to the membrane, is attached to the C-terminus of the protein (Stahl et al., 1987). The two Cys residues 179 and 214 are engaged in the formation of a disulphide bond essential for the stability of the protein. The protein exists as un-, mono- or di-glycosylated, as one or two

oligosaccharidic chains can be linked to two asparagines (N) (residues 181 and 197 in humans) in the C-terminal part of PrPC in the Golgi apparatus during the journey of the protein to the plasma membrane.

At the structural level, PrPC has a long, flexible N-terminal tail (residues 23-128) that is present in all the animal species studied (Prusiner 1998). It contains an octarepeat region (OR) consisting of 5 repeats of the sequence PHGGGWGQ (major binding site for divalent cations), a basic charged region (CC) important for PrPC trafficking and an hydrophobic domain (HR) that can be used by PrPC to assume different transmembrane topologies. Indeed, PrPC presents at least three distinct topological orientations: the fully extracellular form (or (sec)PrP) which is GPI anchored and two transmembrane isoform (called Ntm-PrP and Ctm-PrP) with opposite sequence orientations with respect to the lumen of the endoplasmic reticulum (Nicolas et al., 2009). Following the unstructured N-terminus is a globular C-terminal domain consisting of three α-helices interspersed with two-stranded antiparallel β-sheets that flank the first α-helix. (Figure 7B) (Zahn et al., 2000; Hornemann et al., 2004).

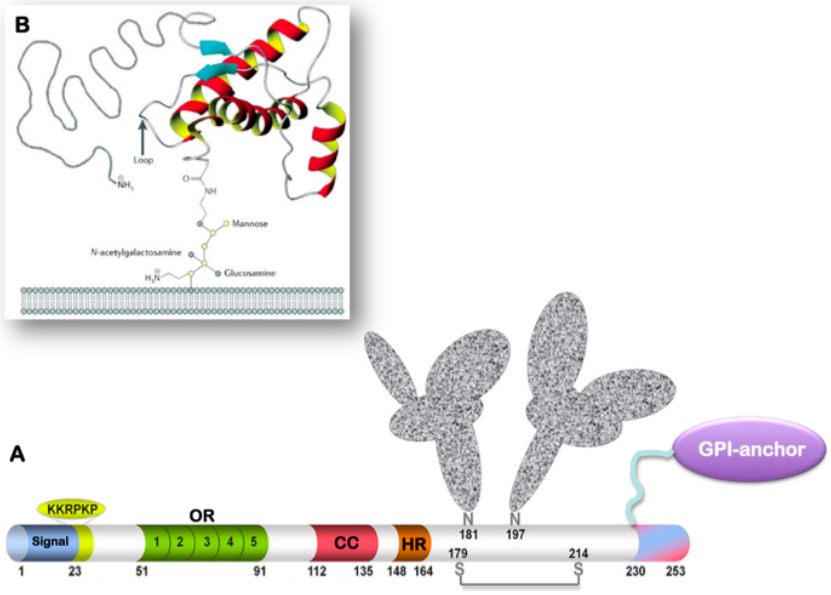

Figure 7 (A) PrPC primary structure and (B) tertiary structure of PrPC. The numbers describe the position of the respective amino acids. CC (red) defines the charged cluster. HR (orange) defines the 'hydrophobic region'. S-S indicates the single disulfide bridge. OR, octarepeat region. *Modified from Lewis and Hooper 2011 and Aguzzi and Heikenwalder 2006*

The structure of several mature PrPC proteins in mice, cattle, humans and Syrian hamsters is very similar (Lysek et al., 2005;

Calzolai et al., 2005), thus suggesting a relevant evolutionary conserved function for this protein.

A plethora of cellular functions have been attributed to PrPC but as already mentioned above its physiological role appears to be redundant, since PrP knock-out mice are vital and do not present severe abnormalities (Bueler et al 1992; Manson et al., 1994a). However, a growing number of studies implicate PrPC in diverse cellular processes (Nicolas et al., 2009) as cellular resistance to oxidative stress (Milhavet and Lehmann, 2002), cell signalling (Mouillet-Richard et al., 2000), copper and zinc metabolism (Pauly and Harris, 1998; Watt and Hooper, 2003), synaptic transmission (Collinge et al., 1994) and cytoprotection through anti-apoptotic activity (Kuwahara et al., 1999; Bounhar et al., 2001). Recently, it has been shown that PrPC is required for the maintenance of myelin sheath around peripheral nerves (Bremer et al., 2010; Benvegnù et al., 2011). In addition, a role for PrPC as cellular receptor of oligomeric forms of amyloid-β, mediating its toxic effect in Alzheimer's disease, has been described by Lauren and co-workers (Lauren et al., 2009). In contrast, other reports have shown that amyloid-β toxicity is independent from PrPC (Balducci et al., 2010; Calella et al., 2010; Kessels et al., 2010). Therefore, its role in Alzheimer's disease is still controversial (see paragraph 6.5). Besides, it has been shown that PrPC is implicated in cell adhesion (Málaga-Trillo et al., 2009), focal adhesion formation and filopodia extention (Schrock et al 2008) . These findings point out towards an additional role of PrPC in cytoskeleton dynamic and remodeling and cell-to-cell communication.
The identification of interacting partners of PrPC is of fundamental importance not only to provide new insights into its role in physiological conditions but also to better understand the basic mechanism of PrPC-PrPSc conversion that leads to neuropathology (see paragraph 6.4). In a recent report, a series of interacting partners for PrPC has been found by using a proteomics approach (Zafar et al., 2011). The results have confirmed 15 interacting partners already shown to interact with both PrPC and PrPSc but 28 new proteins were also identified. A functional categorization of these proteins (Figure 8) confirmed many of the assigned roles for PrPC in highlighting its multi-faceted functionality and involvement as a biological platform for diverse cellular processes.

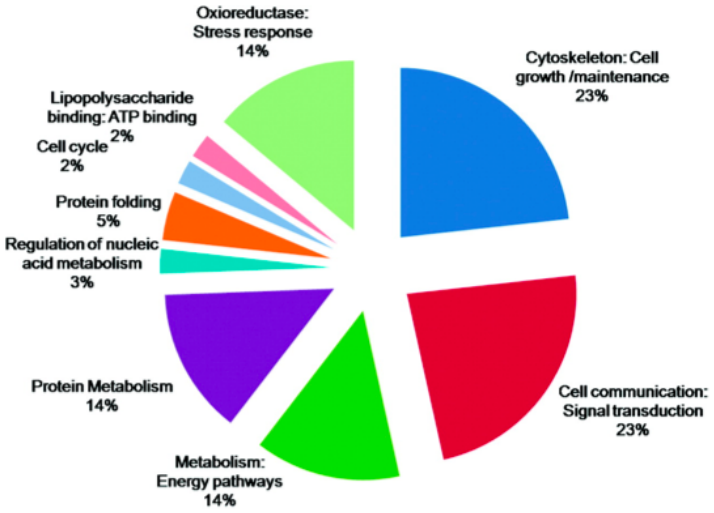

Figure 8 Functional categorization of putative PrPC binding partners. *Modified from Zafar et al 2011.*

2.4 Biochemical and structural properties of PrPSc

Purified full-length PrPSc is insoluble in non-ionic detergents and is partially resistant to proteolytic cleavage. Indeed, proteinase K treatment removes a fragment of about 12 kDa from the N terminus of PrPSc (Parchi et al., 1996) leaving a protease-resistant core that retains infectivity (Cronier et al., 2008) and is referred to as PrP res orPrP27-30 because of the apparent size of the monomer in western blots. Limited protease digestion has been a convenient tool to detect PrPSc because the same treatment fully hydrolyzes the cellular protein PrPC thus allowing the discrimination between the two forms (Figure 9A and B). Furthermore, these observations suggest that the N-terminal region of PrP (up to around amino acid 90) is not essential for self-propagation. Experiments using transgenic mice expressing different PrP truncations confirmed that the minimal region required for sustaining PrPSc *in vivo* propagation starts from residue ~90 all the way up to the C-terminal part of PrP (Supattapone et al., 1999). Also, it has been shown that cathepsin D digestion of the C-terminus of PrPSc and liberation of the glycosylphosphatidylinositol (GPI) anchor results in a fragment that retains prion infectivity (Lewis et al., 2006). Moreover, recently, transgenic mice expressing PrPC lacking a GPI anchor, which normally attaches PrP to the membrane (see paragraph above 2.3), can propagate prions and produce

infectious anchorless PrPSc that is mostly non-glycosylated (Chesebro et al., 2005), thereby suggesting that both GPI anchor and glycans are not a prerequisite component of the infectious prion.

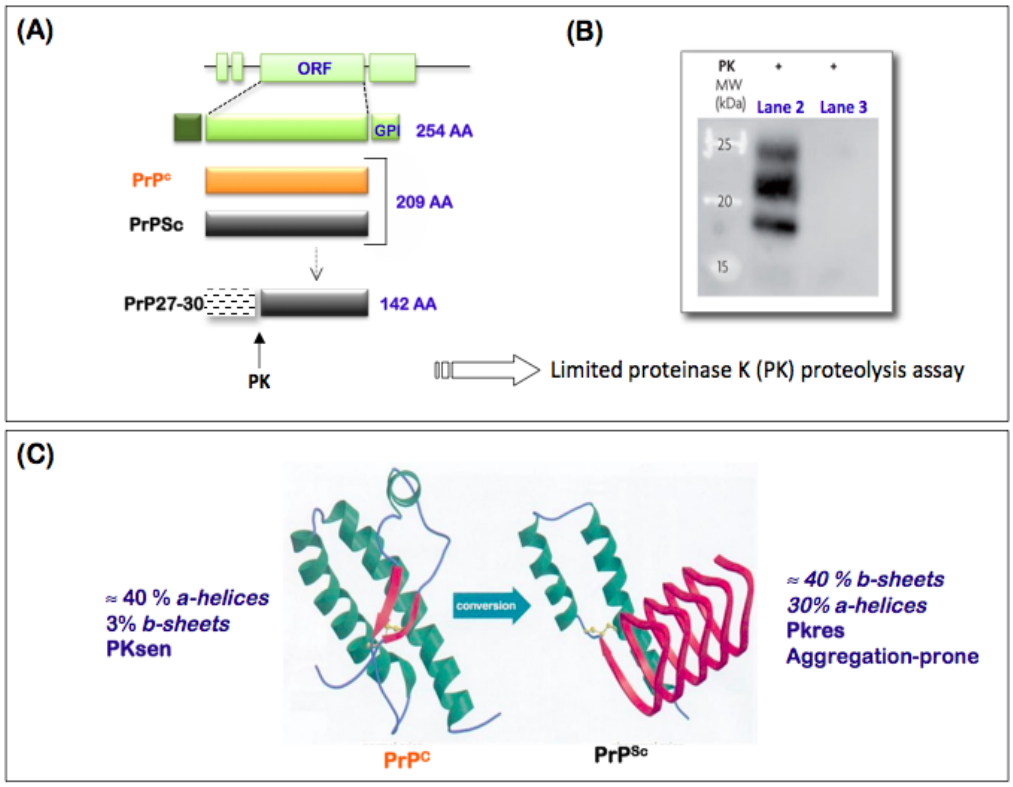

Figure 9 (A) Schematic representation of hamster *Prnp* gene and PrP isoforms. The Prnp ORF encodes a protein of 254 residues, which is shortened to 209 residues during posttranslational processing. PrPSc is an alternate conformation of PrPC with identical primary structure. Limited proteolysis of PrPSc cleaves the amino terminus and produces PrP 27-30, composed of approximately 142 residues. (B) Western blotting of cell lysates from prion-infected (lane 2) and uninfected (lane 3) CAD cells. Samples in lanes 2 and 3 were digested with 50 µg/µl proteinase K for 30 min at 37°C, completely hydrolyzing PrPC , thus allowing to discriminate between the two isoforms of PrP. Blot developed with anti-PrP monoclonal antibody Sha31. *Modified from Prusiner 2004.* (C) Schematic representation of the structures of PrPC and PrPSc (Picture from http://healthmad.com/conditions-and-diseases/scientists-suspect-a-spontaneous-prion-brain-in-contact-with-the-metal/)

Unlike PrPC, which can be readily cleaved from membranes by treatment with phosphatidylinositol-specific phospholipase C (PIPLC) (Stahl et al., 1987), PrPSc is resistant to such treatment (Caughey et al., 1990; Borchelt et al., 1993) suggesting that a conformational change prevents accessibility of PIPLC. Therefore, limited proteolysis and biochemical techniques also provided structural information on PrPSc. As an alternative, several groups have used low-resolution biophysical techniques to gather

structural information on PrPSc because for high-resolution techniques like X-ray crystallization and NMR the properties of prion aggregates pose serious challenges, which are similar for most amyloids.

Initial structural studies by Fourier Transform Infrared Spectroscopy (FTIR) and circular dichroism have demonstrated that unlike PrPC, which is predominantly α-helical, PrPSc isolated from diseased brains is highly enriched in β-sheets (45% compared to 3% in PrPC) (Gasset et al., 1992; Pan et al., 1993) (Figure 9C). Transmission electron microscopy (TEM) (McKinley et al., 1991; Merz et al., 1981) and more recently atomic force microscopy (Sim and Caughey, 2009) studies revealed that brain-isolated PrPSc molecules usually appear as amorphous aggregates of heterogeneous sizes. Upon exhaustive purification procedures, including prolonged protease treatment, the aggregates acquire more defined structures called prion rods. Rods are typically in the range of 10- to 100-nm long and 5-nm wide and are usually shorter than classical amyloid fibrils (Colby et al., 2009). Interestingly, PrPSc deposits are stained with congo red and show green-gold birefringence, typical of amyloids (Prusiner et al., 1983) (see chapter 1, paragraph 1.2 and figure 1). Although PrPSc isolated from the brain of diseased animals does not form crystals amenable to X-ray crystallography, low-resolution diffraction patterns can be obtained by X-ray fiber diffraction (Eanes and Glenner, 1968). This technique has been widely used to study the organization of amyloids, and has revealed the typical motif called cross–β-sheet (see chapter 1 paragraph 1.2 and figure 1). Indeed, a similar motif was identified in PrPSc (PrP27-30) with a typical meridional 4.72 Å cross–β-reflection, whereas the equatorial 10 Å reflection, typical of amyloids, was replaced by 8-Å signal (Wille et al., 2009). Overall, these data suggest that PrPSc has a structure with cross–β-packing similar to that of amyloid fibrils. On the basis of the informations obtained from low-resolution biophysical studies, several structural models of PrPSc have been proposed in the last decade. Among them the β-helix, the β-spiral and the exended in-register β-sheet model (Figure 10). An important difference in these models is the structural fate of the C-terminal domain, which is globular in PrPC, with well-defined and stable α-helices (Figure 7B). In both the β-helical and the β-spiral models, the C-terminal domain retains most of its structure upon misfolding (Figure 10A and B) whereas in the extended in-register-β-sheet model, the entire protein refolds into a mainly β-sheet

conformation, thus explaining the high resistance to proteolytic degradation of the C-terminal part of PrPSc buried inside the polymer (Figure 10C). It is difficult to determine which of these

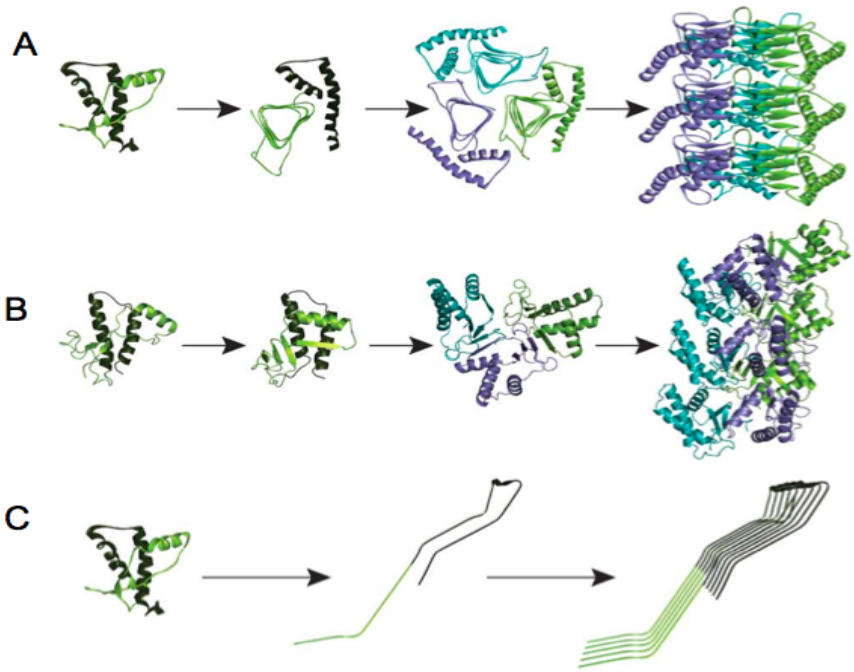

Figure 10 Alternative models proposed for the structure of PrPSc. (A) The β-helical model. (B) The β-spiral model. (C) The parallel in-register extended β-sheet model. The C-terminal region (residues 178–230) is depicted in dark green in the three models. *From Diaz-Espinoza and Soto, 2012.*

models is a closer representation of the PrPSc structure due to the lack of high-resolution biophysical experiments. Furthermore, although the structure of the self-propagating infectious agent is unknown, recent studies have demonstrated that small PrP oligomers of 14-28 molecules are more infective then monomeric or fibrillar PrP (Silveira et al., 2005; Tixador et al., 2010). More recent reports have described the isolation of partially detergent-soluble infectious PrPSc oligomers that are markedly more protease sensitive than classical prions (Pastrana et al., 2006). Solving the structure of PrPSc will provide a major step in understanding how proteins can propagate biological information.

2.5 Prion replication

Two different conformational conversion models have been proposed to explain the phenomenon of prion replication: the 'template-directed refolding' model (Prusiner, 1998) and the 'seeded nucleation' model (Jarrett and Lansbury, 1993). In the 'template-directed refolding' PrPC to PrPSc conversion would occur through "instructions" given by PrPSc to PrPC in order to change the structure of the latter in to the pathological conformer of the protein (Figure 11A). On the other hand, the 'seeded nucleation' model proposes that PrPSc could exist together with PrPC with the equilibrium shifted towards PrPC under physiological conditions. However, the intrinsic instability of PrPSc could lead to aggregation of this conformer in more stable 'seeds' that are prone to incorporate other monomers, thus shifting the equilibrium towards an accumulation of the pathological isoform PrPSc (Jarrett and Lansbury, 1993; Soto et al., 2006; Caughey et al., 2009) (Figure 11B). Most likely prion replication follows the seeding-nucleation model and the spontaneous (unseeded) formation of PrPSc, which would be thermodynamically unfavourable, may explain the low frequency of sporadic disease.

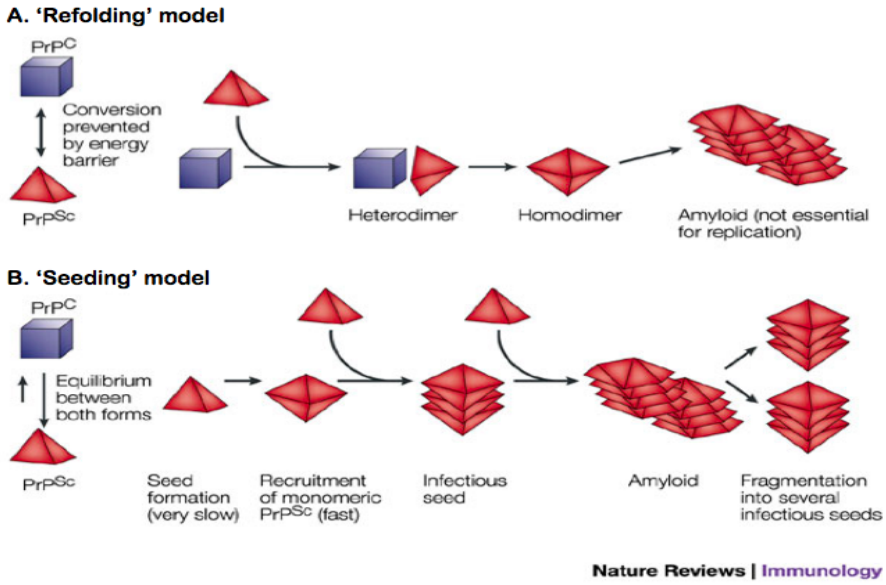

Figure 11 Model of prion replication. (A) The 'refolding' or template-directed assistance model postulates an interaction between exogenously introduced disease-associated prion protein (PrPSc) and endogenous cellular prion protein (PrPC), which is induced to transform itself into more PrPSc. A high-energy barrier might prevent the spontaneous conversion of PrPC to PrPSc. (B) The 'seeding' or nucleation–polymerization model proposes that PrPC and PrPSc are in a reversible thermodynamic equilibrium. So, only if several monomeric PrPSc molecules are mounted in a highly ordered seed can more monomeric PrPSc be recruited and eventually aggregate to form amyloid. In such a crystal-like seed, PrPSc becomes stabilized. Fragmentation of PrPSc aggregates increases the number of nuclei, which can recruit more PrPSc, and so seems to result in replication of the agent. In sporadic prion disease, fluctuations in the local PrPC concentration might (exceptionally rarely)

trigger spontaneous seeding and self-propagating prion replication. *From Aguzzi et al 2001*

However, a precise knowledge of both PrPC and PrPSc structural features is necessary to support one or the other hypothesis.
Recently, the development of an efficient prion-replication system in vitro, termed the protein misfolding cyclic amplification (PMCA) assay had become a powerful tool to provide information on the nature of the infectious agent (Castilla et al., 2008; 2005). In this system, prions are replicated by mixing minute amounts of brain homogenates containing PrPSc with healthy brain homogenates harboring PrPC. The replication of PrPSc can be amplified exponentially, as PrPSc polymers are fragmented by sonication, multiplying the number of seeds for conversion (Soto et al., 2006). Importantly, the newly converted PrPSc has physicochemical properties identical to those of brain-derived PrPSc and is also highly infectious in wild-type animals (Castilla et al., 2005). PMCA allows faithful replication of prion strain properties (Castilla et al., 2008), including complex characteristics such as species barrier (see below pararagraph 2.6) (Figure 12).

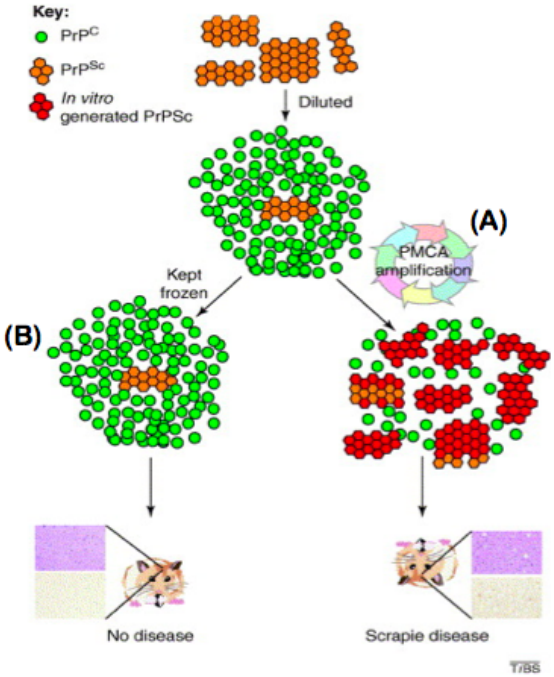

Figure 12 In vitro generation of infectious prions. Subjecting a solution of highly diluted brain-derived PrPSc in an excess of PrPC to many cycles of protein misfolding cyclic amplification (PMCA) resulted in amplification of the amount of PrPSc at the expense of the normal protein (A) . When the in vitro generated PrPSc was inoculated into wild-type hamsters, all of the hamsters developed a disease with clinical, histological and biochemical characteristics typical of scrapie. Control hamsters

inoculated with the original diluted material (B) without amplification remained free of the disease. *From Soto et al 2006*

Nevertheless, the use of brain homogenates limits the usefulness of the PMCA assay in understanding the mechanism of prion replication.

It has been long postulated the possibility that one or more additional factors (generally termed as protein X) are required for the conversion process (Prusiner 1998). Indeed, incubation of purified PrPC and PrPSc does not allow prion replication (Saborio et al 1999) and addition of the bulk of cellular protein, restore the conversion process (Saborío et al., 1999), therefore providing direct evidence that other factors present in the brain are essential to catalyze prion propagation (Soto et al., 2002). Supattapone and colleagues generated infectious prions by using purified PrPC from healthy brains as substrate for PMCA with the addition of synthetic polyanions and the presence of co-purifying lipids (Deleault et al., 2007) . Recently, Wang and co-workers have reported the formation of infectous prions from recombinant PrP in the presence of synthetic lipids and RNA (Wang et al., 2012; Kim et al., 2010). Altogether, these findings clearly indicate that non-protein components participate in prion replication, at least in vitro, pointing towards a role for polyanionic molecules. Indeed, negatively charged molecules (particularly nucleic acids, lipid particles and heparin sulfate proteoglycans) have long been proposed as PrP partners during conversion (Deleault et al., 2007; Cordeiro and Silva, 2005; Caughey, 1994). Cofactor molecules can influence PrP misfolding through at least two different mechanisms (Diaz-Espinoza and Soto, 2012). In the first model, the cofactor may act as a catalytic molecule that binds both the normal and misfolded PrP forms and brings them together, lowering the activation energy for the conversion process (Figure 13A). Upon binding, the cofactor may also induce conformational changes in PrPC and/or PrPSc that facilitate the interaction and conversion process. In the second model, the infectious PrPSc conformation would be stabilized by the cofactor (Figure 13B).

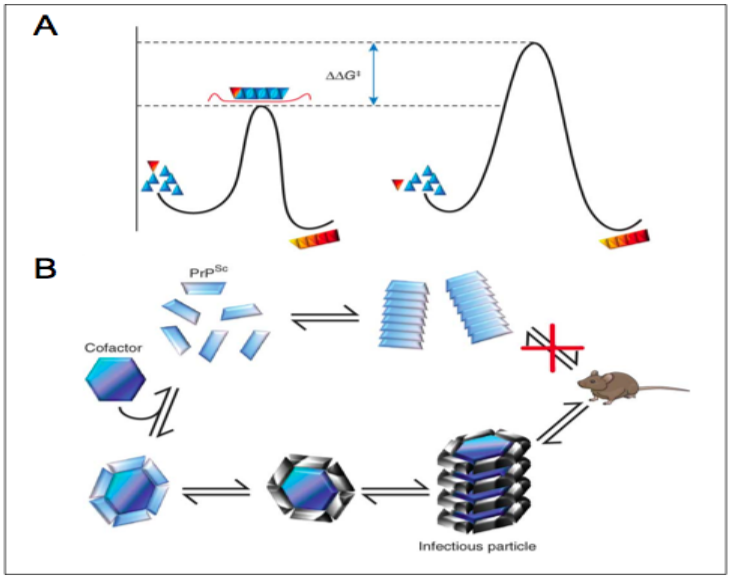

Figure 13 Potential roles of non-PrP cofactor molecules during conversion of PrPC into PrPSc. (A) Template-based conversion of PrPC (blue triangles) into PrPSc (red triangles) requires surpassing a large energetic barrier that may preclude efficient misfolding during experimental timescales. In the presence of certain cofactor molecules (red line), the conversion will be greatly enhanced by reduction in the free energy of activation (ΔΔG‡), as in typical surface-catalyzed chemical reactions. (B) The formation of an infection-competent misfolded PrP conformation depends on permanent binding of a cofactor molecule (blue hexagon) to PrPSc, leading to the stabilization of this structure. The resulting complex is able to propagate and produce disease upon *in vivo* transmission, whereas in the absence of this molecule, PrPSc-only aggregates (blue trapezoids) are unable to propagate *in vivo*. From

In biological terms, the main difference is whether the cofactor is a molecule provided by the host or a component of the infectious particle. In the latter case, the infectious agent would not be 'protein-only' but rather would consist of a complex between PrPSc and the cofactor. Indeed, PrPSc-templated conversion of pure PrPC by PMCA in the presence of light-cleavable nucleotides generated infectious PrP that showed no differences in titer and strain properties when the nucleotides were hydrolyzed after conversion (Piro et al., 2011), suggesting that polyanions act during conversion and do not need to be part of the infectious agent. It is therefore likely that polyanionic molecules act as two-dimensional catalytic scaffolds that efficiently gather PrPC and PrPSc, increasing the likelihood of conversion (Cordeiro, 2005). Finally, though many molecules can be found associated with PrPSc particles, no specific molecules are present in high quantity in the infectious material and amyloid forms of PrP generated from solely recombinant protein can induce transmissible neurodegenerative disease upon inoculation into transgenic mice that overexpress PrP (Colby et al., 2009; Legname et al., 2004)

In addition, differences in the amino acidic sequence can influence the conversion efficiency (Scott et al., 1989) . Also different levels of PrPC can be directly proportional to the rate of PrPSc formation and inversely correlated to the length of the incubation time (Bueler et al 1994). In some cases, the conversion process itself is impaired, a phenomenon known as 'transmission barrier' (see below in paragraph 2.6).
Taken together, these findings support the 'protein-only' hypothesis and highlight the strong identity of the different prion strains that characterized by their diverse biochemical, structural and biological properties (Castilla et al 2008). Still, the lack of high-resolution structural data makes it impossible to rule out the stabilizing role of a cofactor as an integral part of the infectious agent.

2.6 Strains and transmission barrier

Prion strains represent one of the most intriguing features of prion diseases. They are defined as infectious isolates that, when transmitted to identical host, exhibit distinct prion-disease phenotypes that are maintained unaltered for several passages (Aguzzi and Calella 2009). Phenotypic traits associated with different strains include distinct patterns of protein aggregate deposition, incubation times, histopathological lesion profiles and specific neuronal targets. The phenomenon was first noticed when goats where inoculated with "hyper" and "drowsy" isolates from sheep. Indeed, two different phenotypic traits of the disease were observed accordingly with the inoculated isolate derived from infected animals with characteristic disease-associated traits (Pattinson and Millson 1961). On SDS-PAGE, prion strains exhibit specific migration profiles of PrPSc fragments following PK-assay highlighting their conformational diversity (Parchi et al 1999). Also, they can be associated with different glycosylation patterns resulting in different ratios between the glycosylated forms (Prusiner 1998; Collinge 2001). Both PrPC and PrPSc exist in three different glycosylated forms: unglycosylated, mono-glycosylated and di-glycosylated. For example, PrPSc fraction in immunoblots of brain extracts after digestion with PK deriving from individuals affected by variant CJD lead to a specific glycosylation pattern (type 4 pattern), similar to the one given by bovine spongiform encephalopathy-(BSE) affected brains and different to the one deriving from

sporadic CJD and iatrogenic CJD (Type 1, 2 or 3 patterns) (Hill et al., 2000; Parchi et al., 1999) (Figure 14).

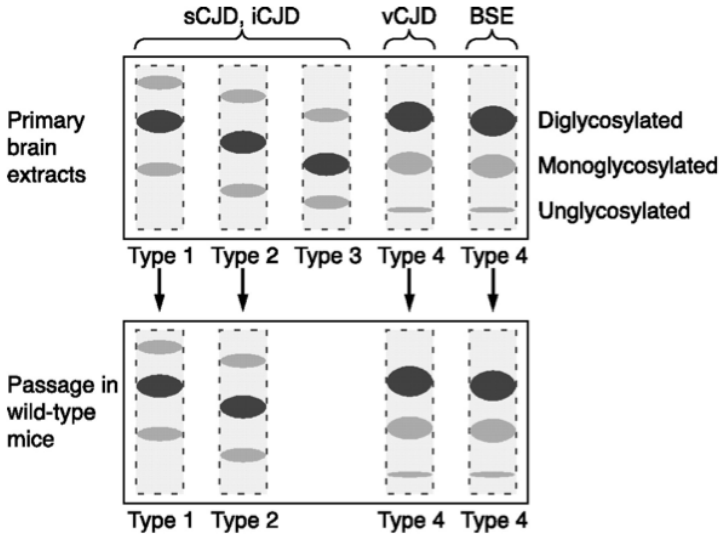

Figure 14 Representation of the three glycosylated PrPSc moieties (un-, mono-, and diglycosylated PrPSc) in immunoblots of brain extracts after digestion with proteinase K. Different inocula result in specific mobilities of the three PrP bands as well as different predominance of certain bands (top panel). These characteristic patterns can be retained, or changed to other predictable patterns after passage in wild-type mice (bottom panel). On the basis of the fragment size and the relative abundance of individual bands, three distinct patterns (PrPSc types 1–3) were defined for sCJD and iCJD cases. In contrast, all cases of vCJD and of BSE displayed a novel pattern, designated as type 4 pattern. *From Aguzzi et al 2009*

It has been proposed that the prevalence of distinct glycoforms may determine the structure of infectious PrP seeds and thereby determine strain properties (Collinge, 2005). However, transmission electro microscopy studies (TEM) analyses do not show appreciable differences between distinct strains (Diaz-Espinoza and Soto, 2012). Instead, sedimentation velocity experiments have shown that size-distribution patterns differ between distinct strains, and the size of the polymers tends to correlate with infectivity properties (Tixador et al., 2010). A plausible interpretation is that prion strain differences lay within unique secondary and/or tertiary structural elements that give rise to strain-specific quaternary arrangements upon *in vivo* spreading (Diaz-Espinoza and Soto, 2012). Therefore, the size distribution of a particular strain will be faithfully recovered upon injection and replication even with low amounts of highly disrupted starting material (Diaz-Espinoza and Soto, 2012). Recent work in mouse models indicate that unique prion strains correlate with the sensitivity of the associated fibrils to in vitro denaturation with less stable prions able to replicate and kill the

host more rapidly (Legname et al., 2006). Prion strains display different organ tropisms. Some of them preferentially propagate in the central nervous system, as bovine prion causing BSE and some others are also detected in secondary lymphoid organs as many scrapie and vCJD strains (Aguzzi and Calella 2009). Yet, this different tropism suggests that cell-specific co-factors, such as RNA species, chaperones or lipids, are required for replicating prion in different physiological environment (Aguzzi and Calella 2009).

'Strain mutations' are also observed upon transmission of prions to the same species carrying a different polymorphism in *PRNP*, or to different species (Wadsworth et al., 2004; Bruce, 1993). Also, many of the inoculated animals have a delay in developing or do not develop the disease (Carlson et al., 1989; Telling et al., 1995, 1994; Tateishi et al., 1996) . This phenomenon is referred to as the 'transmission barrier' and was first noted by Ian Pattison in 1965 (Colby and Prusiner, 2011).

It seems that the most important factor regulating the transmission barrier is the sequence homology between PrPC in the inoculum and PrPC expressed by the host. In fact, mice resistant to a different species prion strain became susceptible to the infection if artificially expressing PrPC of that species (Prusiner et al., 1990). For example, transmission studies of human prion diseases have shown that while classical CJD prions may be efficiently transmitted to transgenic mice expressing human PrPC, they encounter a significant barrier for transmission to wild-type mice. On the other hand, vCJD prions transmit readily to wild-type mice, whereas their transmission to transgenic mice expressing human PrPC is relatively inefficient (Collinge 2001; Collinge and Clarke, 2007).

3. Invasion and Spreading: PrPSc lethal journey to the brain

TSEs as variant of Creutzfeldt-Jacob disease, scrapie or chronic wasting disease can be acquired from consumption of contaminated food. Understanding how exposure to TSE agents, present in the environment, leads to invasion and spreading to the brain of a particular host is of fundamental importance in many different aspects of prion diseases, including the control of the infection, diagnosis, prophylaxis and identification of therapeutic approaches.

From several studies, it is now well accepted that prion infection starts mainly with the uptake of prions by the alimentary tract or through scarification of gums, skin and conjunctiva (Beekes and McBride, 2007). It is interesting to note that the spreading of prions may also depend on their site of entry, strain and species, as well as dose and PrPC genotype of the host (Kovacs and Budka, 2008). Despite the number of variables involved in prion spreading, from substantial data present in the literature reviewed in great detail by Beekes and McBride (2007), it is possible to dissect the routing of TSE agents through the body in precise characteristic stages, summarized in Figure 15.

Particularly: (A) accumulation of prions in lymphoid tissues; (B) neuroinvasion, consisting in the spread from the lymphoid tissues to the peripheral nervous system (PNS); (C) dissemination within the brain and spinal cord (central nervous system, (CNS)) and, (D) centrifugal spread from the CNS to further peripheral sites such as muscles (Beekes and Mc Bride 2007).

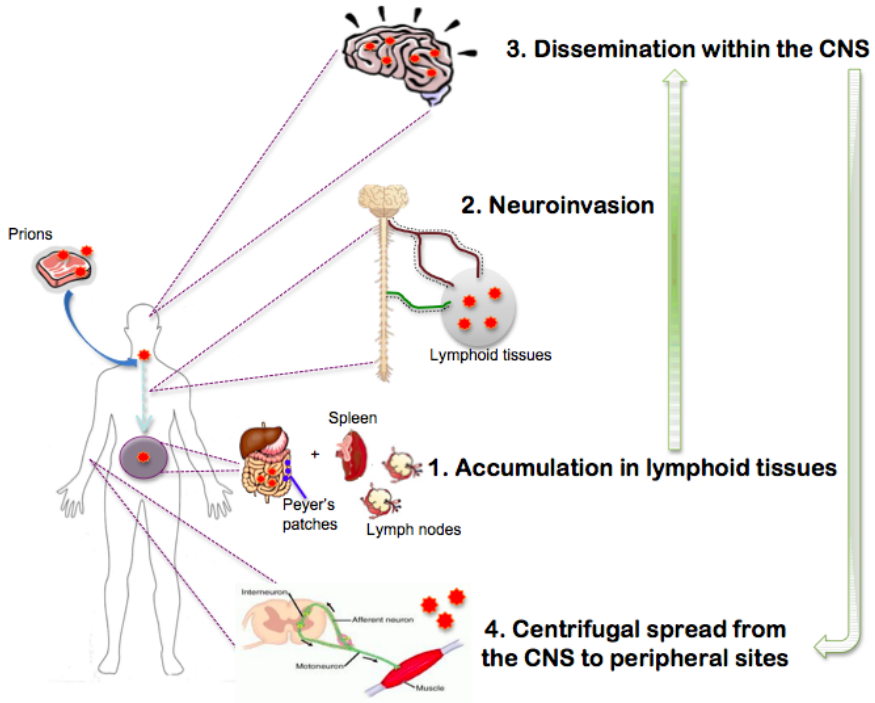

Figure 15 Different stages of prion infection. 1) accumulation of prions in lymphoid tissues; (2) neuroinvasion, consisting in the spread from the lymphoid tissues to the peripheral nervous system (PNS); (3) dissemination within the brain and spinal cord (central nervous system, (CNS)) and, (4) centrifugal spread from the CNS to further peripheral sites such as muscles. *This figure was kindly provided by L. Marzo*

3.1 From the periphery to the central nervous system: which is the route to follow?

Following oral exposure, prions enter the host organism through the gut before invasion of the draining lymphoid tissues where the first amplification of PrPSc (e.g. prion replication) takes place (Andréoletti et al., 2000; Heggebø et al., 2002; Aguzzi, 2003). The mechanism by which prions spread from the gastrointestinal tract to the lymphoid tissues is still not well understood but different players with specific roles have been identified.
From early studies in mice fed with scrapie or BSE agent, it was observed that the first prion deposition may occur in Peyer's patches and mesenteric lymph nodes prior to infection to other lymphoid tissues (Kimberlin and Walker, 1989) and that the spleen does not play a major role in neuroinvasion (Maignien et al., 1999). Instead, gut-associated lymphoid tissue (GALT) and GALT-draining lymph nodes appear to play a more significant role in early pathogenesis (Beekes and Mc Bride 2007).

Different cell types have been implicated in prion transport and replication in lymphoid follicles such as microfold cells (M cells), follicle-associated epithelium (FAE), follicular dendritic cells (FDCs), dome and tangible body macrophages (TBMs) and dendritic cells (DCs) (Beekes and McBride, 2000). In addition, it has been shown that B cells and complement system can have a supporting role that appears not to be essential (Klein et al., 1997, 2001; Mabbott et al., 2001). Also, at later stages of infection, lympho-reticular system (LRS) components seem to accumulate the scrapie agent (Beekes et al., 1996; McBride et al., 2001). A schematic representation with the main players that are thought to be involved in the uptake of prion from the gut to the lymphoid tissues is depicted in figure 16.

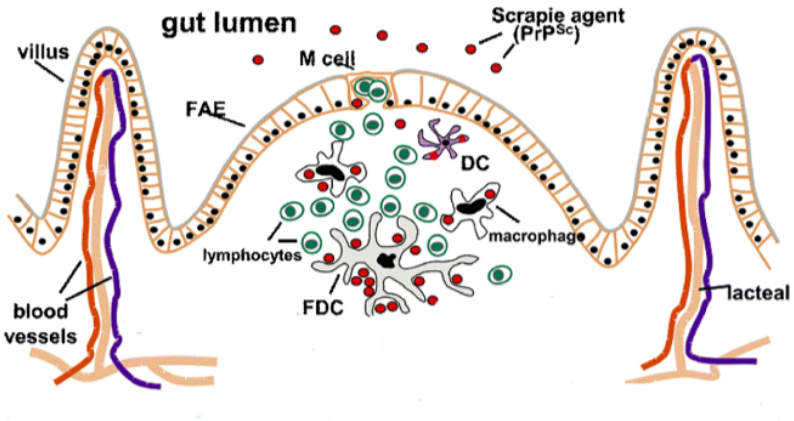

Figure 16 Possible cells involved in the uptake of prion from the gut to the lymphoid tissues. The intestinal epithelium is protected by a single layer of epithelial cells bound by tight junctions. How TSE agents cross this protective barrier is not known, but several mechanisms. have been proposed. Within the epithelium, microfold (M) cells are specialized for the transepithelial transport of macromolecules and particles. One study suggests that M cells are also plausible sites for the transport of TSE agents across the intestinal epithelium. TSE agent transport across the intestinal epithelium might also occur independently of M cells. Alternatively, dendritic cells (DCs) can also acquire antigens directly from the intestinal lumen by opening up the tight junctions that join the epithelial cells and inserting their dendrites between them. Once across the intestinal epithelium, current data suggest that the TSE agent might be acquired by migratory DCs and macrophages. Although DCs are plausible candidates or the delivery of TSE agents to lymphoid tissues, macrophages seem to phagocytose and sequester them (Mabbott and McPherson 2006). *Adapted from Beekes and McBride, 2007*

A consistent number of studies have shown that mainly GALT and, in minor part, other lymphoid tissues play a pivotal role in amplifying prions and acting as a bridge towards the CNS (Aguzzi and Calella 2009). Nevertheless a direct infection of the peripheral nervous system could also take place after oral

exposure, as observed in rodent models lacking a detectable lymphoid infection (Fraser et al., 1996; Race et al., 2000; Oldstone et al., 2002; Bartz et al., 2005).

Of particular interest, a number of studies suggest a major involvement of FDCs, as GALT components, in prion replication (Montrasio et al., 2000; Mabbott et al., 2003, 2000). However, the mechanisms by which prions would spread from the gastrointestinal tract to the FDCs and from lymphoid tissues to the CNS are still undetermined (Mabbott et al 2000; Montrasio et al 2000; Mabbott et al 2003;). FDCs are immobile stromal-differentiated cells that express high levels of PrPC (Brown et al., 1999). They reside in the follicles and germinal centres and possess many fine dendrite processes used for the trapping and retention of antigen in a native state (Imazeki et al., 1992; Kapasi et al., 1993; Shortman and Liu, 2002).

But how can immobile FDCs allow the passage of PrPSc from the intestinal barrier to the peripheral nerves?

Indeed, prion neuroinvasion is initiated in the enteric nervous system and followed by a retrograde transport along the sympathetic and parasympathetic nerve fibers (Kimberlin and Walker, 1989; Beekes and McBride, 2000). Because of the absence of neuroimmune synapses between resident FDCs and nerve fibers, direct prion transfer mechanisms between this two cell types can be excluded (Defaweux et al., 2007; von Poser-Klein et al., 2008; McGovern et al., 2009). FDCs might transfer prion to proximal cells or nerve endings through exosomes or vescicle secretion (von Poser-Klein et al., 2008; Prinz et al., 2003). Alternatively, based on in vitro studies it has been hypothesized that M cells and mobile haematopoietic dendritic cells (DCs) might transfer prions from the intestinal barrier to FDCs and from FDCs to the nerve endings (Mabbott and MacPherson, 2006)

Heppner and colleagues (Heppner et al., 2001) have shown in an in vitro system, consisting of a CaCo-2 epithelial cells monolayer, that microfold cells (M cells) are able to actively transcytose the scrapie agent through the basolateral site of the epithelium like in the case of some pathogenic microorganisms (Neutra et al., 1996). M cells are localized between the villus epithelium and the follicle-associated epithelium of the Peyer's patches and are specialized in transcytosis of macromolecules and particles. Therefore, PrPSc can cross the gut epithelium by this particular cell type, even if this is not the exclusive route.

Dendritic cells (DCs) are situated beneath the M cells in the intraepithelial pocket where they can uptake antigens that has been transcytosed by the M cells; therefore they are specialized in the capture of antigens in the periphery, followed by delivery to the lymphoid organs (Shortman and Liu 2002). Alternatively, DCs can directly uptake antigens from the intestinal lumen by opening the tight junctions of the intestinal barrier and interpolating their dendrites (Rescigno et al., 2001). Huang and co-workers (2002) have shown that DCs are indeed able to transport PrPSc from the gut to the prion-replicative lymphoid tissue. Also, PrPSc deposits have been detected in DCs from Peyer's patches, mesenteric lymph nodes or spleen, after oral exposure to prions (Defaweux et al., 2005; Dorban et al., 2007). Moreover, in mice lacking DCs, neuroinvasion is partially impaired because accumulation of PrPSc in lymphoid tissues does not take place following peripheral prion infection (Aucouturier et al., 2001; Raymond et al., 2007; Cordier-Dirikoc and Chabry, 2008). In addition, DCs can potentially transfer prions to nerve cells both through direct contact with nerve fibers (Defaweux et al., 2005; Dorban et al., 2007) or trough Tunneling Nanotubes (TNTs) (see paragraph below) (Gousset et al., 2009; Dorban et al., 2007). Indeed, Gousset and co-workers from our lab demonstrated that bone-marrow-derived DCs (BMDCs) were able to transfer prions to primary neurons in co-culture condition, resulting in infection (e.g., prion replication) of the neuronal cultures. The authors also showed that BMDCs contacted co-cultured neurons through TNT-like structures (Gousset et al., 2009). Overall, these data indicate that BMDCs can be possible candidates for the passage of PrPSc to FDCs and from FDCs to the PNS (Figure 17) (Gousset and Zurzolo, 2009).

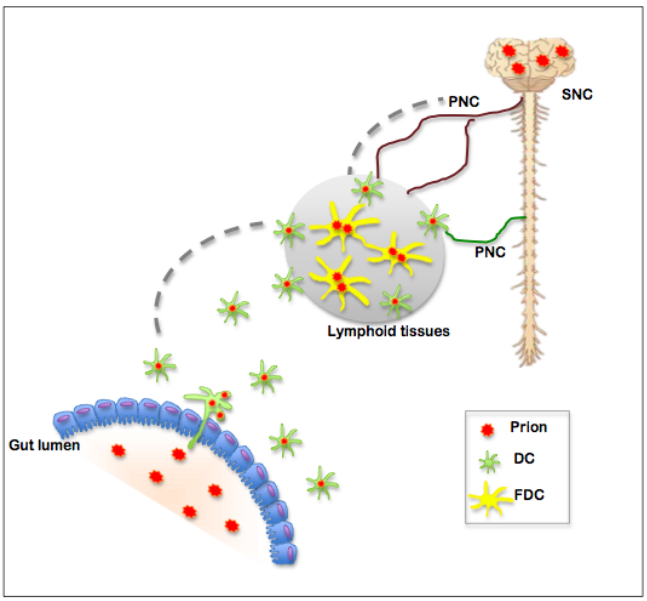

Figure 17 Schematic representation of the passage of TSE agent from the gut lumen to the PNC. Migratory bone-marrow dendritic cells (DC) can then be possible candidates for the passage of PrPSc to FDCs in lymphoid tissues and from FDCs to the PNS. This figure was a kindly provided by L. Marzo.

A further characterization of the role and the mechanisms of BMDCs-mediated prion transfer to primary neurons came from a first part of my PhD work in collaboration with Dr Langevin, (former post-doc in Dr Zurzolo's lab) and resulted in a publication (Langevin et al., 2010) that is described and appended at the end of the session "Results 1".

On the way to the CNS, from the lymphoid tissues, PrPSc get access to the peripheral nerves prior to reaching the brain.
Studies from McBride and co-workers (McBride et al., 2001) suggest that efferent fibres of both sympathetic (as the splanchnic nerve) and parasympathetic nerves (as the vagus nerve) can direct prions to the CNS in a retrograde direction from the enteric nervous system. In the case of parasympathetic nerves, the entry in the CNS occurs independently from the spinal cord highlighting the fact that different routes may be responsible for prion spreading to the CNS (Baldauf et al., 1997).
Once the infection has reached the brain, it can spread along it in both anterograde and retrograde directions (Beekes et al., 1996). For example, from a study in hamsters orally challenged with scrapie, it was observed that substantial amount of PrPSc was present in different muscles, including the tongue, providing the first evidence of a centrifugal spread of infection from the CNS to peripheral locations (Bosque et al., 2002).

3.2 Cell-to-cell spreading

At the different stages of its lethal journey to the CNS (Figure 17), PrPSc is transferred from one cell to another and this passage can involve several mechanisms (not mutually exclusive) probably depending on cell types, strains infecting and hosts.
As depicted in figure 18, prion transmission may occur (A) by cell-to-cell contact through the conversion of recipient PrPC on the cell surface without internalization of donor PrPSc; (B) in association with secreted exosomes; (C) through the release in the medium of a C-terminal truncated form of PrPSc followed by uptake in the recipient cell; (D) by “GPI-painting” and, (E) by spreading through tunneling nanotubes (TNTs).

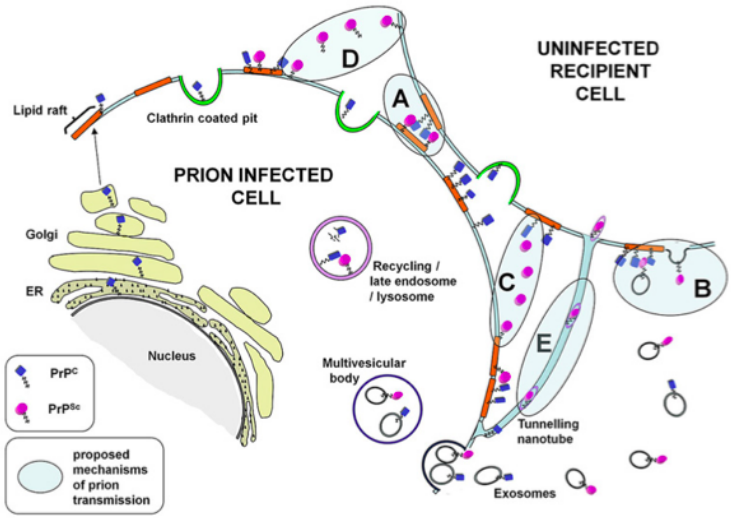

Figure 18 Proposed mechanisms of cell-to- cell spread of prion infectivity. (A) Prion transmission through direct cell-to-cell contact(conversion of recipient PrPC without internalization of donor PrPSc). (B) Transmission of prions through exosomal PrPSc association; both a direct interaction of exosome-associated PrPSc with cell-associated PrPC and incorporation of exosomal membrane with recipient cell membrane are represented. (C) C-terminal truncation of PrPSc allowing release from an infected cell and movement to an uninfected recipient cell. (D) "GPI-painting" mode of prion transfer. (E) PrPSc spread through tunnelling nanotubes, in association with small vesicles of lysosomal origin. Mode (A) is represented by lipid raft associated PrP, but could involve non-raft associated PrP. Mode (D) is depicted by transfer of cell surface PrPSc, but could potentially occur with exosomal PrPSc. *From Lewis and Hooper 2011.*

A brief description of the different means of PrPSc transmission is presented below:
- Cell-to-cell contact

From the works of Kanu and co-workers (2002) and Paquet and colleagues (2007), it has been shown that prion transmission needs a close cell-to-cell contact to occur and that infection can not be transmitted by infected cells when a physical separation between infected and uninfected cells occurs. However, in both reports, the authors have not postulated a model of transmission. The mechanism could involve, for example, PrPC conversion in trans in the recipient cell by contact with PrPSc present on the plasma membrane of an infected cell. Moreover, a transfer of infected apoptotic bodies in uninfected cells could not be totally excluded since dead infected cells are still able to pass the infectivity to naïve cells (Kanu et al., 2002).

- Exosomes

Exosomes are small vesicular carriers with a diameter of 40-100 nm generated by invagination of the limiting membranes of cytoplasmic organelles known as multivesicular bodies (MBVs) and they are released in the extracellular space following the MVB fusion with the plasma membrane. These vesicles are involved in intercellular communication by transferring not only transmembrane proteins but also nucleic acids and other cytosolic components (Simons and Raposo, 2009). It has been shown that cells can release prions in association with exosomes (Vella et al., 2007; Fevrier et al., 2004), moreover, intracerebral injection of purified prion-containing exosomal particles resulted in the infection of healthy mice (Fevrier et al., 2004).

-Microvesicles

Besides vesicles of exosomal origin, a recent report describes the involvement of microvesicles (MVs) in prion spreading (Mattei et al., 2009). MVs are sub-micron membrane-bound vesicles released by healthy or damaged cells, whose number can increase upon injury, apoptosis or inflammation and are normally present in the blood (Ratajczak et al., 2006). Mattei and colleagues (2009) have shown that PrPSc is released from infected murine neuronal cell in association with MVs, resulting in infection both in vitro and *in vivo*. Moreover, it has been demonstrated that blood as well as plasma of animals experimentally infected with TSEs can transmit TSE infection by transfusion (Cervenakova et al., 2003; Ludlam and Turner, 2006). In these cases, MVs could be the vehicles for prion transmission through infected blood.

-Shedding

Alternatively, it has also been reported that around 15% of PrPSc is present in a C-terminal truncated form in hamster brains (Stahl et al., 1990). This form results from the cleavage at the level of

Gly228, part of sequence Gly-Arg-Arg that is a target for proteolysis and release of bioactive peptides (Stahl et al 1990). The presence of this C-terminal truncated form of PrPSc in the medium following the actions of a phospholipase- or protease-like activity could also allow the spreading of PrPSc in neighboring uninfected cells (Lewis and Hooper, 2011).

-GPI painting

GPI painting phenomenon consists in the transfer from one cell to another by re-insertion of a functional GPI-anchored protein in the plasma membrane of the recipient cell and seems to occur both in vitro and *in vivo* (Kooyman et al., 1995; Legler et al., 2005). Baron and co-workers (2002) have suggested that GPI-painting could be one of the possible mechanisms of PrPSc transfer between cells, as described for PrPC in a co-culture system using a PrPC expressing cell line (M17-PrP) and the cell line IA lacking PrPC (Liu et al., 2002).

-Tunneling nanotubes (TNTs)

TNTs and their role in prion spreading are described and discussed largely in the next paragraphs (paragraph 3.3 and 3.3.1) as I focused on this mechanism of intercellular communication in my PhD work to study first the spreading of prions and then spreading of polyglutamine aggregates.

3.3 Tunneling nanotubes (TNTs): structure and function

TNTs were discovered only a few years ago as a novel form of cell-to-cell communication (Rustom et al., 2004). They were first recognized in cultured PC12 neuronal cells as long thin actin-containing bridges that do not contact the substratum and extend up to 100 µm in length with diameters ranging from 50-200 nm (Rustom et al., 2004) (Figure 19A). TNTs have been found in many cell types, from neuronal cells and primary cells to immune and epithelial cells in culture, acting as conduits for the exchange of cytosolic and membrane-bound molecules, organelles and for the spreading of pathogens (Abounit and Zurzolo, 2012; Marzo et al., 2012). In PC12 neuronal cells, two different mechanisms of TNT formation have been described: i) de novo actin-driven formation from directed filopodia-like protrusions (in the majority of the cases) and ii) formation after cell dislogement between cells previously in contact (7% of the cases) (Rustom et al., 2004; Bukoretshliev et al., 2009; Abounit and Zurzolo, 2012) (Figure 19B).

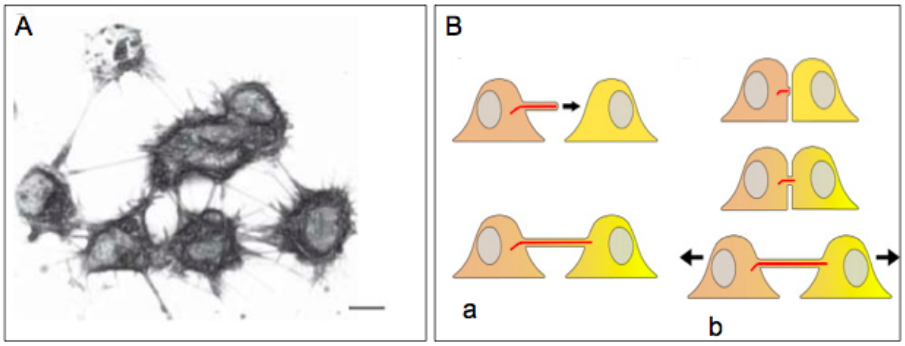

Figure 19 (A) Three-dimensional reconstruction of a network of TNTs in CAD cells (modified from Gousset et al 2009). (B) Model of TNT formation. One cell forms an actin-driven protrusion directed towards the target cell (a). TNTs may form between adjacent cells, which subsequently diverge (b). Red line, F-actin; arrows indicate direction of filopodium in a and cell movement in b. *Modified from Gerdes et al 2007*

In the mouse neuronal CAD cell line, both types of TNT formation were observed (Gousset et al., 2009 data not shown) and, as previously described in PC12 cells (Rustom et al., 2004), they contained actin filaments but no microtubules (Gousset et al., 2009). Recently Wang et al. (2011) have shown that TNT-like structures formed between primary rat astrocytes and neurons contain actin as major cytoskeleton component. As mentioned above, the majority of neuronal TNTs arise from filopodia-like structures, detached from the substratum, suggesting that actin plays an important role in this type of TNT formation. This is also supported by the finding that treatment with the F-actin depolymerizing drug latrunculin abolish neuronal TNTs in PC12 cells (Rustom et al., 2004) and CAD cells (Gousset et al., 2009). Indeed, treatment with latrunculin or Cytochalasin D (another actin-depolymerizing drug) abrogated neuronal TNT formation also in primary cells (Wang et al., 2011). However, differently from filopodia, once formed, TNTs are no longer sensitive to low levels of Cytochalasin D, demonstrating that TNTs are distinct from filopodia in both structure and function (Bukoreshtliev et al., 2009). While filopodia have a major role in sensing the enviroment and in cell motility, TNTs act as conduits for cell-to-cell communication by connecting the cytosol distant cells (for review see Abounit and Zurzolo, 2012; Marzo et al., 2012). In non neuronal cells, in particular immunological and epithelial cells, TNTs were found to contain only actin or both F-actin and microtubules or to be composed of cytokeratin filaments in the case of epithelial cells (for review see Marzo et al., 2012).

Different from neuronal TNTs, formation of TNT-like structures in immune cells relays primarily on detachment after cell-to-cell contact (Davis and Sowinski, 2008). Therefore there is a large disparity both in the cytoskeleton components and mechanisms of formation as well as in the diameter and length in naturally occurring TNT-like structures in neuronal, immunological or epithelial cells (for review see Abounit and Zurzolo, 2012; Marzo et al., 2012).

Together with structural differences, Tunneling nanotubes have revealed a high degree of heterogeneity also from a functional point of view. As reviewed in great details by Abounit and Zurzolo, (2012) and Marzo et al. (2012) different components seems to be selectively transferred via TNTs by different cell types including signals (calcium mediated signals and death signals), organelles and pathogens.

Cargoes can be uni-directionally or bi-directionally transported insides TNTs. Uni-lateral transfer occurs when a donor cell (usually the cell that initiated TNT formation in the case of neuronal TNTs) transfers material to an acceptor cell, whereas bi-lateral transfer happens when both cells mutually exchange materials (found mainly in non neuronal cells). The reasons for these different transport mechanisms can depend on TNTs structural components (actin-only versus both actin- and microtubules). Indeed, unidirectional transfer is found when only actin is present whereas transfer appears to be bi-directional in the presence of both actin and microtubules (for review see Marzo et al., 2012). Tunneling nanotubes have been shown in certain cases to be highways for pathogens transfer, leading to the spreading of infection. Hijacking of these structures can be preceded by induction of TNT formation, as it has been shown for HIV particles, thus optimizing pathogen transfer and spreading. In particular the HIV virus has been shown to spread through TNTs in primary macrophages both surfing on the membrane (Eugenin et al., 2009) or traveling inside associated with endocytic compartments (Kadiu and Gendelman, 2011). Furthermore, Onfelt and colleagues (2006) have shown that M. bovis BCG or clusters of several bacteria can surf on thin membrane nanotubes between macrophages before being internalized by receptor-mediated endocytosis, pointing towards a possible role of these structures in concentrating the pathogen on the entry site for a more efficient bacterial invasion.

3.3.1 TNTs and prion spreading

As described above, the mechanisms of prion spreading from the periphery to the central nervous system (CNS), and subsequently within the CNS, are not completely understood and a number of mechanisms for the intercellular spreading of prions, such as cell-to-cell contact, exosomes and GPI-painting, has been proposed (Kanu et al., 2002; Fevrier et al., 2004; and Baron et al., 2006). Recently, our lab has shown that TNTs formed in neuronal CAD cells were able to transfer the cellular GPI-anchor prion protein PrPC, as well as fluorescently labeled infectious prion particles, PrPSc (e.g. exogenous prions) (Gousset et al., 2009). Moreover, by co-culturing chronically prion infected CAD cells (ScCAD cells) and naïve neuronal CAD cells, we were able to show that endogenous PrPSc is also found in TNTs and that these infectious particles were efficiently transferred from Sc CADs to non-infected cells only in the case where the two cell populations were connected by means of TNT structures (Gousset et al., 2009). In addition, the use of nanomolar concentration of latrunculin blocked TNT formation and abolished the transfer of PrPSc particles in the same experimental setting described above, further demonstrating that TNTs are an efficient route for PrPSc spreading in neuronal cells (Gousset et al., 2009). Since the prion protein is a GPI-anchored protein, it has the possibility of traveling via TNTs either at the surface or within vesicular structures. Further studies in our laboratory have shown that a major fraction of PrPSc (50%) within TNTs colocalize with markers of the endocytic recycling compartment (ERC) in ScCAD cells (Marzo and Zurzolo, unpublished data). Remarkably, the ERC has been shown to be one intercellular site of PrPC-PrPSc conversion (Marijanovic et al., 2009). Moreover, similar to what it has been shown for HIV particles (see paragraph 3.3 above), PrPSc infection per se induces TNT formation as demonstrated by the increased number (about 20%) of TNT-connected cells in ScCAD cells compared to naïve CADs (Marzo and Zurzolo, unpublished data). In addition, the transfer of infectious prion particles via TNTs is not confined to neuronal co-cultures but is also occurring between loaded Bone-Marrow Dendritic Cells (BMDCs) and primary neurons (Gousset et al., 2009). Finally, co-culture of primary neurons with prion-infected BMDCs resulted in the transfer of infectivity to the recipient cells. Altogether these studies suggested that TNTs might play a critical role *in vivo* in

the spreading of prions within the central nervous system (CNS) and at the periphery (Gousset and Zurzolo, 2009)(Figure 20).

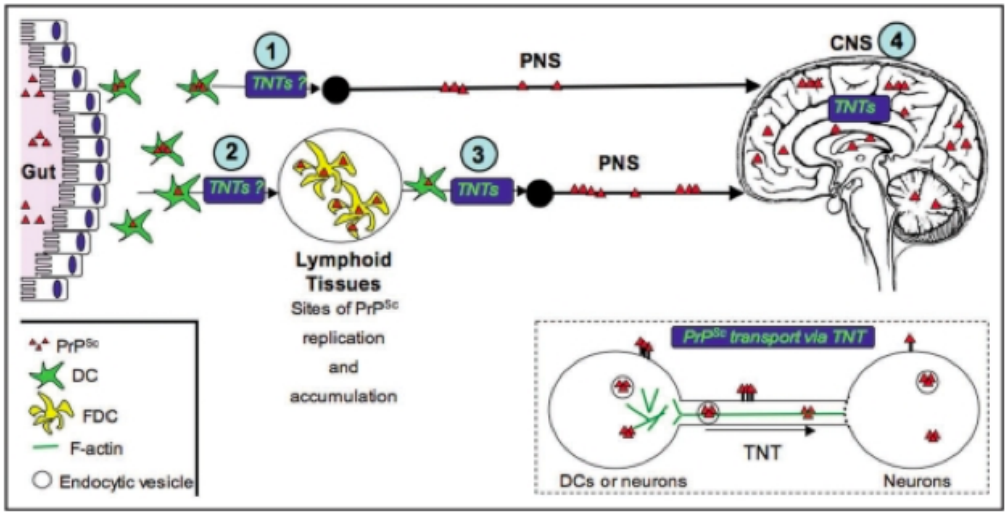

Figure 20 Transport of PrPSc via TNTs, an alternative spreading mechanism during neuroinvasion. Studies in our laboratory suggest that TNTs allow for the intracellular transport of PrPSc between dendritic cells and neurons and between neurons (see inset). The exact mechanism of transport remains to be determined. For instance, it is still not clear, whether PrPSc is strictly transported within endocytic vesicles, or whether it can slide along the surface or be transported as aggregosomes within the tubes. Similarly, the types of motors used, as well as the possible gated mechanisms to enter the recipient cells are not known. Because of the high propensity of DCs to form TNTs with different cell types, we propose that TNTs could play important roles in delivering PrPSc to the proper cell types along the neuroinvasion route. For instance, DCs could deliver PrPSc from the peripheral entry sites to FDCs in the secondary lymphoid tissues (2) or in a less efficient manner, they might occasionally directly transport PrPSc to the PNS (1). They could also bridge the immobile FDC networks and the PNS (3), since we have shown that DCs can form TNTs with nerve cells. Finally, once PrPSc has reached its final destination within the CNS, TNTs might play a final role in the spreading of PrPSc within the brain between neurons and possibly between neuronal cells and astrocytes (4). *From Gousset and Zurzolo, 2009*

Similar to prion diseases, neurodegenerative diseases such as Alzheimer, Parkinson and Huntington appear to be the result of protein misfolding and aggregation, therefore it is tempting to speculate that these diseases might also share some common mechanisms of spreading. Recently, Wang and colleagues have analyzed whether intracellular Aβ particles could spread through TNTs in astrocytes and neurons (Wang et al., 2011). Microinjection experiments demonstrated that intracellular Aβ-fusion proteins were indeed able to quickly spread from cell-to-cell via TNT-like structures. In addition, they looked at the transfer of Aβ toxicity in co-cultures of infected astrocytes and neurons. They showed that increasing the number of TNTs

between the cells by H2O2 treatment led to an increase in neuronal cell death in co-cultures with infected astrocytes compared to the control GFP or non-stressed cells. Thus they speculate that Aβ particle spreading via TNTs within the cultures resulted in an increase in neuronal toxicity leading to cell death. Such observations are very similar to what we found regarding the spreading and infectivity of PrPSc and suggest that prion diseases and other neurological diseases might use TNTs as mechanism of intercellular spreading. If these types of studies can be further extended to Parkinson or Huntington, they might open up new ways of looking at these neurodegenerative diseases and could lead to new strategies to fight them. This has been one of the subject of study of my PhD (see “Results 2”).

4. Generalizing the prion principle

Neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's disease and prion disorders are associated with the deposition of amyloidogenic protein aggregates in the intracellular and/or extracellular space (see chapter 1 of the introduction). Although protein misfolding and aggregation are common features in neurodegenerative diseases, the concept of spreading and infectivity of aggregates in the central nervous system has, until recently, been confined to prion diseases (Lee et al., 2010; Brundin et al., 2010). Indeed, among the diverse neurodegenerative disorders, the uniqueness of prion diseases relays on their infectivity as transmission of the pathology occurs between individuals and across species through exposure in the natural enviroment (e.g. variant CDJ is transmitted from cow to human) (Aguzzi and Calella, 2009). As previously discussed, the molecular mechanism underlying prion infectivity is the ability of prions to self-propagate via PrPSc-templated conversion of endogenous PrPC molecules (Prusiner, 1998).
Like most cases of prion diseases, non-prion neurodegenerative disorders are mainly sporadic with a small percentage being inherited (Prusiner, 1994; Hardy and Orr, 2006). With the exception of AA amyloidosis that has been shown to be transmitted via feces among cheetahs (Zhang et al., 2008), so far inter-organism spread of non-PrP misfolding diseases has not been observed (Guest et al., 2011). The reason may be in the remarkable stability of the cross-β prion form which resists to harsh environments like heat denaturation, detergents and proteases, while most protein aggregates are more fragile and therefore may not be preserved in the external environment (Guest et al., 2011; Miller, 2009; Cushman et al., 2010). Even if most neurodegenerative diseases are not transmitted from one individual to another like true prions, they might propagate in analogous way within a single organism. Indeed, recent studies suggest that intercellular prion-like transmission mechanisms may be responsible for propagation of protein misfolding in non-prion neurodegenerative disorders, involving both secreted proteins such as amyloid-β and cytosolic proteins such as tau, huntingtin and α-synuclein, suggesting the existence of a general pathogenic principle in neurodegenerative proteinopathies (Frost and Diamond, 2010, 2009). These findings blur the distinction

between transmissibility and infectivity and therefore between prion and amyloids (Cushman et al., 2010). Transmissibility, referred to as host-to-host transmission, up to date remains peculiar property of prion diseases while infectivity seems to be a general property of amyloids (Moreno-Gonzales and Soto, 2011). Misfolded protein aggregates are "infectious" if they propagate from one cell to another and, in the recipient cell, they act as a "seed" initiating aggregate formation by recruiting additional unfolded or oligomeric species of the same protein (Brundin et al., 2010).

In addition, prions themselves encode many phenotypic TSE variants, termed prion strains. Prion strains, after inoculation into distinct hosts, cause infection with typical features, such as incubation period, clinical signs, characteristic pattern of neuropathological lesions, and specific PrPSc biochemical features (Aguzzi et al., 2007). Other neurodegenerative diseases also exhibit phenotypic variation (Frost and Diamond, 2010). As the prion strain phenomenon relays on the existence of different conformers of prion fibrils, non prion-aggregates also exhibit conformational diversity as it has been shown in vitro in the case of amyloid-β (Petkova et al., 2005), in vitro and in mouse models in the case of polyglutamine huntingtin (Nekooki-Machida et al., 2009) and in vitro as well as in patients for tau (Frost et al., 2009; Goedert et al., 1992). However, in contrast to PrP (Legname et al., 2004), it has not been possible to induce a transmissible neurodegenerative disease by intracranial injection of pure polyglutamine, amyloid-β, α-synuclein or tau conformers. Auxiliary factors might be more crucial for these amyloid forms to transfer from cell-to-cell and gain access to the native substrate (Cushman et al., 2010).

Neverthless, some features of prions can be extended to other protein conformational disorders. In particular, on the basis of recent evidences described in the next paragraphs, the prion-like phenomenon in other neurodegenerative diseases can be regarded at different levels: (i) tissue level: progressive spreading of the pathology throughout the affected tissue; (ii) intercellular level: propagation of the aggregates between affected cells and their neighbors; (iii) molecular level: seeded aggregation and self-propagation of the aggregates. (Table 2)

Proteins	Molecular recruitment and misfolding induction	Intercellular transmission	Tissue migration	Spreading between organs	Evidence of strains	Protein only sufficient for infection
Amiloid-β	YES: seeded polymerization of native protein by aggregates in vitro	NO:Aggregation takes place extracelllularly	Possibly: characteristic spread of Aβ in AD neuropathological staging	Possibly: transmission in mice on intracerebral inoculation	No evidence to date	Possibly: Aβ oligomers are cytotoxic, unclear if aggregates are cause or consequence
Tau	YES: seeded polymerization of native protein by aggregates in vitro	YES: extracellular aggregates can be taken up by cells	Possibly: outward spread of Tau aggregates from transentorhinal cortex in AD	Possibly: pathology in mice intracerebrally inoculated with misfolded Tau	YES: fibrillar aggregates in brain have range of ultrastructural features	No evidence to date
Huntingtin	YES: polyglutamine peptides exceeding critical length aggregate in vitro	YES: aggregates are internalized by mammalian cells in culture	YES: contiguous spread of pathology through CNS in HD	NO: unlikely as genetic susceptibility in host necessary	NO evidence to date	NO: etiology is underlying genetic mutation
α-synuclein	YES: seeded polymerization of native protein by aggregates in vitro	YES: premature α-synuclein misfolding in transplanted stem cell grafts	YES: contiguous spread of pathology through CNS in PD	NO evidence to date	NO evidence to date	NO evidence to date

Table 2 Prion-like features of protein misfolding in other neurodegeneratives diseases(*modified from Guest et al. 2011)*

4.1 Patterns of neuropathology spread

Neuropathologies of Alzheimer's, Parkinson's, Huntington's disease spread progressively from an initial affected region to other distant areas of the brain following predictable anatomical pathways that are specific for each disorder (Brundin et al., 2010; Frost and Diamond, 2010). This supports the hypothesis that different proteinaceous aggregates can transfer between cells, thus contributing to the spreading of the pathology in the healty tissue (Brundin et al., 2010; Moreno-Gonzales and Soto, 2011) (see paragraph 4.2) (Figure 21).

Figure 21 Principles for progression of neuropathological changes. Three drawings propose principles for how neuropathological changes in Parkinson's,

Alzheimer's and Huntington's diseases spread spatiotemporally during disease progression. The earlier the neuropathology develops in a given brain region, the darker the shading in the diagram. As only one view (mid-sagittal for Parkinson's and Alzheimer's diseases; lateral for Huntington's disease) of the brain is depicted for each disorder, not all relevant anatomical structures and details of the spreading patterns (indicated by arrows) are presented. See text for details. *Modified from Brundin et al., 2010.*

-Parkinson's disease

Braak and co-workers proposed that brainstem and anterior olfactory structures are afflicted by the deposition of α-synuclein aggregates several years before involvement of the substantia nigra (Braak et al., 2004, 2006) and suggested that α-synuclein aggregates spread in a topographically predictable manner as the pathology progresses, following anatomical connections throughout the brainstem, limbic and autonomic systems and neocortex (Braak et al., 2004, 2006).

-Alzheimer's disease

In Alzheimer's disease neurofibrillary tangles have been proposed to spread throughout the brain in a stereotypical manner. Some of the first affected areas include the hippocampus, the basal nucleus of Meynert and the brainstem whereas the neocortex appears to be involved only in advanced stage of the disease (Braak and Braak, 1991; Delacourte et al., 2002). The deposition of intracellular aggregates of hyperphosphorylated tau is suggested to follow anatomical connection (Lace et al., 2009). By contrast the deposition of extracellular amyloid-β seems not to follow anatomical patterns and correlates poorly with the level of cognitive decline (Nelson et al., 2009; Arriagada et al., 1992).

-Huntington's disease

Brain imaging studies showed that cortical degeneration in HD follows a topologically predictable pattern (Rosas et al., 2008) and precedes degeneration in the striatum (Vonsattel and DiFiglia, 1998, Brundin et al 2010). Within the striatum, degeneration progresses following two defined anatomical direction (Vonsattel and DiFiglia, 1998) and striatal projecting neurons are among the first one to be affected (Deng et al., 2004). Inclusions containing huntingtin are present in the regions of the brain that degenerate, although the presence of visible aggregate does not always correlate with cell death (Kuemmerle et al., 1999).

Furthermore, as previously described, prions can disseminate beyond the tissue where they are produced and spread through the entire organism in a peripheral and systemic transmission thanks to cells of the immune system, peripheral nerves and

bloodstream (see chapter 3 of the introduction). Remarkably, most of the disease-associated misfolded proteins are found circulating in the cerebrospinal fluid and plasma, which could facilitate their spread through the body as it has been reported for amyloid-β (Hampel et al., 2004), α-synuclein (Tokuda et al., 2010; El-Agnaf et al., 2003) huntingtin (Weiss et al., 2009) and A amyloid (Tasaki et al., 2010).

4.2 Cell-to-cell aggregate transmission

Recent experimental findings and clinical observation shave shown that cell-to-cell transfer of protein aggregates is occurring in non-transmissible neurodegenerative disorders and this might contribute to the anatomical spreading of the pathology in a prion-like manner (Brundin et al., 2010; Frost and Diamond, 2010).

-Parkinson's disease

Postmortem analysis of Parkinson's disease patient who had received a transplant of healthy embryonic neurons one decade earlier, revealed that grafted neurons were positive for Lewy bodies and Lewy neurites, thus indicating that misfolded α-synuclein was transmitted from the host to the graft cells (Allan et al., 2010; Li et al., 2010, 2008). To further investigate this possibility, Desplats and collaborators injected GFP-labeled mouse stem cells in transgenic mice overexpressing human α-synuclein. One week later intracellular α-synuclein immunoreactivity and occasionally inclusion bodies could be detected within GFP-labeled cells, suggesting that host-graft transfer of α-synuclein had occurred (Desplats et al., 2009). Also, in α-synuclein transgenic mice where expression of α-synuclein was restricted to neurons, prominent accumulation of α-synuclein was detected in glial cells and transmission of α-synuclein from neurons to astroglia was further confirmed in co-culture experiments (Lee et al., 2010). Several in vitro studies have supported the hypothesis that α-synuclein can transfer between neurons. Indeed, exogenous fluorescently tagged α-synuclein is internalized in cultured cells (Desplats et al., 2009; (Lee et al., 2008a) and intracellular human α-synuclein is transferred to GFP-labelled stem cell in co-culture condition (Desplats et al., 2009; Lee et al., 2008). Furthermore, extracellular GFP-α-Synuclein when internalized in primary cortical neurons is seeding polymerization of an intracellular red fluorescent tagged version of the protein

resulting in the formation of double positive aggregates (Danzer et al., 2009, 2007). Moreover, Hansen et al (2011) showed that transmitted α-synuclein interacts with α-synuclein expressed in the recipient cell and forms aggregates. Taken together, these findings suggest that α-synuclein spreads from one cell to another, throughout the CNS, during the course of Parkinson's disease in accordance with the stereotypical progression of the pathology described by Braak.

-Alzheimer's disease

Injection of mouse brain homogenates containing aggregates of a human disease-associated mutant tau in to transgenic mice expressing wild-type human tau (Clavaguera et al., 2009) induced misfolding and aggregation of the intracellular, otherwise soluble protein. Moreover, the aggregates appearance increases over time and they spread to anatomically connected regions (Clavaguera et al., 2009), consistent with the stereotypical progression of neurofibrillary tangles in Alzheimer's disease patients (Guest et al., 2011; Cushman et al., 2010; Brundin et al., 2010). Recently, it has been shown in cell culture experiments that externally applied tau aggregates once internalized can induce the seeded polymerization of intracellular tau (Frost et al., 2009; Nonaka et al., 2010; Guo and Lee, 2011). Furthermore, intracellular tau aggregates can transfer from cell to cell in co-culture experiments (Frost et al., 2009). Recently, two independent studies provided evidences for a trans-synaptic spreading of tau pathology in transgenic mouse models selectively expressing a pathological human tau in the entorhinal cortex (Liu et al., 2012; de Calignon et al., 2012). Indeed, human tau immunoreactivity was detected over time along synaptically connected neuronal circuits (Liu et al., 2012; de Calignon et al., 2012) and was found to co-aggreagate with endogenous mouse tau (de Calignon et al., 2012).

Intracerebral injection of brain extracts from alzheimer's disease patients in transgenic mice expressing human amyloid precursor protein induced deposition of extracellular amyloid-β plaques, suggesting that amyloid-β has "infectious" properties and is capable of seeding Alzheimer's pathology in mouse models (Meyer-Luehmann et al., 2006; Kane et al., 2000). Recently, Wang and colleagues demonstrated that intracellular amyloid-β fusion proteins were able to quickly spread from cell-to-cell in cultured primary rat astrocytes and neurons (Wang et al., 2011). In addition, extracellular amyloid-β is uptaken by astrocytes and

transferred to primary neurons in co-culture conditions (Wang et al., 2011).

-Huntington's disease.

In the case of Huntington's disease (HD), cell culture experiments have shown that synthetic polyglutamine peptides or recombinant fragments of mutant Htt when applied externally to cultured cells are readily taken up (Yang et al., 2002; Ren et al., 2009) and they can seed polymerization of a soluble huntingtin reporter (Ren et al., 2009). Moreover, these assemblies persist for over 80 generation in prolonged cell culture, despite their dilution in dividing cells, suggesting a self-sustaining seeding and fragmentation process similar to prion replication (Ren et al., 2009). Currently, the relevance of these observations in HD pathogenesis is unclear. Indeed, fetal grafts of striatal tissue in HD patients brains have shown, upon autopsy, to be susceptible of disease-like neurodegeneration, but abnormal huntingtin aggregation was not observed within a decade from the transplant (Cicchetti et al., 2009), contrary to what has been found in grafted cells from Parkinson's disease patients (Allan et al., 2010; Li et al., 2008). In addition, Ren and colleagues reported that natural cell-to-cell transmission of Htt, measured indirectly from the seeded-polymerization of a cytoplasmic huntintin reporter, was rather inefficient in co-cultured HEK293 cells, and could be drastically increased by selective lysis of the donor cells (Ren et al., 2009). Yet, it is unknown whether and how huntingtin misfolding progresses through the brain, in order to explain the topologically predictable progression of HD. A contribution in evaluating the occurrence and the mechanisms of polyglutamine huntingtin transfer between intact neuronal cells came from one part of my PhD work and is described in the results section (Result 2, Article 2).

4.3 Mechanisms of aggregates release and uptake

All the evidences reported above for the different disease-associated protein aggregates suggest a prion-like process in the propagation of the underlying neurodegenerative disorders, putting them in the same framework of canonical prions. Since I have already described (see chapter 3 of the introduction) the mechanisms of intercellular prion spreading, here I provide a brief insight in the mechanisms of intercellular transfer of these prion-like aggregates based on the evidences currently available in the

literature. Cell-to-cell transfer of protein aggregates requires two consecutive steps: (i) exit from the donor cell, (ii) entry in the recipient cell.

(i) Aggregates can be passively released from degenerating neurons upon cell death in a non specific manner as a result of the toxicity of protein aggregation (Brundin et al., 2010). For example, as discussed above, it seems that polyglutamine huntingtin can induce nucleation of a soluble huntingtin reporter in co-cultured cells only upon selective induced donor cell lysis (Ren et al., 2009). Alternatively, cells can release aggregates via exocytosis, exosomes or trans-synaptic transmission at the axonal terminals (Brundin et al., 2010). As an exemple, α-synuclein can be secreted via a non-classical vesicle-mediated exocytic mechanism (Lee et al., 2005; Jang et al., 2010) and in association with exosomes (Emmanouilidou et al., 2010) (Figure 22)

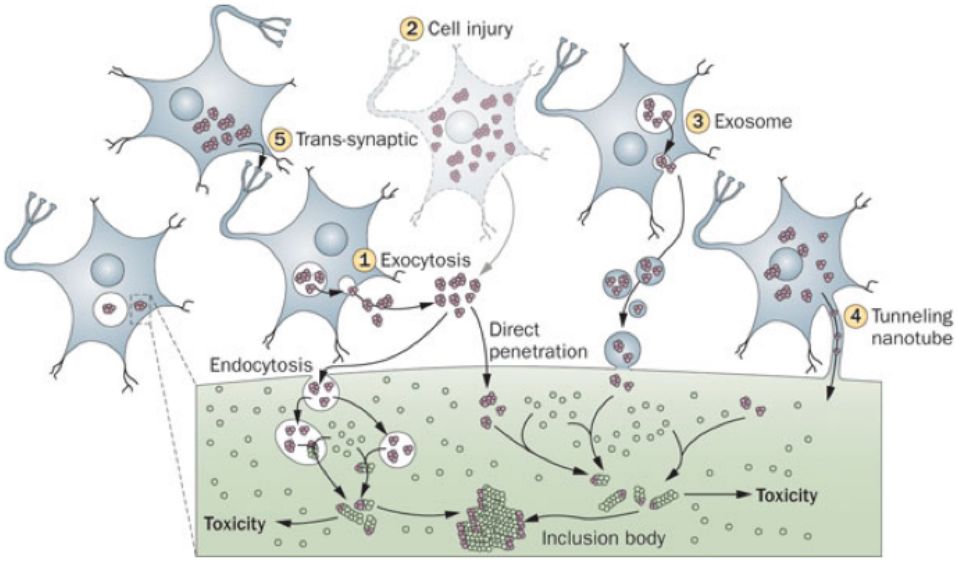

Figure 22 Models of cell-to-cell transmission of misfolded and aggregated proteins. Proteins might be released from neurons via vesicle-mediated exocytosis (1) or leakage through damaged membranes (2) and be internalized into neighboring neurons via endocytosis or direct membrane penetration. Alternatively, proteins could be transmitted to neighboring neurons by packaging into exosomes (3) or through tunneling nanotubes (4). Different mechanisms might work simultaneously, with specific proteins preferring certain pathways. Mechanisms might act between the cell bodies or trans-synaptically (5). Internalized aggregates (pink) might act as seeds for aggregation of endogenous native proteins (green). Seeded aggregation may produce toxic aggregate species, leading to formation of pathological inclusion bodies. *From Lee et al., 2010*

(ii) Entry in the recipient cell can relay on passive diffusion through the plasma membrane, endocytosis and exosome-

mediated transfer or TNTs (Figure 2). Internalized tau was found to localize with dextran, implying an endocytic method of entry rather then direct penetration of the membrane (Frost et al., 2009). Similarly α-synuclein aggregates internalization differentiated SH-SY5Y cells has been reported to be sensitive to temperature and to require dynamin-1 pointing towards a role for the endocytic pathway in the entry mechanism (Desplats et al., 2009). Thus, internalized aggregates of α-synuclein and tau are likely packaged into membrane-bound vesicles from where they have to escape to gain access to the cytoplasm with mechanisms that are not yet understood. However, α-synuclein seems also to be able to breach the plasma menbrane upon passive diffusion (Lee et al., 2008b; Park et al., 2009). Deep-etch electron microscopy of exogenous synthetic polyQ aggregates uptaken in HEK293 cell culture revealed no evidence of surrounding membranous structure, suggesting the absence of a vesicular uptake and therefore direct penetration of the plasma membrane (Ren et al., 2009).

PrPSc like HIV was reported to use tunneling nanotubes (TNTs) as infection highways to transfer from one cell to another (Gousset et al., 2009; Gousset and Zurzolo, 2009; Davis et al., 2008; Sowinski et al., 2008). Of interest, recently Wang et al. (2011) have shown that extracellular amyloid-β once internalized in primary astrocytes is found in TNTs established with co-cultured neurons. Therefore, it is important to investigate whether other self-templating aggregates could also spread from cell-to-cell via this route. However, the different nature of the disease-associated proteins should be taken in to account: while PrPSc and amyloid-β are anchored to membranes, α-synuclein, tau and huntingtin are cytosolic. In the case of α-synuclein and tau, after uptake endocytic vesicles could shuttle these aggregates in TNTs. Then once the aggregate reach the recipient cell, they should escape from vesicles in order to seed the misfolding of endogenous cytosolic proteins (Marzo et al., 2012). On the other hand, a cytosolic passage as aggresomes through TNTs could also be envisaged (Marzo et al., 2012). Interestingly α-synuclein and huntingtin can interact with acidic phospholipids enriched on the cytoplasmic membrane leaflet (Kegel et al., 2005, 2009; van Rooijen et al., 2008) suggesting the additional possibility of a "surfing" process along the inside of TNT membranes (Marzo et al., 2012).

In both inherited neurodegenerative disorder and in the more common idiopathic ones, the low probability of spontaneous

misfolding and aggregation might explain the mid-life or later onset of these disorders. Once misfolding and aggregation are initiated in a stochastic manner in a subpopulation of cells, prion like-transmission mechanisms seems likely to contribute to the gradual spreading of the neuropathological changes in the brain of afflicted individuals (Brundin et al., 2010; Guest et al., 2011). In this frame, aggregation and progression of the disease in the brain can be non-cell autonomous. Therefore the current data indicate that the classic model of cell autonomous disease in which protein misfolding arises separately but simultaneously in a large population of cells is less likely (Brundin et al., 2010; Guest et al., 2011). Furthermore, if misfolded proteins seed themselves through the central nervous system, they satisfy the molecular, cellular and tissue definition of prions so that we could state that "the uniqueness of prions is diminished, but their importance has never been greater" (cit. Guest et al., 2011)

5. Huntington's disease

Huntington's disease (HD) is a fatal neurodegenerative disorder with an autosomal dominant mode of inheritance. It progresses slowly over years with symptoms beginning typically, but not always, in midlife between the ages of 35 and 50. Although relatively rare, juvenile cases, before age 20 and later-onset cases, after age 65, are also described. Death occurs from 15 to 20 years after the onset of the symptoms. HD is caused by the expansion of a CAG repeat in the exon 1 of the huntingtin gene, resulting in an expanded polyglutamine (polyQ) tract in the N-terminal part of the encoded protein.
In population of European descent, the prevalence of HD is approximately 4-9/100,000. Although HD is relatively uncommon, the economical and social impact of HD is disproportioned to its prevalence because of the middle age onset and the slow progression of the disease.

5.1 Symptoms and Neuropathology

In 1872, physician George Huntington reported a familial form of chorea noted previously in Long Island by his father and grandfather, also physicians. Huntington described chorea as "the dancing propensity of those... affected", in whom there "seems to exist some hidden power... upon the will" (Huntington 1973; reprinted in Huntington 2003)
The clinical features of the disease, now carrying his name, can be resumed in HD clinical triad (Figure 23)

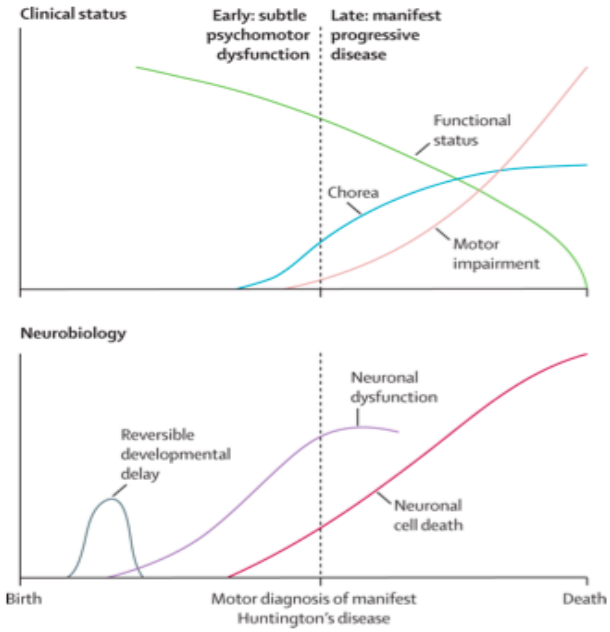

Figure 23 Progression of Huntington's disease over a patient's lifespan.Subtle signs and symptoms of Huntington's disease begin years before a motor diagnosis can be made, and correlate with neurobiological changes such as striatal atrophy, giving rise to the concept of a Huntington's disease prodrome. Chorea is often the earliest motor feature noted clinically, but motor impairment or bradykinesia and incoordination are more disabling. Early in the disease course, neuronal dysfunction is likely to be important, but later, neuronal cell death in vulnerable regions of the brain is predominant and correlates with motor impairment and functional disability. From Ross and Tabrizi, 2011

-Disturbances in motor function

Chorea is the classic motor sign of HD. Derived from the Greek word for “dance”, it consists of involuntary movements of the proximal and distal muscles of the body. Together with chorea, that by itself it is not greatly disabling, HD patients develop progressive impairment of coordination of voluntary movements. The inability to sustain voluntary muscular efforts contributes mainly to the physical disability of individuals affected by HD. In patients with juvenile HD, motor symptoms are somewhat different; they include bradykinesia (slowness of the movements), rigidity and dystonia (involuntary contraction of the muscles) and chorea may be completely absent. Epileptic seizures are also common in affected children.

-Cognitive disturbances

The cognitive difficulties usually begin with a slowing of intellectual processes and can be present much before the first motor symptoms appear (Aylward et al., 2004, 2000). HD is classified as a subcortical dementia characterized by difficulty in initiating thought processes, difficulties with executive functions,

although memory in general is well preserved (Paulsen et al., 1995; Rohrer et al., 1999)
-Behavioral disturbances
Behavioural disturbances include most notably depression and apathy (Paulsen et al., 2001) but also obsessive-compulsive behaviors and irritability (Duff et al., 2007). These symptoms are present already at the moment of the diagnosis but they evolve and worsen in the course of the disease (Duff et al., 2007)
HD is characterized by a massive loss of GABAergic medium spiny neurons in the striatum (caudate nucleus and putamen) of the basal ganglia, which is responsible for the typical symptoms of the disease (Reiner et al., 1988). However, it is now well established that a more widespread degeneration occurs in the diseased brains and progresses in a topological predictable manner involving cortical structures even before the onset of clinical symptoms (see paragraph 4.1). In the late stages, globus pallidus, thalamus, subthalamic nucleus, substantia nigra, white matter and the cerebellum are markedly affected (Vonsattel and DiFiglia, 1998).

5.2 The huntingtin gene

From the first description of HD in 1987, the identification of the causative gene and related mutation arrived only in 1993 and required the effort of a collaborative group of scientists for over 10 years. They found that the disease was linked to the IT15 gene that contained in the exon 1 a polymorphic repetitive trinucleotide element: C (cytosine) A (adenine) G (guanine). Indeed, in non-HD controls the number of CAG repeats varied from 6 to 35, a phenomenon that was described as “instability of the trinucleotide repeat”. In HD patients, the number of CAG repeats was always 40 or more indicating that the expansion of the trinucleotide repeat in the IT15 gene, now renamed the huntingtin gene, was responsible for HD (HD collaborative research group, 1993; Gusella et al., 1983)(A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington’s disease chromosomes. The Huntington’s Disease Collaborative Research Group, 1993; Gusella et al., 1983). Further studies revealed that some individuals with no symptoms had “intermediate-size repeats” ranging from 27 to 35 and were at risk of transmitting the disease to their children with a risk that was higher if the intermediate allele was inherited from the

father (Ranen et al., 1995). This phenomenon is known as "genetic anticipation" and relays on the fact that the expanded CAG triplets are not stable and tend to further expand from one generation to another, probably because of replication slippage during mitosis (Pearson, 2003). Therefore, the risk of expansion is more frequent in spermatogenesis then oogenesis and individuals that inherit the disease gene from the father may have a longer CAG tract and develop symptoms at an earlier age (Ranen et al., 1995). Indeed, there is a positive correlation between the length of the CAG repeat (within the expanded range) and the onset of the disease with longer repeats causing early onset of HD (Duyao et al., 1993). Consistently, extremely large CAG repeats from 60 on are often associated with juvenile HD (Andrew et al., 1993).
The gene encoding for huntingtin in vertebrates is composed of 67 exons and covers a region of about 170 kb. It is highly conserved among all animals from sea urchin to insect and mammals with 80% conservation among the most divergent vertebrate species (i.e., Homo sapiens and the Fugu fish) (reviewed in Cattaneo et al., 2005; Zuccato et al., 2010). Remarkably, the N-terminal fragment of the protein containing the polyglutamine (polyQ) tract encoded by the CAG repeats, is the most recently evolved part of huntingtin, and seems to have appeared for the first time in fishes and it has been maintained during vertebrate evolution becoming highly polymorphic only in humans (Tartari et al., 2008). Therefore, it has been hypothesized that the N-terminal domain may be endowed with newly acquired neuronal activities while the C-terminal domain may be endowed with primordial activities in non neuronal tissues (Tartari et al., 2008).

5.3 Huntingtin Structure

Huntingtin is a 348-kDa protein. A schematic of the amino acid sequence of the protein is depected in Figure 24
The polyQ stretch in the human protein begins at the 18th amino acid and, as previously described, in unaffected individuals contains up to 35 glutamine residues (HD collaborative group, 1993). In 1994 the Nobel Laureate Max Perutz showed that the polyQ forms a polar zipper structure and suggested that its physiological function was to bind transcription factors also containing a polyQ region (Perutz et al., 1994). Since then, it has

been shown that huntingtin interacts with a large number of partners and that the polyQ tract is a key regulator of huntingtin binding to its interactors (Harjes and Wanker, 2003). In addition, X-ray crystallography at atomic resolution has shown that the polyQ region adopts multiple flexible conformations (a-helix, random coil and extended loop) (Kim et al., 2009). Therefore, huntingtin might be able to assume specific conformation and activities depending on its binding partners, subcellular localization, cell type and tissue, and, serving as a scaffold protein, the binding of different set of interactors may account for wild-type huntingtin function during development and in adulthood (Zuccato et al., 2010).

In higher vertebrates and therefore in mammals, the polyQ region is followed by a polyproline (polyP) stretch that has evolutionary emerged in concomitance with longer polyQ tracts (Steffan et al., 2004). Structural studies have suggested that the polyP domain may function in stabilizing the polyQ tract by preventing its conformational collapse and keeping it soluble (Kim et al., 2009).

Downstream the polyQ, three main different clusters of HEAT repeats have been identified (MacDonald, 2003). They are about 40 amino acids long sequence that usually occur multiple time with in a protein and are involved in protein-protein interactions (Andrade and Bork, 1995), further suggesting that huntingtin may exert its physiological function by binding different protein partners (see paragraph 5.4)

Huntingtin contains well-defined consensus sites for proteolytic cleavage of different enzymes, such as caspases (caspase 3 and 7, caspase 6 and caspase 2), calpain and aspartyl proteases, that generate a wide-range of protein fragments. The different cleavage sites and respective amino acid positions are depicted in Figure 2. The exact contribution of wild-type huntingtin proteolysis to cell functioning is still not clear. However, as discussed in the next section, reduction of mutant huntingtin proteolysis by caspases and calpains also diminish toxicity of the mutant protein and delay disease onset and progression (reviewed in Zuccato et al., 2010).

At the C-terminal of the protein both a nuclear export signal (NES) and a nuclear localization signal (NLS) are present, indicating that the protein shuttles between the nucleus and the cytosol and might be involved in transporting molecules from the nucleus to the cytoplasm (Xia et al., 2003). Moreover, the first 17 N-terminal amino acids before the polyQ stretch do form an amphipatic a-helical membrane-binding domain that is necessary

and sufficient for huntingtin association to mitocondria and for its colocalization with Golgi and endoplasmic reticulum, therefore regulating its subcellular localization. This sequence also enhances aggregate formation and promotes disregulation of calcium homeostasis (Atwal et al., 2007; Rockabrand et al., 2007)(see paragraph 5.4).

Huntingtin is subjected to several post-translational modifications. The protein is ubiquitinated at the N-terminal lysines K6, K9 and K15 and targeted to the proteasome (Figure 24) (DiFiglia et al., 1997; Kalchman et al., 1996).

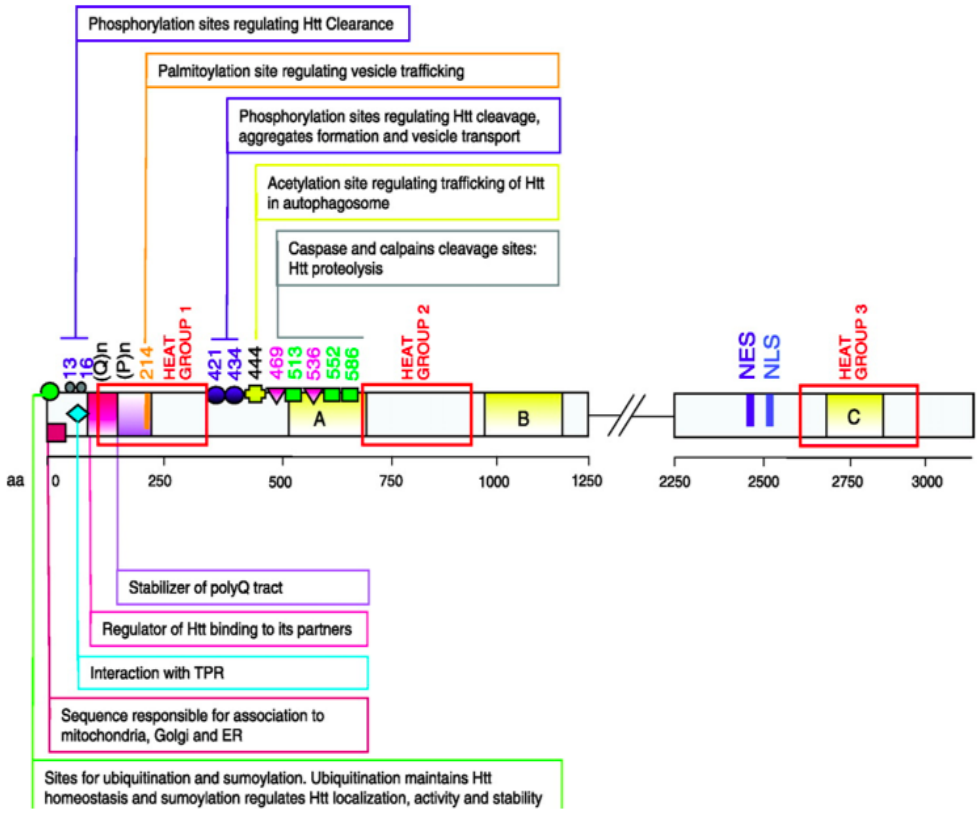

Figure 24 Schematic diagram of the huntingtin amino acid sequence. (Q)n indicates the polyglutamine tract, which is followed by the polyproline sequence (P)n; the red emptied rectangles indicate the three main groups of HEAT repeats (HEAT group 1, 2, 3). The small green rectangles indicate the caspase cleavage sites and their amino acid position (513, 552, 586), while the small pink triangles indicate the calpain cleavage sites and their amino acid positions (469, 536). Boxes in yellow: B, regions cleaved preferentially in the cerebral cortex; C, regions of the protein cleaved mainly in the striatum; A, regions cleaved in both. Posttranslational modifications: ubiquitination (UBI) and/or sumoylation (SUMO) sites (green); palmitoylation site (orange); phosphorylation at serines 13, 16, 421, and 434 (blue); acetylation at lysine 444 (yellow). NES is the nuclear export signal while NLS is the nuclear localization signal. The nuclear pore protein translocated promoter region (TPR, azure) is necessary for nuclear export. Htt, huntingtin. ER, endoplasmic reticulum. *From Zuccato C et al. Physiol Rev 2010;90:905-981*

In the presence of mutant huntingtin this process is impaired causing proteasomal dysfunction and accumulation of toxic huntingtin fragments. Phosphorylation of specific serine residues (depicted in figure 24), seems to confer neuroprotective properties to wild-type huntingtin and is responsible for huntingtin mediated transport of vesicles in neurons. Furthermore, phosphorylation of mutant huntingtin on different serine residues has been shown to reduce caspase and calpain-mediated cleavage resulting in reduced toxicity (reviewed in Zuccato et al., 2010).

5.4 Huntingtin functions and mutant huntingtin "disfunctions"

Huntingtin is ubiquitously expressed in humans and rodents, with the highest level in the CNS. It has a widespread subcellular distribution and associates to a variety of organelles including the nucleus, Golgi complex, endoplasmic reticulum and mitocondria. It is found within neurites and at the synapses where it is associated with different vesicular structures such as clathrin-coated vesicles, caveolae, endosomal compartments as well as microtubules.
Several studies reviewed in great details by Zuccato and colleagues (Zuccato et al., 2010) indicate that huntingtin has a variety of cell functions that are impaired and disregulated in presence of the mutant protein.

5.4.1 Embrionic development and prosurvival functions

Huntingtin is required at different steps of the embryonic development as total absence or a reduction of more that 50% generates very early phenotypes in mice. Knock out mice are not viable with death occurring at embryonic day 7.5 (before gastrulation and the formation of the nervous system) due to defects in the organization of extraembrionic tissues (Dragatsis et al., 1998). With the progression of embryonic development, in addition to its early extraembrionic functions, huntingtin becomes important for the formation of the CNS as shown by the fact that more then 50% reduction of the protein after gastrulation results in defects of the epiblast (the structure that will give rise to the neural tube) (Auerbach et al., 2001). In particular huntingtin seems critical for establishing and maintaining cortical and striatal

neuronal identity (Reiner et al., 1988; Godin et al., 2010) Human mutant huntingtin (e.g. via transgenic expression) can complement loss of function of endogenous huntingtin (e.g. via knockout) during development rescuing embryonic lethality, suggesting that huntingtin's function during development is independent of the polyQ length. HD patients, indeed, develop normally with the disease arising only in adulthood, however, it is not possible to exclude that a transient developmental defects might rend cortical and striatal neurons more vulnerable to the toxic effect of the mutant protein (Godin et al., 2010; Ross and Tabrizi, 2011) .
In post-mitotic cells and in adulthood wild-type huntingtin has a prosurvival role. In vitro studies have shown that overexpression of huntingtin confers protection against toxic stimuli whereas its depletion results in an increased sensitivity to apoptotic cell death with higher levels of caspase 3 activity (Zuccato et al., 2010). Indeed, it has been shown that huntingtin physically interacts with active caspase 3, thereby inhibiting its proteolytic activity (Zhang et al., 2003). In addition, neuronal inactivation of the huntingtin gene in adult mice caused apoptotic neuronal degeneration in different brain regions and the appearance of a phenotype (e.g. limb clasping upon tail suspension) that resembles the one of HD mouse models (Dragatsis et al., 2000). Also, Huntingtin seems to activate prosurvival pathways controlled by the serine/threonine kinase Akt (Humbert et al., 2002).

5.4.2 Gene regulation

Huntingtin directly interacts with protein of the transcriptional machinery and enzyme involved in chromatin remodeling. In HD the presence of mutant huntingtin results in a large number of gene expression changes (reviewed in Zuccato et al., 2010). A schematic of wild-type huntingtin interactions and of their dys-regulation in HD is proposed in figure 25.

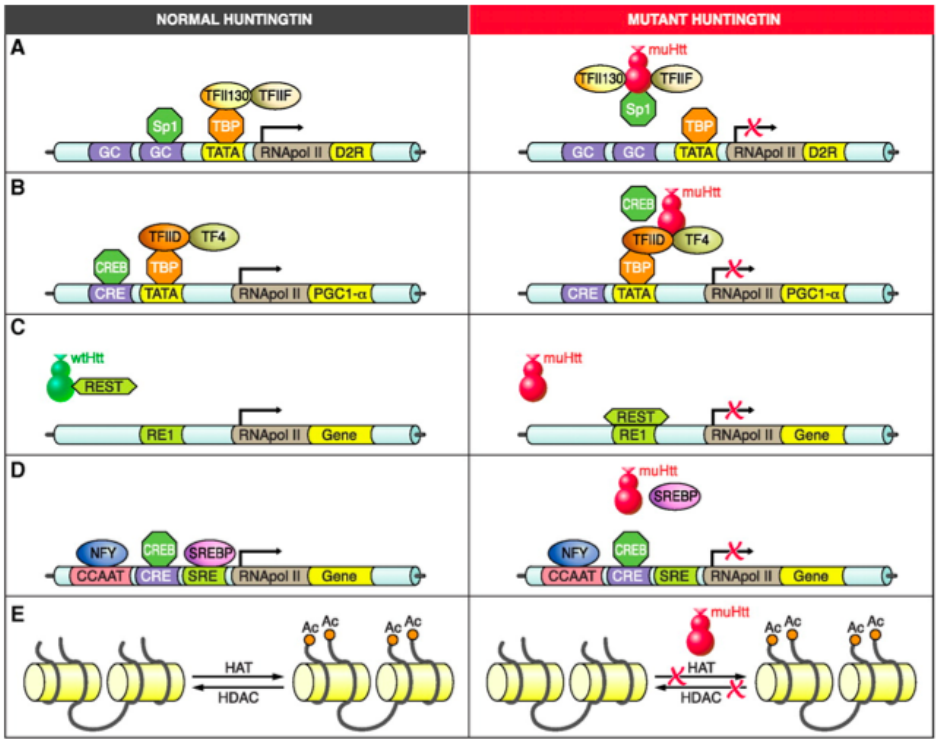

Figure 25 Transcription factors, DNA target sequences, and chromatin structure in HD. A: expanded polyQ in huntingtin represses transcription of Sp1-dependent promoters (i.e., D2R gene) by abnormally interacting with specific transcription cofactors such as Sp1 itself, TFIIF, and TFII130. B: the transcription factor cAMP-responsive element (CRE)-binding protein (CREB) binds to DNA elements that contain a CRE sequence, as in the promoter of the PGC1-α gene, a master regulator of genes involved in mitochondrial function and energy metabolism. Mutant huntingtin interferes with CREB and TFIID, leading to reduced activation of PGC1-α gene, reduced PGC1-α protein levels, and consequently, downregulation of its mitochondrial target genes. C: the transcription factor REST/NRSF binds to RE1/NRSE elements in neuronal gene promoters such as in the BDNF gene. Wild-type huntingtin sustains the production of BDNF by interacting with REST/NRSF in the cytoplasm, thereby reducing its availability in the nucleus to bind to RE1/NRSE sites. Under these conditions, transcription of BDNF and of other RE1/NRSE regulated neuronal genes is promoted. Mutant huntingtin fails to interact with REST/NRSF in the cytoplasm, which leads to increased levels of REST/NRSF in the nucleus. Under these conditions, REST/NRSF binds avidly to the RE1/NRSE sites, suppressing the transcription of BDNF and of other RE1/NRSE regulated neuronal genes. D: SREBP binds to SRE to regulate the transcription of genes involved in the cholesterol biosynthesis pathway. Under physiological conditions, SREBP is transported from the endoplasmic reticulum to the Golgi region, where it is cleaved to obtain a fragment that enters the nucleus and activates cholesterogenic genes. In the presence of mutant huntingtin, this mechanism is impaired, which leads to the reduced expression of SREBP-dependent genes and decreases the biological effects of cholesterol biosynthesis. E: levels of histone acetylation at specific lysine residues are determined by concurrent reactions of acetylation (Ac) and deacetylation, which are mediated by histone acetylases (HATs) and histone deacetylases (HDACs). Histone acetylation is vital for establishing the conformational structure of DNA-chromatin complexes suitable for transcriptional gene expression. Mutant huntingtin leads to disruptions in HAT and HDAC balance, leading to general transcriptional repression. wtHtt, wild-type huntingtin; muHtt, mutant huntingtin. *From Zuccato C et al. Physiol Rev 2010;90:905-981*

5.4.3 Regulation of BDNF production and vesicle transport

Brain-derived neurotrofic factor (BDNF) is a neurotrophin that is particularly important for the survival of striatal neurons and of the cortico-striatal synapses (Zuccato and Cattaneo, 2007). Most of the striatal BDNF is produced in the cerebral cortex and delivered to the striatal neurons via anterograde transport along the cortico-striatal afferents (Fusco et al., 2003; Baquet et al., 2004).
As depicted above in figure 25B, wild-type huntingtin promotes the expression of BDNF by acting at the level of BDNF gene transcription, thereby stimulating cortical BDNF protein production. In 2004 the group of Frederic Saudou proposed that huntingtin has also a role in promoting BDNF vesicles transport along the cortical-striatal afferents (Gauthier et al., 2004). Indeed, huntingtin was found to be part of the motor complex that drives anterograde and retrograde transport of BDNF vesicles along microtubules. In particular, huntingtin by forming a molecular complex with the huntingtin associated-protein 1 (HAP1) interacts with the p150Glued subunit of dynactin, thereby promoting retrograde transport, or alternatively with kinesin, thereby promoting anterograde transport of BDNF vesicles (Gauthier et al., 2004). The phosphorylation of huntingtin at serine-421 by Akt Kinase seems to be a crucial factor in the regulation of BDNF movement. Indeed, when phosphorylated at this position, huntingtin promotes anterograde transport whereas BDNF vesicles are more likely to undergo retrogade transport when huntingtin is not phosphorylated (Colin et al., 2008). In HD cortical BDNF is reduced both in mRNA and protein levels indicating a reduced production of the neurotrophin. As depicted in figure 25B, mutant huntingtin fails to sequester REST/NRSF (repressor element 1 silencing transcription factor/neuron-restrictive silencing factor) in the cytoplasm leading to its translocation in the nucleus and thereby activating the RE1/NSRE silencer with a consequent reduction of BDNF gene transcription. Moreover, mutant huntinting binding to HAP1 reduce HAP1/p150Glued association and affects also the complex with Kinesin resulting in a reduced BDNF vesicle transport (reviewed in Zuccato et al., 2010). In contrast with the report of Gauthier and colleagues, a recent study suggests that the alteration of BDNF vesicles transport in HD is not attributable

to a disruption of these motor protein complexes but rather may result from altered regulation of the intact compexes (Her and Goldstein, 2008). Regardless of the exact mechanisms, the reduction of BDNF synthesis and transport in HD is a fact and because of the importance of BDNF for medium spiny neurons in the striatum (the most affected population in HD) it has been proposed that loss of BDNF in the cerebral cortex may contribute to the striatal (and cortical) vulnerability in HD (see also chapter 6, paragraph 6.3).

5.4.4 Synaptic activity

Huntingtin is involved in the cell machinery that controls synaptic transmission interacting with a number of cytoskeletal and synaptic vesicle proteins that are essential for exo and endocytosis at synaptic terminals (Smith et al., 2005). One key molecule in synaptic transmission is PSD95 (postsynaptic density protein 95), a member of the membrane associated guanylate kinase family of proteins that binds the NMDA (N-methyl-D-aspartate) and kynate receptors at the postsynaptic density (Sheng and Kim, 2002). Wild-type huntingtin directly binds PSD95 (Leavitt et al., 2001). The decreased interaction of mutant huntingtin with PSD95 indicates that more PSD95 is released in HD, thus affecting the activity of NMDA receptor leading to their over activation or sensitization and exitotoxicity (Leavit et al., 2001). In addition mutant huntingtin has been reported to induce phosphorylation of the NR2B subunit of the NMDA receptor (NMDAR), promoting its overactivation. NR2B subunits are particularly abundant in the striatum and this may be one part of the preferential vulnerability of these cells in HD. Indeed, excitotoxicity was the first identified pathogenic mechanism proposed to explain the selective striatal vulnerability in HD. Excitotoxicity is defined as an excessive stimutation of excitatory amino acid receptors, expecially NMDAR, that leads to cell death. In the context of HD, excititoxicity results from processes that take place in the degenerating neurons due to the expression of the mutant protein (as discussed above), but also from dysfunction of neuronal interaction and circuits at the corticostrial synapse due to increased glutamate release from cortical afferents or reduced uptake of glutamate by glia (reviewed in Zuccato et al., 2010). This non-cell autonomous

pathogenic mechanism will be discussed in more details in the next chapter.
A schematic of the altered signaling pathways that results from cell autonomous and non cell autonomous overactivation of NMDARs is proposed in figure 26. They include alteration in calcium homeostasis, mitochondrial disfunctions, and activation of apoptotic pathways.

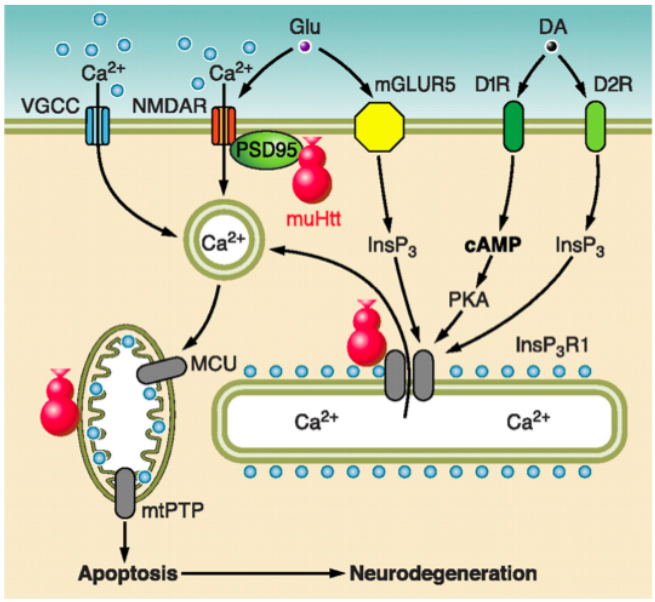

Figure 26 Dysfunction of Ca^{2+} signaling in HD. Mutant huntingtin causes cytosolic and mitochondrial Ca2+ overload and apoptosis of HD MSN. Mutant huntingtin perturbs Ca2+ signaling by enhancing NMDAR function, possibly through decreased interaction with the PSD95-NR1A/NR2B complex. Moreover, mutant huntingtin binds strongly to InsP3 R1, causing Ca2+ release through the InsP3 R1. Dopamine (DA) released from midbrain dopaminergic neurons stimulates D1 and D2 receptors (D1R, D2R). D1R are coupled to activation of adenyl cyclase, increase in cAMP levels, and activation of PKA. PKA potentiates glutamate-induced Ca2+ signals by facilitating the activity of NMDAR and InsP3 R1. D2R are coupled directly to InsP3 production and activation of InsP3 R1. Supranormal Ca2+ signals activate calpain, which cleave huntingtin and other substrates. Excessive cytosolic Ca2+ signals result also in mitochondrial Ca2+ uptake, which eventually triggers mtPTP opening and apoptosis. The mitochondrial Ca2+ handling is further destabilized by direct association of mutant huntingtin with mitochondria. muHtt, mutant huntingtin; MCU, mitochondrial calcium uniporter; mtPTP, mitochondrial permeability transition pore; VGCC, L-type voltage-gated calcium channel. *From Zuccato C et al. Physiol Rev 2010;90:905-981*

5.5 The process of aggregate formation

The pathological hallmark of HD is the formation of intracellular aggregates of mutant huntingtin called inclusion bodies (IBs). They occur both in the nucleus and the cytoplasm of affected neurons and show ubiquitin immoreactivity (Davies et al., 1997). The role of inclusion bodies as well as of protein aggregates in

other neurodegenerative disease is debated and will be discussed in chapter 6.
Two major aggregation pathways are in competition with each other and explain how the polyQ expansion can facilitate aggregation (Figure 27).

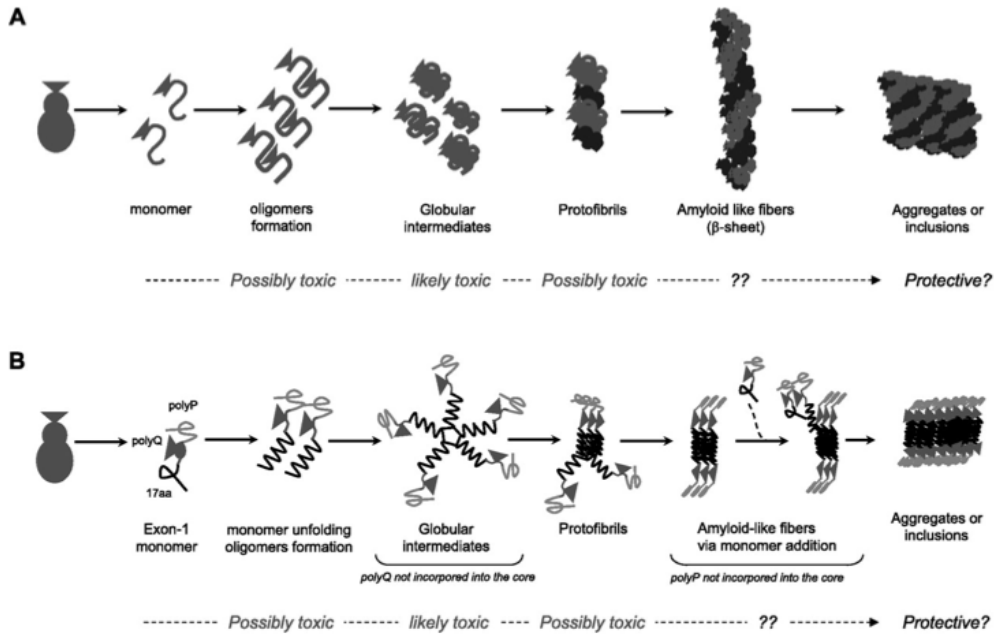

Figure 27 The process of aggregate formation. Two major aggregation pathways are in competition with each other and explain how the polyQ expansion can facilitate aggregation. A: in the first pathway, mutant huntingtin undergoes covalent modifications (posttranslational modification or cleavage), determining the conversion of the protein to an abnormal conformation. The mutant protein forms oligomer intermediates that then give rise to globular intermediates from which protofibrils are generated. Protofibril intermediates associate to produce amyloid like structures, resulting in aggregates or inclusions. B: in the second pathway, oligomers having the first 17 amino acids of the protein in its core and polyQ sequences exposed on the surface are formed. As the polyQ increases, the structure decompacts and oligomers or protofibrils rearrange into amyloid-like structures capable of rapidly propagating via monomer addition and producing aggregates. *From Zuccato C et al. Physiol Rev 2010;90:905-981*

The first pathway, described almost 10 years ago, is mediated by aggregation of the polyQ stretch (Bates, 2003; Ross et al., 2003) (Figure 27A). PolyQ aggregation displays kinetics of nucleated-growth polymerization with a prolonged lag-phase required for forming an aggregation nucleus, followed by a fast extension phase during which additional polyglutamine monomers rapidly join the growing aggregate. The aggregates consist of β-sheet-rich fibrils aligned side-by-side to form ribbonlike structures and exhibit several defining features of amyloid, such as binding to thioflavin T, Congo red birefringence, and reactivity with a generic antiamyloid antibody (reviewed in Zuccato et al., 2010).

The second pathway, recently elucidated by Ron Wetzel's group at the University of Pittsburgh (Thakur et al., 2009), depends on the first 17 NH2-terminal amino acids and involves intermediate structures. It is characterized by the formation of oligomers having the first 17 amino acids of the protein in its core and polyQ sequences exposed on the surface. As the polyQ increases, the structure decompacts and oligomers or protofibrils rearrange into amyloid-like structures capable of rapidly propagating via monomer addition (Thakur et al., 2009) (Figure 27B).
Cells have compensatory mechanisms against unfolded and abnormal proteins (Figure 28).

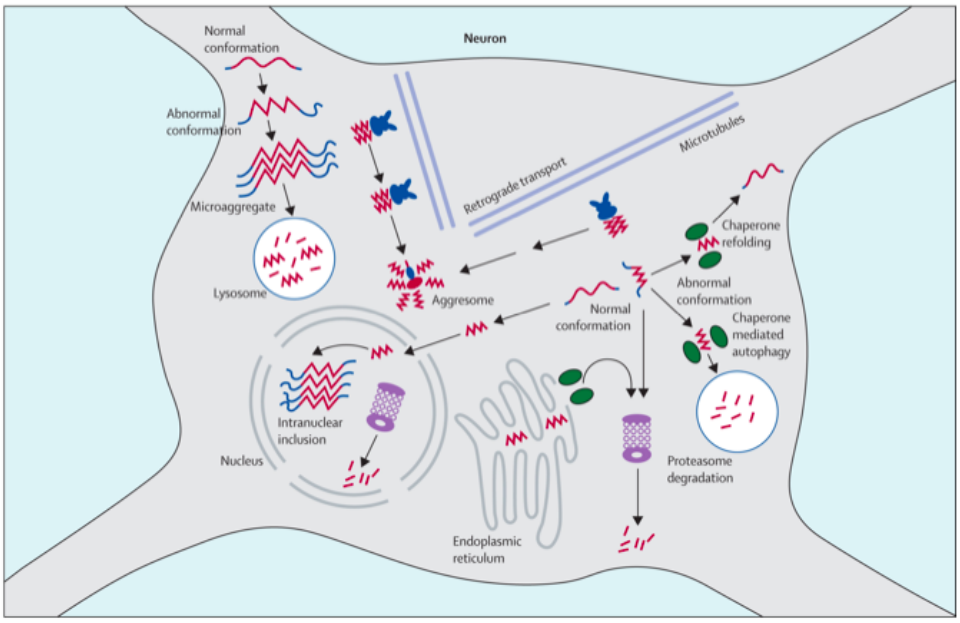

Figure 28 Cellular pathways possibly used as compensatory mechanisms in Huntington's disease. Cells can clear mutant HTT by proteasomal degradation, chaperone-mediate refolding, chaperone-mediated autophagy, and macro-autophagy. Active transport of aggregated HTT leads to inclusions in the cytoplasm; the mechanism of nuclear inclusion formation is less well understood. *From Ross and Tabrizi, 2011.*

Misfolded and aggregated proteins are recognized and coupled to the retrograde microtubule motor dynein for transport to a perinuclear aggresome (Kopito, 2000). Two major cellular pathways for degradation of misfolded proteins are the ubiquitin-proteasome system and autophagy (Kaganovich et al., 2008). Researchers have postulated that a toxic effect of mutant HTT could be to compromise ubiquitin-proteasome activity, thus leading to inclusion bodies formation (Bennett et al., 2005). Indeed, changes in the ubiquitin system in Huntington's disease mouse model and human post-mortem brain tissue have been reported (Bennett et al., 2007). Autophagy is a bulk degradation

process in which a portion of the cytosol and its content is enclosed by double-membrane structures called autophagosomes/autophagic vacuoles, which ultimately fuse with lysosomes for the degradation of the contents. It has been shown that mutant HTT can also interfere with target recognition and compromise autophagic clearance (Martinez-Vicente et al., 2010). Molecular chaperones can promote refolding of misfolded proteins. Indeed, overexpression of one or both of the chaperones HSP104 and HSP27 can suppress mutant HTT-mediated neurotoxicity in mouse and rat models of Huntington's disease (Perrin et al., 2007; Vacher et al., 2005).

In conclusion the current data indicate that wild-type huntingtin has beneficial activities in mature brain. It is therefore possible that its loss in HD reduces the ability of neurons to survive and counteract the toxic effects of the mutant protein. Loss of function (of the normal protein) as well as gain of function (of the mutant protein) are involved in HD pathogenesis. A schematic recapitulation of mutant huntingtin disfunction is presented in figure 29.

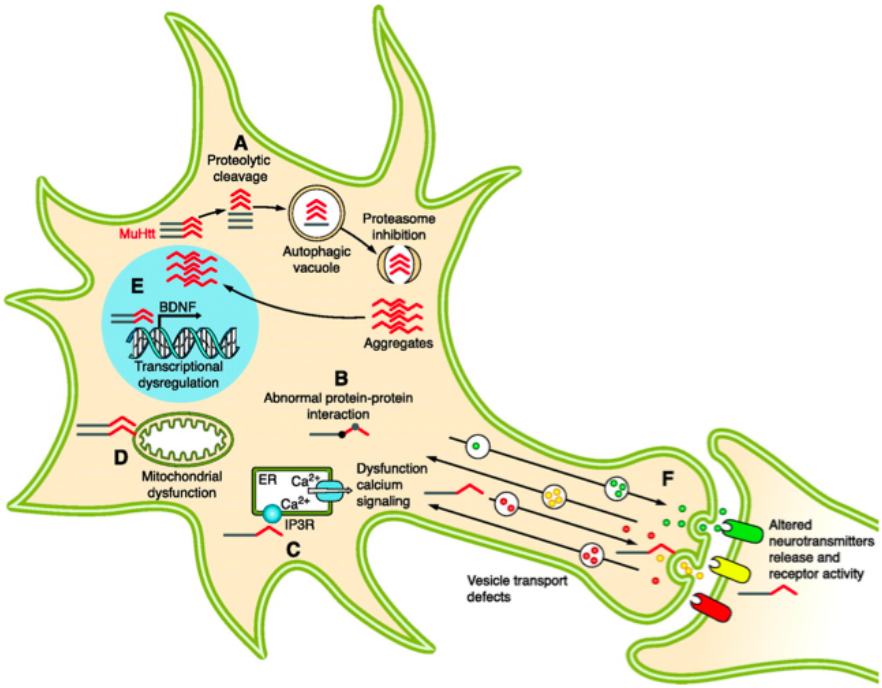

Figure 29 Key cellular pathogenic mechanisms in Huntington's disease (HD). Multiple cellular pathways have been implicated in the pathogenesis of HD. These mechanisms could be exclusive or, more likely, have a high degree of cross-talk. A: the mutation in huntingtin causes a conformational change of the protein that leads to partial unfolding or abnormal folding of the protein, which can be corrected by molecular chaperones. Full-length mutant huntingtin is cleaved by proteases in the cytoplasm. In an attempt to eliminate the toxic huntingtin, fragments are ubiquitinated and targeted to the proteasome for degradation. However, the proteasome becomes

less efficient in HD. Induction of the proteasome activity as well as of autophagy protects against the toxic insults of mutant huntingtin proteins by enhancing its clearance. B: NH2-terminal fragments containing the polyQ strech accumulate in the cell cytoplasm and interact with several proteins causing impairment of calcium signaling and homeostasis (C) and mitochondrial dysfunction (D). E: NH2-terminal mutant huntingtin fragments translocate to the nucleus where they impair gene transcription or form intranuclear inclusions. F: the mutation in huntingtin alters vesicular transport and recycling. muHtt, mutant huntingtin. *From Zuccato C et al. Physiol Rev 2010;90:905-981*

6. From protein aggregation to neurotoxicity: an imperfect fit

The concept of spreading and infectivity of protein aggregates in the central nervous system has been recently extended beyond prion diseases (for review see Lee et al., 2010; Brundin et al., 2010; Cushman et al., 2010; Frost and Diamond, 2010). As previously described in chapter 4, recent studies suggest that intercellular prion-like transmission mechanisms may be responsible for propagation of protein misfolding in non-prion neurodegenerative disorders. This might involve both secreted proteins such as amyloid-β and cytosolic proteins such as tau, huntingtin and alpha sinuclein, suggesting the existence of a general pathogenic principle in neurodegenerative proteinopathies. Yet, whether and how the propagation of protein aggregates is linked to neurotoxicity is not completely understood (Brundin et al., 2010; Lee et al., 2010). In Alzheimer's, Parkinson's, Huntington's disease and prion disorder neurodegeneration affects distinct regions of the brain reflecting a disease-specific vulnerability of particular neurons (reference from Ellison et al., 2004). However, in all the diseases mentioned above, the correlation between the brain regions that degenerate and the presence of protein aggregates deposition is weak (Lee et al., 2010; Treusch et al., 2009). Indeed, the presence of visible aggregates does not always correlate with cell death (Ross and Poirier, 2004, Treusch et al., 2009). For the purpose of this section, that is to introduce the second part of my PhD work, I will focus mainly on Huntington's disease and prion disorders. I will discuss the role of inclusion bodies and the mechanisms of toxicity.

6.1 Aggregates: toxic or protective species?

Studies in Huntington's disease brains have revealed a surprising discrepancy between the vulnerability of specific subsets of neurons and the localization of inclusion bodies (IBs) containing aggregated huntingtin. Indeed, it has been reported that at early stage of the disease, when cortical neuronal loss is low but striatal degeneration is already significant, IBs are much more common in the cerebral cortex then in the striatum (Gutekunst et

al., 1999). Furthermore, within the striatum, which is the most affected brain region IBs are enriched in the population of the large interneurons, which are spared, rather than in the medium spiny projecting neurons (MSNs) that are selectively lost (Kuemmerle et al., 1999). Therefore, neurons with inclusions do not correspond exactly to the neurons that degenerate. In addition, post-mortem autopsy of transplanted HD patients revealed that fetal grafts of striatal tissue were susceptible to neurodegeneration displaying increased caspase-3 activation, vacuolization and decreased structural integrity in the absence of abnormal huntingtin aggregation (Cicchetti et al., 2009). Thus, it appears that IB formation is dissociated from the vulnerability of different neuronal types and affected regions of HD brains (Arrasate and Finkbeiner, 2005; Ross and Poirier, 2004). However, in HD there is a positive correlation between the length of the CAG repeat and the density of the aggregates (Scherzinger et al., 1999) and within the expanded range, longer repeats cause early onset (Duyao et al., 1993), consistent with the hypothesis that aggregation of the protein is related to pathogenesis.

Which is then the role of inclusion bodies in HD? Are IBs toxic, incidental or the result of a beneficial coping response of affected cells?

One argument in favor of the toxic function of huntingtin (and in general of protein aggregates) is that they can physically sequester proteins critical for cell homeostasis that consequently may lose their physiological function (Preisinger et al., 1999). In particular IBs of mutant huntingtin have been shown to sequester transcriptional factors (McCampbell et al., 2000; Nucifora et al., 2001; Steffan et al., 2000), proteasomes or other ubiquitin-proteasome system (UBS) components (Donaldson et al., 2003). However, several studies reported that the extent of sequestration of these components into IBs was not biologically significant (Bennet et al., 2005; Yu et al., 2002) and that functional sequestration of transcription factor and UPS impairment can occur prior to IB formation (Bennet et al., 2005; Mitra et al., 2009; Schaffar et al., 2004) due to the presence of soluble mutant huntingtin (see chapter 5).

Moreover, several studies suggest that the formation of IBs is beneficial for cell survival. In primary striatal neurons, the formation of IBs was not sufficient to induce apoptosis. On the contrary, inhibition of the ubiquitinylation of mutant huntingtin prevented IB formation and actually increased cell death (Saudou

et al., 1998). Also, in a study by Steven Finkbeiner's group (Arrasate and Finkbeiner, 2005; Finkbeiner et al., 2006) a robotic microscope was used to follow the fate over time of thousands of individual primary neurons expressing the exon 1 fragment of huntingtin fused to GFP. It was shown that neurons bearing aggregates survived significantly longer than those without aggregates. In addition, studies in mouse model of HD expressing a CAG expanded huntingtin gene truncated after intron II, called Short-Stop mice, showed a widespread and frequent IB formation, but no evidence of neuronal dysfunction and degeneration (Slow et al., 2005), further confirming that huntingtin aggregation and toxicity are not connected.

6.2 Where does the toxicity come from? Is there a particular toxic structure?

Recent evidences coming from several independent studies of different proteins indicate that oligomers might be the most toxic species in pathogenesis (reviewed in Caughey and Lansbury, 2003; Glabe, 2006; Walsh and Selkoe, 2007). Soluble oligomers are small assemblies of misfolded proteins that are present in the soluble fraction of tissue extracts and usually include structures ranging in size from dimers to 24-mers (Glabe, 2006). Indeed, both synthetic and natural oligomers have been shown to induce apoptosis in cell cultures at very low concentrations (Demuro et al., 2005; Bucciantini et al., 2005; Simoneau et al., 2007), block long term potentiation in brain slice cultures (Wang et al., 2002) and impair synaptic plasticity and memory in animals (Cleary et al., 2004; Shankar et al., 2008). Fibrils can also elicit toxicity in cultured cells, but usually at much higher concentrations than oligomers and protofibrils (Caughey and Lansbury, 2003).
In the case of HD, in the same studies mentioned above by Steven Finkbeiner's group (Arrasate and Finkbeiner, 2005; Finkbeiner et al., 2006) the authors demonstrated that diffuse (soluble) form of mutant huntingtin (and not IBs) predicted neuronal death. Moreover, a decrease in soluble huntingtin was observed upon IB formation, suggesting that IBs may be a beneficial coping response that reduces the levels of toxic misfolded proteins in the soluble fraction by sequestering them in insoluble aggregates. Consistently, as mentioned above, the neurons bearing IBs had significant longer survival compared to those without aggregates (Arrasate and Finkbeiner, 2005;

Finkbeiner et al., 2006). Other studies also supported the hypothesis that small aggregates or even aberrantly folded monomeric forms of mutant huntingtin are toxic to cells (Arrasate et al., 2004; Bennett et al., 2007). The two possible pathways leading to aggregation of mutant huntingtin have been described before (see paragraph 5.5). They both involve the formation of intermediate globular assemblies that likely correspond to the toxic species (Zuccato el al., 2010).

Analysis of IBs purified from HD brains demonstrated the presence of a broad range of N-terminal fragments of mutant huntingtin (DiFiglia et al., 1997; Hoffner et al., 2005). Indeed, huntingtin is a substrate of proteolytic cleavage of several proteases including caspase-3, caspase-6 and calpain (see paragraph 5.3). The cleavage event generates specific N-terminal fragments containing the expanded polyQ tract that localize either in the nucleus or the cytoplasm (Lunkes et al., 2002). Biochemical analyses of nuclear and cytoplasmic inclusions showed that nuclear aggregates are composed mostly by the NH2 terminus of mutant huntingtin fragments (Hackam et al., 1998; Martindale et al., 1998; Cooper et al., 1998). On the other hand, extranuclear neuronal inclusions contain both full-length mutant and truncated huntingtin (Cooper et al., 1998; Hackam et al., 1998; Martindale et al., 1998). Interestingly, caspase 6 mediated cleavage seems to be a crucial event in HD pathogenesis. Transgenic mice expressing a full-length mutant huntingtin resistant to caspase 6 cleavage do not develop striatal atrophy (Graham et al., 2006). Accordingly to the toxic fragment hypothesis, the activation of caspase 6 may be a primary event in the proteolytic process of mutant huntingtin leading to the production of toxic fragments and to the activation of additional proteolytic caspase activities (e.g; activation of caspase 2 and 3) in a vicious cycle that exacerbate neurodegeneration and contribute to the appearance of the disease phenotype (Zuccato et al., 2010).

Conformational diversity of polyQ aggregates has been shown in a mouse model of HD (Nekooki-Machida et al., 2009). Aggregates purified from different brain regions showed distinct conformation once propagated in vitro by seeding reactions. Moreover, distinct conformers showed distinct toxicity when introduced in neuronal cells stably expressing a soluble expanded-polyQ fragment with conformers coming from the most affected brain regions (e.g. striatum) showing the higher toxicity (Nekooki-Machida et al., 2009). Interestingly, microarray

experiments showed that mRNA levels of chaperones and transcription factors that bind to expanded-polyQ htt are different between distinct brain regions (Hodges et al., 2006). Therefore, it is possible that different expression levels and types of chaperones and htt-interacting proteins modulate the extent of folding and dynamics of htt in distinct brain regions (Hodges et al., 2006). These differences, in turn, could lead to a range of htt conformations that have distinct cytotoxicity when htt protein misfolds. Taken together, these findings suggest that conformational differences of htt amyloids or most likely of soluble aggregated species may dictate the regional specificity of HD.

6.3 Cell autonomous and non cell autonomous degeneration

Besides the specific role of large visible protein aggregates, protein misfolding is a critical step in neurodegeneration. Indeed, once the capacity of neurons to handle misfolded protein species is exceeded, protein misfolding initiate a cascade of pathological events that includes mitochondrial disfunction, increase oxidative stress, alteration in calcium homeostasis and result in cell death (Saxena and Caroni, 2011; Williams and Paulson, 2008). As mentioned before, mutant proteins are ubiquitously expressed by neurons and non neuronal cells in the CNS, yet for each disorder neurodegeneration occurs in selective vulnerable cell population. Furthermore, mutant proteins are expressed throughout the patient's lifetime, but the related disease develops mainly in middle life or adulthood.
For many years, the events leading to neurodegeneration were believed to be entirely cell-autonomous, that is occurring independently in many cells (reviewed in Brundin et al., 2010; Garden and La Spada, 2012). However, the current hypothesis is that the disease-associated mutant proteins "result in cell-type specific dysfunction which individually do not cause the full spectrum of disease symptoms, but in concert and over time will result in the distinct patterns of neurological dysfunction and/or neurodegeneration that characterize a given disorder" (cit. Garden and La Spada, 2012). This synergistic form of cellular dysfunction via cell-cell interaction may account for both selective neuron loss and age dependence for a given disorder. This hypothesis is supported by numerous studies suggesting that the pathogenesis of neurodegenerative diseases involves

(mis)communication between different cell type and is consistent with the recent evidence of prion-like spreading of pathogenic misfolded proteins from cell-to-cell (Garden and La Spada, 2012). In this frame, prion like-transmission of protein misfolding (e.g toxic soluble oligomers) might contribute to the gradual spreading of the pathology in the brain of afflicted individuals leading to cell-type specific dysfunctions that in concert will determine the selective pattern of neurodegeneration characteristic for each disorder. When cell types other then the dying neurons themselves are critically involved in the degenerative process we are indeed in presence of a non-cell autonomous degeneration (Figure 30).

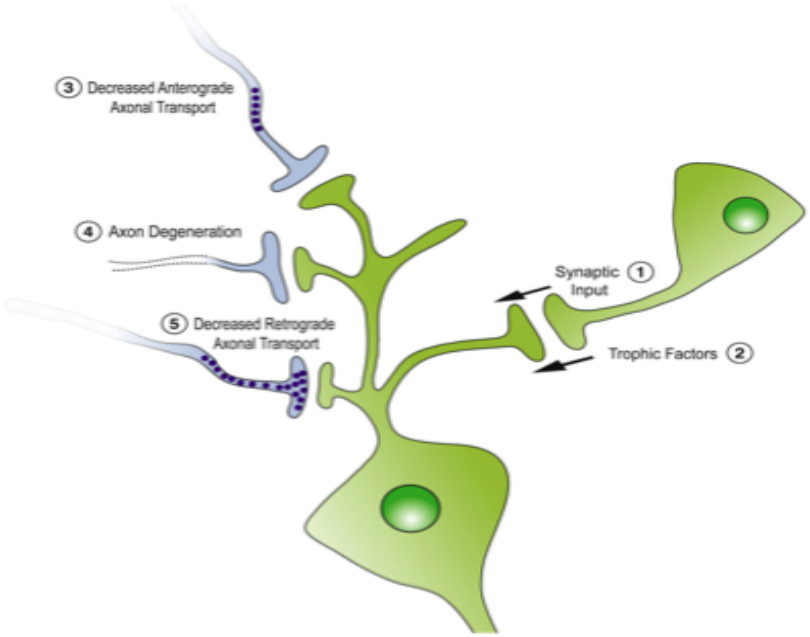

Figure 30 A variety of non-cell autonomous factors influence neuronal survival Neurons receive synaptic input (1), delivering both neurotransmitters and neurotrophic factors (2) that sustain neuronal survival. Neurodegenerative diseases can alter synaptic input by inhibiting anterograde axonal transport (3) and/or axon degeneration (4), resulting in decreased release of transmitters and neurotrophic peptides. (5) Failed retrograde transport as well as molecular dysfunction in target neurons or in non-neural target organs (e.g., muscle and blood vessels) can also damage presynaptic neurons, mimicking events that occur during development, when pathways of target-dependent neuronal survival are active. *From Garden and La Spada, 2012.*

Indeed, when the expression of a disease-associated protein was restricted to the respective vulnerable neuron population, the respective mouse models did not develop the complete disease phenotype (Brown et al., 2008; Yvert et al., 2000; Gu et al., 2005). On the contrary, widespread expression of the disease genes in mouse models recapitulates the features of the human

diseases, sometimes even when the disease gene is not expressed in the vulnerable neuronal population (Garden and La Spada, 2012). In the specific case of HD, when mutant huntingtin protein was expressed in a cell-type specific manner, cortical degeneration could not be achieved in a cell-autonomous manner (Gu et al., 2005).

Different types of interaction occur between neurons involving presynaptic input from one population of neurons to the postsynaptic target cells. Presynaptic inputs influence the funcion and health of their target neurons in a variety of ways including delivery of neurotrophic factors and regulation of synaptic activity. In the case of HD, neuronal interaction and circuits at the corticostriatal synapse are dysfunctional. Striatal neurons, the most affected neuronal population in HD, depend for their survival and activity on BDNF and glutamate release from the cortical afferents (Figure 31).

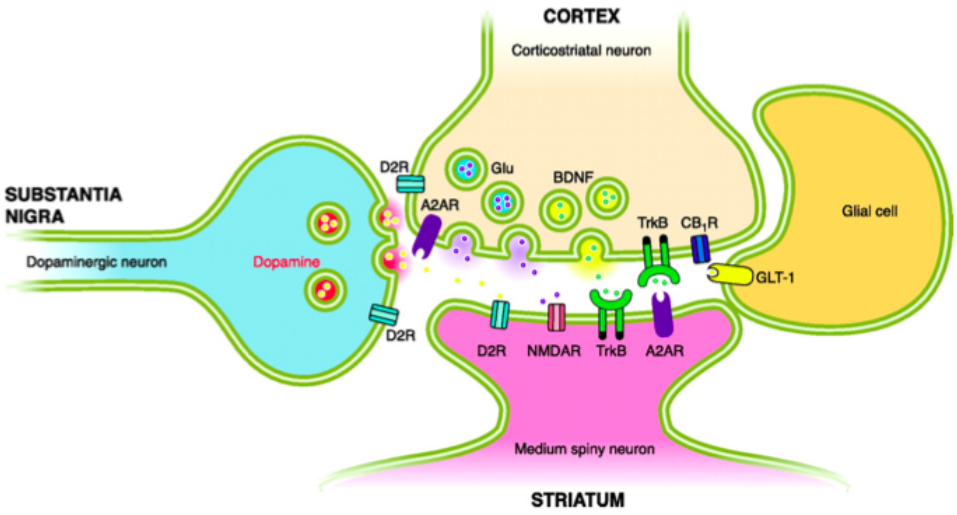

Figure 31 Neurotransmitter systems and growth factors that are dysfunctional at the corticostriatal pathway. Neuronal death may depend on excitotoxicity that results from increased glutamate (Glu) release from cortical neurons and increased activity of the glutamate receptor (NMDAR). In addition to glutamate, other neurotransmitter systems that control the activity of the corticostriatal synapse can contribute to render striatal neurons more sensitive to excitotoxic stimuli. Adenosine A2 receptors (A2AR) and cannabinoid receptors (CB1R) are particularly abundant on the corticostriatal terminals, where, when activated, they increase glutamate release. A crucial input to the striatum comes from the substantia nigra pars compacta, whose fibers represent the main striatal source of dopamine. Dopamine can directly regulate glutamate release from corticostriatal terminals by stimulating the D2 receptors (D2R) located on the cortical afferents. Glial cells may also play important roles through cell-cell interactions. For example, decreased glutamate uptake in glial cells by GLT-1 contributes to increased neuronal vulnerability and neuronal excitotoxicity in neurons. Reduced BDNF production and release from the cortical afferents contribute to neuronal death. Striatal BDNF is produced in the cortex where its transcription is downregulated in the presence of mutant huntingtin. In addition, mutant huntingtin

reduces BDNF vesicle transport. Both mechanisms result in loss of BDNF trophic support to striatal neurons. *From Zuccato C et al. Physiol Rev 2010;90:905-981*

As mentioned before (see paragraph 5.3), most of the striatal BDNF is produced in the cerebral cortex and delivered to the striatal neurons via the cortico-striatal afferents. Both HD patients and mouse models demonstrate reduced BDNF in the caudate and putamen (Canals et al., 2004; Ferrer et al., 2000), which result from the effect of mutant huntingtin on BDNF gene trascription and/or the anterograde transport of BDNF to the presynaptic terminal (Gauthier et al., 2004; Zuccato et al., 2001). A dysfunctional synaptic activity at the corticostriatal synapse, result in excessive activation of glutamate receptor, a phenomenon known as excitotoxicity, due to an increase glutamate release from cortical neurons and increased activity of the glutamate receptor (NMDAR) (Zuccato et al., 2010; Garden and La Spada, 2012) (Figure 31).
In the CNS non neuronal cell are also present and is now well established that astrocytes are not only support cells but they can sense and respond to neuronal activity as they possess receptor for neurotransmitters (Jourdain et al., 2007) and they facilitate rapid and efficient removal of neurotransmitters from the synaptic clefts. In particular, astrocytes play a role during glutammatergic transmission (Rossi and Volterra, 2009). Indeed, GLT1, the glial Na+-dependent transporter of glutamate is responsible for the removal of most extracellular glutamate, and there is mounting evidence that GLT1 actively participates in the regulation of synaptic transmission (Tzingounis and Wadiche, 2007). Decreased GLT1 mRNA and deficient glutamate uptake has been reported in post mortem brain tissues taken from HD patients (Arzberger et al., 1997; Hassel et al., 2008) as well as mouse models of HD (Behrens et al., 2002; Estrada-Sánchez et al., 2009; Shin et al., 2005). Therefore, aside from altered glutamate release from cortical afferents, impaired clearance of glutamate from glial cells at the synaptic cleft may contribute to enhance excitotoxic neurodegeneration in HD. A schematic of the non-cell autonomous mechanism responsible for selective neuron vulnerability in HD is proposed in figure 31.

6.4 From PrPC – PrPSc conversion to neurotoxicity: what is the link?

Plaque deposition may also be beneficial in the case of prion disease. Indeed, PrPres isoforms, the protease resistant forms of

PrP that include amyloid, are not toxic on their own (Brandner et al., 1996). PrPC knockout (Prnp0/0) mice are completely resistant to prion infection upon intracerebral injection of even very high doses of PrPSc from infected brain homogenate (Bueler et al., 1993). Equally striking, in early studies transgenic (Tg) mice expressing low levels of a secreted form of PrP, GPI anchor-less PrP(ΔGPI) did not develop signs of spontaneous neurologic illness, but accumulated massive plaque-like amyloidogenic PrP(ΔGPI) deposits after exposure to prions in absence of any clinical manifestations of prion disease (Chesebro et al., 2005). Notably, it has been recently shown that Tg mice expressing high levels of PrP(ΔGPI) (almost 2 folds compared with PrPC expression in wild-type mice) develop a late onset, spontaneous neurologic illness accompanied by widespread amyloid deposition in the brain and disease is accellerated by co-expression of full-length wild-type PrPC, with incubation times inversely correlated to PrPC expression levels (Stöhr et al., 2011). Furthermore, using human tissue samples, the Baron laboratory showed that the accumulation of certain forms of PrPSc did not result in spongiform degeneration (Piccardo et al., 2007). Brain extracts from two cases of familial prion disease were used to test the transmission of disease to transgenic mice. One of the samples exhibited PrPSc deposits and spongiform changes, while the other presented PrPSc deposits and no spongiform changes. Brain extract from the patient without spongiform degeneration did not result in disease transmission but elicited PrPSc deposition in large multicentric plaques. Based on these evidences, the author concluded that PrPres was rendered nonpathogenic by its sequestration in amyloid plaques (Piccardo et al., 2007). Thus, as for Huntington's diseases and other non-prion neurodegenerative disorders also in prion diseases the presence of large amyloid deposits is distinct from neuronal degeneration with prion toxicity possibly linked to a heterogeneous "toxic cloud" of oligomeric species (Aguzzi and Falsig, 2012).

Understanding how PrPSc actually leads to neurodegeneration following neurotoxicity is still an open question in prion biology (Aguzzi and Falsig, 2012). Despite the fact that apoptosis and oxidative stress have been shown to contribute to TSE pathology (Milhavet and Lehmann, 2002), little is known about primary events causing damage (Aguzzi et al., 2008). Indeed, PrPSc requires physiologically active PrPC on the cell membrane to exert his toxicity (Harris and True, 2006). Thus, PrPC has a dual role in the pathogenesis of prion diseases by virtue of its ability to be

required both for the generation of PrPSc molecules and for PrPSc-induced neurodegeneration. Moreover, it is still unclear weather PrPSc toxicity is the result of a gain of function of PrPSc or of a loss of function of PrPC and which is the responsible factor for the neuropathological changes induced by prions. Some reports that have addressed this question rather support a loss of PrPC function (Nazor et al., 2007). But, based on the mild phenotype of PrPC knockout mice, other groups belive that a gain of function is more conceivable (Westergard et al 2007; Aguzzi et al., 2008). A possibility is also that the normal neuroprotective role for PrPC would be needed during brain damage and therefore could be missing in prion diseases due to PrPC conversion in the pathological counterpart. A third possibility could be that in presence of PrPSc, the cellular PrPC function is subverted and it could trigger toxic signals through pathways not related with its physiological function (subversion of function) (Figure 32).

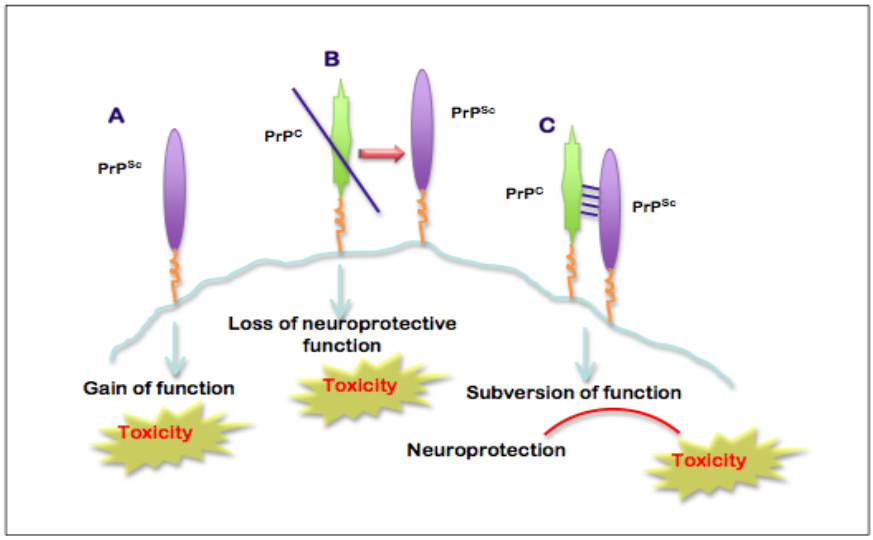

Figure 32 PrP-mediated neurotoxicity. (A) Toxic gain-of-function mechanism. PrPSc possesses a novel neurotoxic activity that is independent of the normal function of PrPC. (B) Loss-of-function mechanism. PrPC possesses a normal, physiological activity, in this case neuroprotection, that is lost upon conversion to PrPSc. (C) Subversion-of-function mechanism. The normal, neuroprotective activity of PrPC is subverted by binding to PrPSc . *Modified from Harris and True 2006*

To date, several reports suggest that these three possibilities might co-exist to different extent in the diverse forms of prion diseases and they all lead to neurodegeneration.

6.4.1 Gain of function through formation of PrPSc

Although the presence of PrPSc is the hallmark of prion diseases, it is highly debated whether prion pathology could really be attributed only to a toxic gain of function. In this context, newly formed PrPSc presents novel properties unrelated with the physiological role of PrPC and PrPSc deposits might interfere with synaptic transmission or block of the axonal transfer (Westergard et al 2007). Some reports have suggested that both full-length PrPSc (Hetz et al., 2003) and shorter PrP peptides are toxic to primary neuronal cultures in vitro (Forloni et al., 1993), but their relevance to *in vivo* pathogenesis is under debate. Nonetheless, from other experimental evidences it is unlikely that accumulation of extra-neuronal PrPSc aggregates is the only responsible factor for neurotoxicity. Indeed, when neural tissue over-expressing WT PrPC is grafted into mice lacking PrP, prion infection of the mice leads to increase in PrPSc levels and neurodegeneration only in the PrPC-expressing graft (Brandner et al., 1996). Furthermore, acute ablation of endogenous PrPC in prion-infected mice has been demonstrated to reverse early spongiform change preventing neuronal loss and progression to clinical disease, even in presence of extra-neuronal PrPSc (Mallucci et al., 2003).
Moreover, as mentioned before, prion-infected transgenic mice expressing PrPC without a GPI anchor produce infectious prions, accumulate extracellular PrP amyloid plaques, but do not succumb to the disease (Chesebro et al., 2005), while Tg mice expressing high level of anchor-less PrP developed a spontaneous neurological illness that is accelerated by co-expressing full-length wild-type PrPC (Stöhr et al., 2011). Finally, it has also been described that in some cases PrPSc accumulation does not lead to clinical symptoms (Hill et al., 2000; Race et al., 2002; Hill and Collinge, 2003).

6.4.2 Loss or subversion of PrPC function

A plethora of cellular functions have been attributed to PrPC but its physiological role appears to be redundant, since PrP knock-out mice are vital and do not present severe abnormalities (Bueler et al., 1993; Manson et al., 1994b). However, a growing number of studies implicates PrPC in diverse cellular processes (Nicolas et al., 2009) like cellular resistance to oxidative stress (Milhavet and Lehmann, 2002) and cytoprotection through anti-

apoptotic activity (Kuwahara et al., 1999; Bounhar et al., 2001) involving both its N terminal and central regions. PrPC has also been involved in cell signalling (Mouillet-Richard et al., 2000), copper and zinc metabolism (Watt and Hooper, 2003; Pauly and Harris, 1998), synaptic transmission (Collinge et al., 1994), myelin homeostasis (Bremer et al., 2010; Benvegnù et al., 2011) and in cytoskeleton dynamic and remodeling and in cell-to-cell adhesion (Malaga-Trillo 2009; Schrock et al., 2009; Chiesa and Harris, 2009). This is in agreement with previous observations made for mammalian PrPC in which a role for this protein in neurite outgrowth and cell-cell interaction, respectively in hippocampal neurons and neuroblastoma cells has been reported (Schmitt-Ulms et al., 2001; Santuccione et al., 2005).

A loss in any of these functions could theoretically lead to neurodegeneration. In particular, loss of its anti-apoptotic role could directly be related to toxicity and neuronal death. For example, neurons derived from mice lacking PrPC were originally reported to be more susceptible to apoptosis mediated by serum deprivation and this phenotype could be rescued by over-expressing either PrPC or B-cell lymphoma protein 2 (Blc2) (Kuwahara et al., 1999). Also, overexpression of Bax, a stimulator of the apoptotic pathway, together with PrPC leads to a decrease in the rate of apoptosis in human neurons (Bounhar et al 2001).

A third possibility is that alteration in PrPC normal function is achieved by contact with PrPSc, thus leading to a toxic signal cascade and inducing a subversion of its normal activity. Consistent with this hypothesis, cross-linking of PrPC at the cell surface with anti-PrP antibodies induces apoptosis of the CNS neurons *in vivo* (Solforosi et al 2004). Additionally, binding of PrPSc could interact with specific region of PrPC necessary for its normal function, thus stimulating altered activities. Accordingly, transgenic mice overexpressing PrP harboring a deletion in a portion in the N-terminal tail (Tg(PrPΔ105-125)) exhibit a severe neurodegenerative illness that is lethal within one week of birth (Li et al 2007). This toxic PrPC mutant (PrPΔ105-125) has been shown to induce a nonselective transmembrane ion current in HEK293 cells that was suppressed by coexpression of full-length PrPC or by co-deletion of the N-terminal charged domain (PrPΔ23-32) (Solomon et al., 2010). Indeed, the N-terminal polybasic domain (residues 23-31) has been implicated in the neurotoxicity of PrP mutants (Biasini et al., 2012). Ion currents were induced by PrP mutants in cells of many different species,

suggesting the formation of PrP-mediated pores across membranes that may account for neurotoxicity (Solomon et al., 2011, 2012). Yet currently no evidence exists that PrP can induce pore formation *in vivo* following prion infections. It is interesting to notice that a common feature of transgenic mice expressing PrP deletion mutant is that co-expression of wild-type PrPC abrogates clinical symptoms and neuropathology with more toxic mutations requiring higher doses of wild-type PrPC to rescue the phenotype (Biasini et al., 2012). Indeed, given that PrPC is an extracellular GPI-anchored protein, this highly conserved region could be an important binding site for a putative cell-surface interactor mediating PrPC function that in presence of PrPSc is masked or subverted (Westergard et al 2007) (Figure 33).

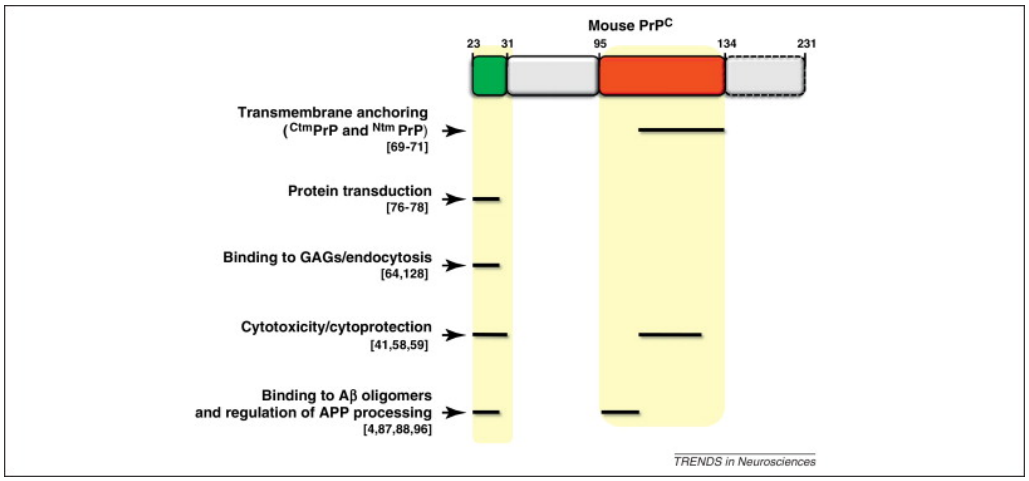

Figure 33 PrP-mediated neurotoxicity.(A) Toxic gain-of-function mechanism. PrPSc possesses a novel neurotoxic activity that is independent of the normal function of PrPC. (B) Loss-of-function mechanism. PrPC possesses a normal, physiological activity, in this case neuroprotection, that is lost upon conversion to PrPSc. (C) Subversion-of-function mechanism. The normal, neuroprotective activity of PrPC is subverted by binding to PrPSc . Modified from Harris and True 2006

Interaction of PrP with a number of proteins have been described including NMDA and GABA receptor subunits (Aguzzi and Calella 2009; Khosravani et al., 2008; You et al., 2012) et al., 2008; You et al., 2012). Although none of these proteins were proven to be involved in prion-induced neurodegeneration (Aguzzi and Falsig, 2012), some of them might have a role in PrPC-mediated toxicity in non-prion neurodegenerative diseases (You et al., 2012) (see below, paragraph 6.5). Many of the alleged interactor partners localize to different cellular compartments, suggesting that atypical PrP topologies may participate to the pathogenesis (Aguzzi and Falsig, 2012). Indeed, abnormal topology or altered

trafficking of PrPC could in part explain PrP-related neuronal toxicity in the absence of PrPSc formation (Aguzzi and Calella, 2009). For example, targeting of PrPC to the cytosol results in rapid lethal neurodegeneration (in the absence of PrPSc) and proteasome inhibition induces a slightly protease-resistant PrP species in cultured cells (Ma and Lindquist, 2002; Ma et al., 2002). Moreover, two topological variants of PrPC have been described, designated CtmPrP and NtmPrP, with their N- or C-termini respectively on the extracellular side of the membrane (Hegde et al., 1998; Stewart and Harris, 2003). Expression of these mutants in transgenic mice induces neurodegenerative phenotypes (Stewart and Harris, 2003; Rane et al., 2008) Interestingly, the phenotype associated with one of these mutants is dependent on the co-expression of wild-type PrPC, suggesting that CtmPrP subverts the normal function of PrPC function to generate toxicity (Stewart et al., 2005). Although numerous studies have provided important information about the function of PrPC, this issue has not been clarified. Until we have a clear understanding of the function of PrPC it will be difficult to understand the mechanism that leads to the pathogenesis of the disease. Therefore more studies at single cell level to understand the cell biology of PrPC and PrPSc are needed.

6.5 Is PrP the road to ruin? Lesson from β -Amyloid

Recent data have suggested that PrPC is a receptor for β-amymoid (Aβ) oligomers and may also mediate the toxic effects of these assemblies (rewied in Biasini et al., 2012). In the initial study by Lauren and coworkers (Lauren et al., 2009), PrPC was identified from an expression cloning screening as a high (nanomolar) affinity receptor for Aβ oligomers. Interestingly, PrPC binding was specific for oligomers since was not observed with Aβ monomers or fibrils. Importantly, PrPC was also found to be a mediator of Aβ-induced totoxicity. Indeed, hippocampal slices derived from PrPC knockout mice did not develop Aβ oligomer-induced suppression of long-term potentiation (LTP), a characteristic feature of amyloid-β toxicity. The same group moved from in vitro and ex vivo approaches to *in vivo* study by crossing PrPC knockout mice with a transgenic mouse model AD (Gimbel et al., 2010). The authors showed that PrPC was required for both the cognitive deficits and reduced survival in AD mice, although the presence of PrPC did not influence the

rate of Aβ plaque formation in the brain. Together these findings further support the distinction between protein aggregates deposition and toxicity with the latter being dependent on small olygomeric assemblies rather then large amyloids (Benilova et al., 2012). In addition, the cellular prion protein appears to be an essential mediator of the Aβ neurodegenerative process upon binding of the toxic oligomeric species.
Several studies confirmed the initial finding that PrPC plays a role in mediating the toxicity (e.g. suppression of LTP) induced by Aβ oligomers (Bate and Williams, 2011) and have provided additional evidence showing that immunotargeting PrPC in vitro or *in vivo* can rescue Aβ-dependent toxic effects (Chung et al., 2010; Freir et al., 2011; Barry et al., 2011). Interestingly one of the proposed mechanisms by which Aβ oligomers induce PrP-mediated toxicity is by crosslinking PrPC molecules similarly to what is found in prion diseases (Bate and Williams, 2011).
By contrast, several other studies reported opposite results. In particular, Forloni and colleagues showed that synthetic Aβ oligomers injected intraventricularly into mice impaired consolidation of long-term recognition memory regardless of the expression of PrPC (Balducci et al., 2010). In two other recent studies, genetic ablation of PrPC had no effect on Aβ-induced inhibition of hippocampal LTP in brain slices (Calella et al., 2010) or learning and memory deficits in a line of AD transgenic mice expressing mutant APP (reviewed in Biasini et al., 2012)

Several possibilities may explain the contrasting data emerging from these studies. First the preparation of synthetic Aβ oligomers is notoriously challenging and the product obtained can differ from one laboratory to another (Rahimi et al., 2008; Finder and Glockshuber, 2007). It is possible that only a specific conformation or size of synthetic oligomers operates through a PrPC-dependent mechanism and only same preparation, but not others, may contain the toxic species. Regarding the *in vivo* results the use of different transgenic models of AD might explain the contrasting results. Given these uncertainties, the role of PrPC in mediating the synaptic toxicity of Aβ requires further clarification (Biasini et al., 2011; Benilova et al., 2012).
Neverthless, all the studies mentioned above confirmed the ability of synthetic Aβ oligomers to bind PrPC with high affinity. Based on deletion analysis, antibody inhibition and biophysical techniques, two distinct Aβ oligomer binding sites have been identified on PrPC corresponding respectively to the residues 95–

105, the central domain, and 23–27, the N-terminal polybasic domain (Lauren et al., 2009). Remarkably, these two sites are coincident with or included in the regions that are important determinants of PrPC activity and toxicity (as discussed above in paragraph 6.4.2). Moreover, the N-terminal polybasic domain has been implicated in internalization of PrP via clathrin-mediated endocytosis (Taylor et al., 2005). Indeed, the same structural domains that govern PrPC function, cellular trafficking and toxicity are also involved in the binding to Aβ oligomers (Figure 33). Interestingly, another recent study showed that Aβ oligomers can affect the localization of PrPC by increasing the formation of clusters of PrPC on the cell surface (Caetano et al., 2011).
Furthermore, a recent study provided evidence that PrPC could mediate not only the toxicity of Aβ oligomers, but also of other β-sheet-rich protein conformers and that toxicity was prevented by NMDA receptor antagonists (Resenberger et al., 2011) (Figure 34).

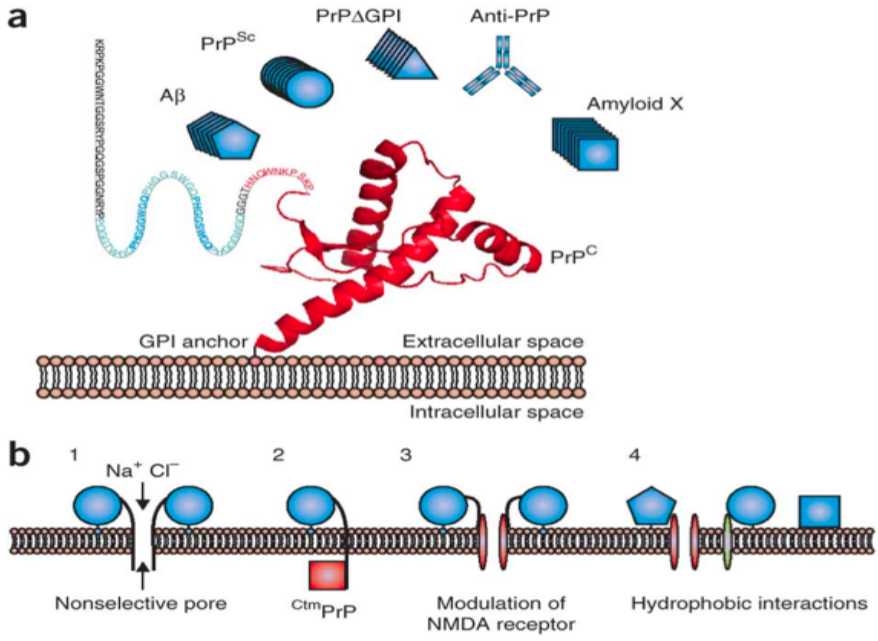

Figure 34 PrP-mediated amyloid toxicity (a) Potentially toxic ligands of PrPC binding to amino acids 95–105 (letters highlighted in red). PrPΔGPI, GPI-deficient PrP. (b) Possible mechanism of PrP toxicity. (1) PrP-mediated pore formation leading to nonselective conductance of ion across neuronal membrane. (2) CtmPrP spanning the lipid bilayer, resulting in the corruption of the normal function of cytosolic proteins by aberrant PrP interactions. (3) Specific modulation by PrP of NMDA receptor function, leading to excitotoxicity. (4) Hydrophobic, nonselective interactions of amyloids of undefined length with membrane proteins and lipids leading to a general corruption of membrane homeostasis. *From Aguzzi and Falsig, 2012.*

As discussed before (see chapter 1), different unrelated disease-assosiated protein aggregates share similar conformation properties consistent in ordered assemblies of β-sheets or cross-β spine. Moreover, the most toxic species appear to be small oligomeric assemblies rather than large fibrils (reviewed in Caughey and Lansbury, 2003; Glabe, 2006; Walsh and Selkoe, 2007). Therefore it is tempting to speculate the existence of a common mechanism for toxicity with PrPC functioning as a "danger sensor" (Aguzzi and Falsig, 2012). Indeed, it is possible that oligomeric forms of several different neurotoxic proteins could exert their effects by blocking, enhancing or subverting the normal function of PrPC as its pathogenic counterpart, PrPSc, does. Thus, binding of either oligomeric Aβ or PrPSc or other pathogenic aggregates to cell-surface PrPC may initiate toxic signals that lead to neuronal loss and/or synaptic dysfunction (Aguzzi and Falsig, 2012) (Figure 34).

In this scenario, two different studies from Zamponi's group have recently demonstrated the functional and physical interaction between PrPC and NMDA receptors (NMDARs) (Khosravani et al., 2008; You et al., 2011). Indeed, excessive NMDAR activity resulting in calcium overload and excitotoxicity, has been implicated in the pathophysiology of several neurodegenerative disorders including Huntington's disease (as described above in paragraph 6.3) and Alzheimer's disease (Lipton and Rosenberg, 1994; Kalia et al., 2008). In particular, hippocampal neurons from PrP knockout mice were found to display enhanced NMDA-induced currents, an effect that was reversed by overexpression of PrPC (Khosravani et al., 2008). Indeed, PrPC has been shown to co-immunoprecipitate with the NRD2 subunits of NMDARs (which are enriched in the striatum) as well as with NR1 subunits (which are common to all subtypes of NMDARs) suggesting a direct modulation of NMDA receptors by PrPC and the existence of an NMDAR–PrPC-signaling complex. (Khosravani et al., 2008; You et al., 2011). It appears that under physiological conditions, PrPC, in its copper-loaded state, reduces glycine affinity for the NMDA receptor complex, thus enhancing NMDAR desensitization and limiting calcium flux through the receptor. When copper is chelated or when PrPC is absent or functionally compromised by binding to Aβ oligomers for exemple, glycine affinity is enhanced, reducing receptor desensitization and producing pathologically large, steady-state currents that contribute to neuronal damage (You et al., 2011). Therefore a physiological role for PrPC may be

to limit excessive NMDAR activity preventing neuronal damage. This findings provides a molecular mechanism whereby toxic oligomeric species mediate neuronal and synaptic injury, at least in part, by disrupting the normal copper-mediated, PrPC dependent inhibition of excessive activity of this highly calcium permeable glutamate receptor.

AIMS OF THE STUDY

Aims of the study

The pathogenesis of most neurodegenerative diseases, including transmissible diseases like prion encephalopathies, inherited disorders like Huntington's disease, and sporadic diseases like Alzheimer's and Parkinson's diseases, is intimately linked to the formation of fibrillar protein aggregates. For many years, the concept of spreading and infectivity of the aggregates has been confined to prion diseases. Misfolded protein aggregates are "infectious" if they propagate from one cell to another and if in the recipient cell they act as "seeds" initiating aggregate formation by recruiting additional unfolded or oligomeric species of the same protein (Brundin et al., 2010). Both these characteristics are typical of prions and are essential for the self-replication (Caughey and Lansbury, 2003). For example, spreading of the misfolded prion protein PrPSc from the gut to the brain and within the brain is required for prion replication and diffusion of spongiform pathology (Prusiner 1994; Aguzzi and Calella, 2009). It is becoming increasingly appreciated that both extracellular (e.g. amyloid-β) and intracellular (α-synuclein, tau, huntingtin) protein amyloids are able to move and possibly replicate within the brains of affected individuals thereby contributing to the spread of pathology in a prion-like manner (Brundin et al., 2010; Jucker and Walker, 2011, Aguzzi and Rajendran, 2009; Soto et al., 2006).

Recently another intriguing connection has been made between prions and other aggregation proteinopathies as it was suggested that the cellular prion protein, PrPC, whose pathological counterpart is responsible for prion diseases, possibly mediates the toxicity of Aβ, the pathogenic protein in Alzheimer's disease, and of other β-conformers independently of infectious prion propagation (Biasini et al., 2011; Bate and Williams, 2011; Resenberger et al. 2011).

Despite the intense research, many questions in prion and non-prion neurodegenerative diseases are still open regarding both the mechanism of protein aggregate spreading and the mechanism of toxicity. Furthermore, exploring some of these aspects in prion biology could also provide a better understanding of non prion neurodegenerative disorders.

My PhD work has been focused on two major parts concerning respectively the mechanism of spreading and toxicity of prion and Huntington's disease as follows:

PART 1 Characterization of the role of dendritic cells in prion transfer to primary neurons: an insight in the mechanisms of prion spreading.

PART 2

- Project 1: Characterization of the mechanisms of polyglutamine aggregates transfer in neuronal cells and primary neurons
- Project 2: Role of the cellular prion protein in the pathogenic pathways of Huntington's disease.

PART 1:

Characterization of the role of dendritic cells in prion transfer to primary neurons: an insight in the mechanisms of prion spreading

After oral exposure, PrPSc accumulates into lymphoid tissues, such as the spleen, lymph nodes or Peyer's patches, prior to neuroinvasion (Beekes and McBride, 2000; Prinz et al., 2003). The exact mechanisms and specific cell types involved in the spreading from the gastrointestinal track to the lymphoid system and to the peripheral nervous system (PNS), leading to neuroinvasion of the CNS are not completely understood. A number of studies have demonstrated that FDCs play a critical role during spreading of infection (reviewed in Mabbott and MacPherson, 2006). However, because FDCs are immobile cells, it is not clear how they may acquire PrPSc and how it could spread from the FDCs to the PNS, since there is no physical contact between the gut and FDCs and between FDCs and nerve periphery (Defaweux et al., 2005). Dendritic cells (DCs) have been proposed to play a critical role in the transport of PrPSc from the gut to FDCs (Huang et al., 2002). In addition, DCs might also be able to promote prion transfer to nerve cells by direct contact with peripheral nerve fibers (Defaweux et al., 2005; Dorban et al., 2010) or through tunneling nanotube (TNT)-like structures (Gousset et al., 2009; Dorban et al., 2010).

In this context, in the first part of my PhD work, I pursued three specific objectives in order to better understand whether and by which mechanisms DCs would contribute to prion spreading:

a. To characterize the kinetic of prion uptake and degradation in bone-marrow-derived DCs (BMDCs)
b. To investigate the mechanisms by which BMDCs can transfer PrPSc to primary neurons
c. To examine whether BMDCs efficiently transfer prion infectivity to primary neurons

This first part of my results have been performed in collaboration with Dr Langevin, a former post-doc in Dr Zurzolo's lab and resulted in a publication in Biochemical Journal that is described and appended (Langevin et al., 2010) in the session "Results 1".

PART 2:

Huntington's disease (HD) belongs to a family of dominantly inherited neurodegenerative diseases and it is caused by expansion of CAG tracts in the exon 1 of the huntingtin gene. The mutant gene encodes a variant of the huntingtin (htt) protein containing a homopolymeric tract of polyglutamine (polyQ) in excess of the pathogenic threshold of ~35Q (HD collaborative research group, 1993) (see chapter 5 of the introduction).

In the second part of my PhD, I have worked on two distinct but closely related projects in order to understand the mechanisms of Htt aggregates spreading and toxicity and the possible role of PrPC in these events.

Project 1: Characterization of the mechanisms of polyglutamine aggregates transfer in neuronal cells and primary neurons

Despite the rapidly accumulating evidence supporting a role for intercellular transmission of protein aggregates in the pathological spread of neurodegeneration in animal and cell culture disease models, little is known about whether and how huntingtin misfolding progresses through the brain. One

possibility to explain the topologically predictable progression of HD (see chapter 4 of the introduction) is through the spreading of the aggregated forms of the pathogenic protein, polyQ aggregates that must be released from and taken up by neighbouring cells in the brain.
Interestingly, uptake of externally applied synthetic polyQ peptides and recombinant fragments of mutant huntingtin has been reported in cell cultures (Yang et al., 2002; Ren et al., 2009) as well as the ability of the internalized aggregates to seed polymerization of a soluble huntingtin reporter, a phenotype that persisted in prolonged cultures of dividing cells (Ren et al., 2009). However, cell-to-cell transmission of mutant Htt was only measured indirectly by analyzing the seeded-polymerization of a cytopasmic huntintin reporter and it was suggested to be rather inefficient in co-culture experiments (Ren et al., 2009).
Therefore, a direct evaluation of the capacity of intracellular polyQ aggregates to transfer from one cell to another and of the underling mechanisms is needed to allow a better understanding of the stereotypical spread of HD pathology within the brain of affected individuals and also for developing potential therapeutical approaches.

To this aim, I divided this project in the following objectives:

a. To investigate whether spontaneous cell-to-cell transfer of polyQ aggregates occurs in co-cultured neuronal cells and primary neurons.
b. To characterize the mechanism of intercellular transfer and evaluate a possible role for Tunneling nanotubes (TNTs).
c. To examine whether cell-to-cell transfer of polyQ Htt induces nucleation and aggregation of endogenous wild-type Htt.

This part of my PhD work has been appended as manuscript in which I am the first author (see “Results 2”) and will be submitted for publications at beginning of September 2012.

Project 2: Role of the cellular prion protein in the pathogenic pathways of Huntington’s disease.

Whether and how the propagation of protein aggregates is linked to neurotoxicity is not completely understood (Brundin et al., 2010; Ross and Poirier, 2004). Recent studies provided evidence

that PrPC could mediate the toxicity of Aβ oligomers and other β-sheet-rich protein conformers by acting as a receptor for soluble ligands (Biasini et al., 2011; Bate and Williams, 2011; Resenberger et al. 2011) (see introduction, paragraph 6.5). It is possible that oligomeric forms of several different neurotoxic proteins could exert their effects by blocking, enhancing or subverting the normal function of PrPC as it has been suspected for its pathogenic counterpart, PrPSc (Westergard et al., 2007). Thus, binding of either oligomeric Aβ or PrPSc or other pathogenic aggregates to PrPC at cell-surface may initiate toxic signals that may lead to neuronal loss and/or synaptic dysfunction (Aguzzi and Falsig, 2012). Contrasting data are present in the literature on the possible role of PrPC in HD pathogenesis (see "Results 3", paragraph 3.2). Therefore, a more systematic analysis of the role of the cellular prion protein in other aggregated proteinopathies is needed as it tempting to speculate the existence of a common mechanism for toxicity with PrPC functioning as a "danger sensor" (Barton and Caughey, 2011; Aguzzi and Falsig, 2012).

In this context, I divided this project in the following objectives:

a. To Evaluate the effect of PrPC on polyQ-huntingtin aggregation and cell-to-cell transfer
b. To Evaluate the effect of PrPC on the toxicity induced by mutant huntingtin

These two objectives are described and discussed in the last part of this thesis ("Results 3").

MATERIALS AND METHODS

Materials and methods

Cell lines, mouse lines, primary cell cultures

CAD cells (mouse catecholaminergic neuronal cell line, Cath.a-Differentiated) were a gift of Dr. Laude H. (Institut National de la Recherche Agronomique, Jouy-en-Josas, France) and were cultured in Opti-MEM (Gibco) with the addition of 10% FBS (fetal bovine serum). Primary cultures were established from C57BL/6J mice provided by Charles River Laboratories and from the transgenic mouse lines PrP0/0 [PrP-KO (knockout) mice] (Zurich I) (Bueler et al., 1992) and tga20 (mouse *Prnp*a allele) (Fischer et al., 1996) provided by the CDTA (Cryopréservation, Distribution, Typage et Archivage animal). All experiments were performed according to national guidelines.
Primary cultures of CGNs (cerebellar granule neurons) were established as previously described (Cronier S. et al., 2004). CGNs were cultured for the indicated time on poly-D-lysine (10 ug/ml; Sigma) pre-coated coverslips at a density of 400 000 cells/coverslip in DMEM (Dulbecco's modified essential medium; Gibco) supplemented with 10% (v/v) FBS, 20 mM KCl, penicillin (50 units/ml), streptomycin (50 µg/ml; Gibco) and complemented with B27 and N2 supplement (Gibco).
Primary astrocytes were prepared from 1-day-old C57BL/6J mice following a previously described procedure for primary cultures of rat astroglia cells (Kaech and Banker, 2006). Briefly, astrocytes were isolated from the cortices of newborn mouse pups by enzymatic and mechanical dissociation. They were plated at a density of 7.5 × 106 cells per 75-cm2 flask coated with 0.1 mg/ml poly-L-lysine (Sigma) and cultivated in MEM containing 10% horse serum (v/v; Gibco), glucose (0,6% w/v), penicillin (50 units/ml) and streptomycin (50 µg/ml).
All cultures were incubated at 37°C in a humidified atmosphere with 5% CO2.

Plasmids and transfection procedures

GFP-HttQ68 and GFP-HttQ17 were a kind gift of Dr. Humbert S. (Institut Curie - UMR 146 du CNRS, Centre Universitaire Orsay, France). pGFP vector and mCherry vector were from Clontech.

CAD cells were transfected at 50% confluence with the indicated construct using Lipofectamine 2000 (Invitrogen), according to the producer's protocol.
CGNs were transfected with the appropriate construct in suspension immediately after isolation using the Amaxa nucleofector system and the amaxa electroporation transfection reagent VPG-1001 (Lonza) according to the manufacturer's procedure.

Western Blots

Following incubation for the times indicated, CGN were washed in PBS before lysis in TL1 buffer [50 mM Tris/HCl (pH 7.4), 0.5% sodium deoxycholate and 0.5% Triton X-100].
CAD cells were seeded 1.000.000 in 25 cm flasks. The following day, cells were transfected with 4 µg of GFP-HttQ68 or GFP-HttQ17 as described above. After 48 h, cells were washed in D-PBS and lysed in 0.5% Triton X-100, 0,5% sodium deoxycholate, 100 mM NaCl, 10mM Tris-HCl (pH 8). After a short centrifugation (3000g for 5min), 40 µg of cell lysate were resolved by SDS-PAGE either on a 7,5% acrylamide gel and Western blot with MAB2166 anti-huntingtin antibody (1:5000) or on a 12% acrylamide gel and probed with antibodies against cleaved caspase 3 [(Asp175) (5A1E); Millipore] and cleaved PARP [(Asp214) (7C9); Millipore], as markers of apoptosis. Blots were stripped and re-probed with mouse anti-tubulin (mouse monoclonal antibody, 1:5000) (Sigma). HRP-conjugated secondary antibodies and ECL TM reagents from Amersham (GE Healthcare) were used for detection.

Flow Cytometry

CAD cells were transfected separately with GFP-HttQ68, GFP-HttQ17, and mCherry constructs in 25 cm flasks as descrided above.
For co-culture experiments, 1-day after transfection, mCherry-expressing CAD cells were co-cultured with cells expressing either GFP-HttQ68 or GFP-HttQ17 at a ratio 1:1 in 35 mm dishes. After 24h co-cultures, cells were scraped in D-PBS plus 1% FBS, passed through 40 µm nylon cell strainers and fixed in 2% paraformaldehyde overnight prior to flow cytometry analysis (BD Biosciences LSRFortessa cell analyzer) Each experiment was

performed in triplicate and repeated three times. 10.000 cells were counted each time.
GFP-HttQ68 or GFP-HttQ17 expressing cells were also plated on 0.4 µm filters (Costar) placed on top of mCherry expressing cells in order to inhibit cell-cell contact. After 24h co-cultures, the filters were removed and the mCherry expressing cells were analyzed by flow cytometry as described above.
In order to test supernatant involvement in transfer, CAD cells were transfected separately with GFP-HttQ68 and GFP-HttQ17. After 24h, cells were gently washed with D-PBS and fresh medium was added for additional 24h. Then, GFP-HttQ68 or GFP-HttQ17 CADs medium was used to culture mCherry expressing CAD (transfected the day before). After 24h incubation, mCherry expressing cells were analyzed by flow cytometry as described above.

CGN co-cultures

For co-culture experiments, CGNs transfected with mCherry construct were mixed with GFP-HttQ68 transfected neurons at a ratio 1,5:1 immedialely after nucleofection and plated on coverslips as described above. For CGN-astrocytes co-cultures, astrocytes were harvested as previously described (Kaech and Bancher., 2007) an plated on on poly-D-lysine (10 ug/ml; Sigma) pre-coated coverslips at a density of 80 000 cells/coverslip. When astrocytes reached 40-70% confluence, fresh CGNs were prepared and plated on the astrocyte layer as described above.

Immunofluorescence

At the indicated times post-transfection, cells were washed in D-PBS (Dulbecco's Phosphate Buffered Saline; Gibco) and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) The cells were permeabilized with 0.1% Triton X-100 and labeled with mouse anti-huntingtin antibody (1:300, for 18 h at 4°C) (MAB2166; Millipore). The Alexa Fluor® 633 secondary antibody was purchased from Invitrogen. When indicated, CAD cells were stained with HCS CellMask™ Blue (1:10 000, for 20 minutes at R.T.) (Invitrogen), Wheat Germ Agglutinin (WGA)-rhodamine or WGA-Alexa Fluor® 350 conjugate (1:300, for 20 minutes at R.T) (Invitrogen). CGNs were also stained with DAPI (1:5000) (Sigma). The cells were washed and mounted with Aqua-Poly/Mount (Polysciences).

Images were acquired with a wide-field microscope (Zeiss Axiovert 200M) controlled by Axiovision software. All Z-stacks were acquired with Z-steps of 0.4 µm. When indicated, random mosaics of (3 × 3 fields) were obtained using a 63× objective Plan-Apochromat objective [1.4 NA (numerical aperture)]. Representative tiles are presented.
For CAD cells, the HCS CellMask™ staining was used to set the autofocus module, providing single focal plane images. Images of CAD cells used for 3D reconstruction and TNTs (tunneling nanotubes) detection were acquired with an optimal Z-step of 0.25 µm covering the whole cellular volume.

TNTs (Tunneling nanotubes) detection

CAD cells were transfected with the indicated constructs in 25 cm flasks. The following day or 12 h post-transfection, cells were plated on µ-Dish35 mm, high (Ibidi ®) and fixed at the indicated time with a solution of 2% paraformaldehyde, 0.05% gluteraldehyde and 0.2 M Hepes in D-PBS for 20 min, followed by a second 20 min fixation with 4% paraformaldehyde and 0.2 M Hepes in D-PBS. Then cells were gently washed in D-PBS and stained as indicated. In order to detect TNTs in CGNs, they were co-cultured with astrocytes as described above.

Image processing and quantification

Raw data were processed with Axiovision 4.8 software. The auto-scaling (min/max) of signal detection was applied to all images. When indicated, images were deconvolved using 3D Huygens Deconvolution software and three-dimensional reconstructions were performed with Imaris software.
To quantify the percentage of CAD cells with huntingtin aggregates and to evaluate the number of TNT-connected cells, a manual analysis was performed as previously shown (Gousset et al., 2009). Experiments were made in triplicate and repeated three times.
FACS raw data were analyzed by Kaluza® Flow Cytometry software (Beckman Coulter, Inc.).

Image Analysis using Acapella™ software

In order to evaluate and quantify the transfer of polyQ aggregates from donor (GFP-HttQ68 transfected) to acceptor

(mCherry transfected) CGN in co-culture experiments, we used the Acapella™ image analysis software (version 2.3 - Perkin Elmer Technologies) provided by the Plate-forme Imagerie Dynamique (Institut Pasteur) that allowed detecting in an automated manner Htt aggregates (GFP-tagged) in mCherry labeled neurons.
The script is subdivided in four object segmentation subroutines and required the setting of several input parameters:

- Segmentation of the nuclei in the channel 305 (DAPI staining) (nuclei_detection)
- Automated detection of the cell body of acceptor cells (mCherry labeled neurons) in the channels 305 (nuclei, DAPI staining) and 546 (mCherry signal) by applying a mask that allowed to select only the cell bodies labeled in both channels (DAPI/mCherry overlap).
- Neurite detection. Starting from the selected cell bodies, the application of a specific module of the Acapella software (neurite_detection) allowed to automatically draw the neuritic arborization corresponding to each cell body that, at this stage, appeared as “lines” in the 546 channel (mCherry signal). Then, to gain the thickness, a dilatation filter (radius = 3) was applied to the neuritic arbors.
- Spot and small object detection. In order to detect Htt aggregates two different algorithms were applied: spots and small object detection in both 488 (GFP-HttQ68 signal) and 633 (anti-Htt MAB2166) channels. While the spot detection is based on a local intensity analysis with each spot corresponding to a local intensity maximum, the small object detection takes in to account not only the global intensity but also shape and size. Spot and small objects were scored as “within neurite” only in presence of a shape overlap with the neurite of at least 70%. We consider only spot and small object that were positive in both 488 (GFP-HttQ68 signal) and 633 (anti-Htt MAB2166) channels (based on a shape overlap) and we reported the presence within neurites of spots and small object detected only in the 633 channel (not GFP positive), thus indicating aggregation of the endogenous protein.

The input parameters were optimized with feasibility studies in collaboration with image analysis experts at Plate-forme Imagerie Dynamique (Institut Pasteur). Different versions of the script corresponding to parameter adjustment were validated and

included the use of GFP vector transfected neurons (versus GFP-HttQ68) as negative control.

RESULTS AND DISCUSSION

Results 1:

Characterization of the role of dendritic cells in prion transfer to primary neurons: an insight in the mechanisms of prion spreading.

1.1 Objectives:

a. To characterize the kinetic of prion uptake and degradation in bone-marrow-derived DCs (BMDCs)
b. To investigate the mechanisms by which BMDCs transfer PrPSc to primary neurons
c. To examine whether PrPSc transferred from BMDCs to primary neurons results in prion infection

1.2 Summary of the results and discussion

TSEs (transmissible spongiform encephalopathies) are neurodegenerative diseases caused by pathogenic isoforms (PrPSc) of the host-encoded PrPC (cellular prion protein) (Prusiner, 1998). After consumption of contaminated food, prions enter the host organism trough the gut and rapidly accumulate in lymphoid tissues before invasion of the CNS (central nervous system) (Andreoletti et al., 2000, Heggebo et al., 2002, Aguzzi 2003). However, the mechanisms of prion spreading from the periphery to the nervous system are still unclear. A number of studies suggest that FDCs could play an important role in prion replication, the mechanisms of prion spreading from the gastrointestinal tract to the FDCs and from lymphoid tissues to the CNS are still undetermined (Mabbott et al., 2000; Montrasio et al., 2000; Mabbott et al., 2003) and there is a lack of physical contact between the gut and FDCs and between FDCs and nerve periphery (Defaweux et al., 2005). Dendritic cells (DCs) have been proposed to play a critical role in the transport of PrPSc from the gut to FDCs (Huang et al., 2002). In addition, DCs could promote prion transfer to nerve cells by direct contact with nerve fibers (Defaweux et al., 2005; Dorban et al., 2010) or through tunneling nanotube (TNT)-like structures (Gousset et al., 2009).
In the first part of my PhD in collaboration with Dr Langevin, I have investigated the role of DCs (dendritic cells) in the

spreading of prion infection to neuronal cells. First, using immunofluorescence analysis and three-dimensional reconstruction, we characterized prion uptake by BMDCs (bone-marrow-derived DCs) challenged with scrapie brain homogenate. We observed prion internalization between 2 and 18 h post-exposure resulting in a progressive shift of localization of PrPSc from the plasmamembrane to the cytosol. Next, we observed a progressive degradation of the internalized prion aggregates, leading to the disappearance of PrPSc signal between 96 and 168 h post-exposure. Similar experiments performed with BMDCs isolated from KO (knockout) mice or mice overexpressing PrP (tga20) indicate that both PrPSc uptake and catabolism are independent of PrPc expression in these cells. Then, using an in vitro approach, we characterized the transfer of PrPSc from BMDCs to primary neurons and the resulting infection of the neuronal cultures. Interestingly, the transfer of PrPSc was triggered by direct cell–cell contact when prion-loaded BMDCs were co-cultured with cerebellar primary neurons (CGN). As a consequence, BMDCs retained the prion protein when cultured alone, and no transfer to the recipient neurons was observed when a filter separated the two cultures or when neurons were exposed to the BMDC-conditioned medium, thus excluding the involvement of PrPSc secretion in our condition (e.g short co-culture time low BMDC/CGN ratios). Additionally, fixed BMDCs also failed to transfer prion infectivity to neurons, suggesting an active transport of prion aggregates. Furthermore, by microscopy approaches we could show that after overnight co-cultures BMDCs were either in close contact with dendrites or directly linked to neurons via TNTs. In addition because at the time of the co-cultures (after 18 h uptake) all of PrPSc aggregates are in the cytosol of BMDCs and not at the cell surface, these data suggest a transfer from the cytosol possibly via TNTs, while a transfer through plasma membrane to neighbouring cells is unlikely. Of interest, a parallel and independent study also suggested prion transfer through TNT-like structures shown to connect BMDCs to dorsal root ganglia and excluded the involvement of PrPSc secretion (Dorban et al., 2010). Consistent with the transfer experiments, when we followed up the neuronal cultures, we found that prion replication in the recipient neurons (infection) was only detected after direct co-culture conditions and did not occur if cells were separated by filters or when co-cultures were performed with aldehyde-fixed BMDCs. In conclusion, data suggests that DCs could be important players during prion

spreading. Furthermore our co-culture system will allow further characterization of prion spreading from the periphery to the nervous system of different scrapie strains, which could lead to a better understanding of the species barrier phenomenon.

The publication on "Biochemical Journal" related to this first part of my PhD work in collaboration with Dr Langevin is appended at the end of this section.

ARTICLE 1

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multimedia online data

Characterization of the role of dendritic cells in prion transfer to primary neurons

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TSEs (transmissible spongiform encephalopathies) are neurodegenerative diseases caused by pathogenic isoforms (PrPSc) of the host-encoded PrPc (cellular prion protein). After consumption of contaminated food, PrPSc deposits rapidly accumulate in lymphoid tissues before invasion of the CNS (central nervous system). However, the mechanisms of prion spreading from the periphery to the nervous system are still unclear. In the present study, we investigated the role of DCs (dendritic cells) in the spreading of prion infection to neuronal cells. First, we determined that BMDCs (bone-marrow-derived DCs) rapidly uptake PrPSc after exposure to infected brain homogenate. Next, we observed a progressive catabolism of the internalized prion aggregates. Similar experiments performed with BMDCs isolated from KO (knockout) mice or mice overexpressing PrP (tga20) indicate that both PrPSc uptake and catabolism are independent of PrPc expression in these cells. Finally, using co-cultures of prion-loaded BMDCs and cerebellar neurons, we characterized the transfer of the prion protein and the resulting infection of the neuronal cultures. Interestingly, the transfer of PrPSc was triggered by direct cell–cell contact. As a consequence, BMDCs retained the prion protein when cultured alone, and no transfer to the recipient neurons was observed when a filter separated the two cultures or when neurons were exposed to the BMDC-conditioned medium. Additionally, fixed BMDCs also failed to transfer prion infectivity to neurons, suggesting an active transport of prion aggregates, in accordance with a role of TNTs (tunnelling nanotubes) observed in the co-cultures.

Key words: cerebellar granule neuron, dendritic cell, intercellular transfer, prion infection, tunnelling nanotube.

INTRODUCTION

TSEs (transmissible spongiform encephalopathies) as variant of Creutzfeldt–Jakob disease, scrapie or chronic wasting disease can be acquired from the consumption of contaminated food. Following oral exposure, prions enter the host organism through the gut before invasion of the draining lymphoid tissues, where the first prion amplification takes place [1–3]. Prions subsequently spread to the CNS (central nervous system), where a characteristic neurodegeneration process is engaged concomitantly with the prion aggregate deposition in the brain [4–6]. Prior to prion neuroinvasion, PrP (prion protein) deposits are mainly visualized in tangible body macrophages and FDCs [follicular DCs (dendritic cells)] of the secondary lymphoid tissues (Peyer's patches, mesenteric lymph nodes, spleen) [7–12].

Although a number of studies suggest that FDCs could play an important role in prion replication, the mechanisms of prion spreading from the gastrointestinal tract to the FDCs and from lymphoid tissues to the CNS are still undetermined [13–15]. Prion neuroinvasion is initiated in the enteric nervous system and followed by retrograde transport along the sympathetic and parasympathetic nerve fibres [16–18]. Because of the absence of neuroimmune synapses between resident FDCs and nerve fibres, direct prion transfer mechanisms between these two cell types can be excluded [19–22]. Based on *in vitro* studies of intercellular prion transfer mechanisms, different hypotheses have been suggested. FDCs might passively transfer prion to proximal cells or nerve endings through exosomes or vesicle secretion [21,23]. Alternatively, mobile haematopoietic DCs might transfer PrPSc (pathological form of PrP) from the gut to FDCs, or possibly directly to nerve fibres. Indeed, different studies have characterized the role of DCs in the prion infection process [24–27]. DCs are mobile cells, which can directly uptake antigens by insertion of dendrites through the tight junctions of the intestinal epithelium cells [28] or after prion transepithelial migration through microfold cells [29,30]. Following antigen capture, DCs can retain proteins in native form for a sufficient time to facilitate their subsequent migration to the targeted lymphoid tissues [31]. Furthermore, after TSE infection by the oral route, PrPSc deposits have been identified in DCs from Peyer's patches, mesenteric lymph nodes or spleen [32,33]. Finally peripheral prion infection performed in mice devoid of DCs failed to accumulate PrPSc in lymphoid tissues and the subsequent neuroinvasion was partially impaired [24,27,34]. Overall, these data strongly point to DCs as potentially important candidates in prion transport from the gut to the lymphoid tissues, even though the subsequent neuroinvasion mechanisms are still undetermined. In addition, in contrast with FDCs, DCs can theoretically promote prion transfer to nerve cells by direct contacts with nerve fibres [32,33,35,36] or through TNT (tunnelling nanotube)-like structures [37,38]. Indeed BMDCs (bone-marrow-derived DCs) are able to form TNT-like structures *in vitro* when co-cultured with primary neurons, and can transfer PrPSc and infection to these cultures [37,38].

In the present study, we have characterized the role of BMDCs in the transfer of prions to primary neurons using an *in vitro* approach. First, we analysed the uptake and the fate of scrapie

Abbreviations used: BMDC, bone-marrow-derived dendritic cell; CGN, cerebellar granule neuron; CNS, central nervous system; DC, dendritic cell; ECL, enhanced chemiluminescence; FCS, fetal calf serum; FDC, follicular DC; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; KO, knockout; NA, numerical aperture; PK, proteinase K; PrP, prion protein; PrPc, cellular PrP; PrPres, protease-resistant PrP; PrPSc, pathological form of PrP; TNT, tunnelling nanotube; TSE, transmissible spongiform encephalopathy.

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homogenate in BMDC cultures. We demonstrated that BMDCs rapidly internalize PrPSc aggregates and retain them for several hours, independently of the PrPc (cellular PrP) expression levels. Next, we characterized the transfer of PrPSc from prion-loaded BMDCs to primary neurons using short-time co-cultures. We found that BMDCs begin to transfer PrPSc as early as 4 h after cell cultures have been established and that this transfer is triggered by cell–cell contact. Furthermore, we show that PrPSc transfer results in prion infection (e.g. prion replication) of primary neurons. Overall, the present study demonstrates that DCs can discharge prions to target cells upon direct cell–cell contact, and confirms that TNTs could be a major transfer mechanism in the passage of prions from the periphery to the CNS.

EXPERIMENTAL

Mouse lines

Primary cultures were established from C57BL/6J mice provided by Charles River Laboratories or the transgenic mouse lines PrP0/0 [PrP-KO (knockout) mice] (Zurich I) [39] and tga20 (mouse *Prnpa* allele) [40] provided by the CDTA (Cryopréservation, Distribution, Typage et Archivage animal). All experiments were performed according to national guidelines.

Cell culture

CGNs (cerebellar granule neurons)

Primary cultures of CGNs were established as described previously [41]. Briefly, CGNs were extracted from brains of 6-day-old C57BL/6 mice by enzymatic and mechanical dissociations. They were plated at a density of 800 000 cells/well in 12-well plates coated with 10 μg/ml poly-D-lysine (Sigma) and cultivated in DMEM (Dulbecco's modified Eagle's medium; Gibco) containing 10 % FCS (fetal calf serum), 20 mM KCl, penicillin (50 units/ml), streptomycin (50 μg/ml; Gibco) and complemented with B27 and N2 supplement (Gibco). Cultures were incubated at 37 °C in a humidified atmosphere with 5 % CO_2 and were complemented weekly with 1 mg/ml glucose and 10 μM of the anti-mitotics uridine and fluorodeoxyuridine (Sigma) to avoid proliferation of astrocytes. As negative controls, CGN cultures were established from PrP0/0 mice.

BMDCs

BMDCs were differentiated from bone marrow cells from 6–8-week-old C57BL/6 mice according to a method adapted from Méderlé et al. [42]. Briefly, bone marrow cells were seeded at 5×10^6 cells per 100 mm diameter Petri dish (Falcon, Becton Dickinson Labware) in 10 ml of Iscove's modified Dulbecco's medium (RPMI 1640; Gibco) supplemented with 10 % heat-inactivated FCS, 20 ng/ml GM-CSF (granulocyte/macrophage colony-stimulating factor; R&D Systems), penicillin (50 units/ml), streptomycin (50 μg/ml) and 50 μM 2-mercaptoethanol. Cultures were incubated at 37 °C in a humidified atmosphere with 5 % CO_2. On day 3, 10 ml of complete RPMI 1640 was added. On day 6, cells in suspension and loosely adherent cells were harvested. The recovered cells were further cultured under the same conditions as described above. On day 10, cells were harvested with EDTA as above and distributed in CellBIND six-well plates (Corning) at a concentration of 1×10^6 cells/well in 3 ml of complete RPMI 1640.

moRK13 cells were provided by Dr Andrew Hill (Bio21 Institute, University of Melbourne, Melbourne, Australia). Cells were maintained at 37 °C in 5 % CO_2 in Opti-MEM medium (Gibco) supplemented with 10 % FCS, penicillin (50 units/ml) and streptomycin (50 μg/ml).

Prion loading of BMDCs

Brain homogenates were prepared from the brains of mice terminally affected with the mouse 139A strain, from an original 139A-affected brain provided by Dr M. Baier (Robert Koch Institute, Berlin, Germany). Homogenates were diluted to a final concentration of 20 % (w/v) in a 5 % (w/v) glucose solution, sonicated in RPMI 1640 medium and a suspension equivalent to 2.5 mg of infected brain tissue was added to the wells of BMDCs for the times indicated.

BMDC–CGN co-cultures

BMDCs were loaded with the equivalent of 2.5 mg of infected brain tissue for 18 h. At 2 days after plating, CGNs were co-cultured with prion-loaded BMDCs overnight (CGN/BMDC, 4:1). BMDCs were removed from the CGN cultures by extensive washing before analyses of PrPres (protease-resistant PrP) in CGNs after short times (30 min–4 h) or after 2 and 3 weeks post-co-culture. Then, 50 μg of protein was treated with 0.5 μg of PK (proteinase K) for 30 min at 37 °C before methanol precipitation. Samples were then subjected to SDS/PAGE and Western blot analysis with the Sha31 anti-PrP antibody (SPI-Bio). The same amounts were methanol-precipitated without PK treatments and analysed by SDS/PAGE and Western blot analsyis using an M5/114 antibody (directed against MHC class II proteins specifically expressed in BMDCs) or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody.

moRK13 cells were co-cultured with prion-loaded BMDCs for 18 h at a 4:1 ratio. After overnight co-cultures, BMDCs were removed from the moRK13 cells by extensive washes. Then, 20 μg of protein from BMDC or moRK13 cell extracts was treated with 0.5 μg of PK for 30 min at 37 °C before methanol precipitation. Samples were then subjected to SDS/PAGE and Western blot analysis with the Sha31 anti-PrP antibody.

Co-incubations with fixed BMDCs were performed as indicated above after BMDC fixation with a solution of 2 % paraformaldehyde, 0.05 % gluteraldehyde and 0.2 M Hepes in PBS for 20 min, followed by a second 20 min fixation with 4 % paraformaldehyde and 0.2 M Hepes in PBS. The cells were then washed thoroughly and added to the neuronal cells.

For the filter experiments, BMDCs were plated on 0.4 μm filters (Costar) on top of CGN cultures at 2 days post-plating. After overnight co-cultures, the filters were removed and the neuronal cultures were analysed at different time points post-incubation. For the supernatant experiments, BMDCs were loaded with the equivalent of 2.5 mg of infected brain tissue as described above overnight. Loaded BMDCs were centrifuged at 1440 ***g*** in RPMI 1640 and the supernatant was added to CGN cultures 2 days post-plating. After overnight incubations with the supernatant, CGNs were lysed and analysed as described above.

Imaging prion uptake in BMDCs

At 10 days post-dissection, 1×10^6 BMDCs were plated overnight on Ibidi dishes (Biovalley) coated with fibronectin (Sigma). Cells were then exposed to 2.5 mg of 139A scrapie brain homogenate for the times indicated, washed thoroughly in RPMI 1640 and fixed in 4 % paraformaldehyde. The cells were permeabilized with 0.1 % Triton X-100, treated with 3 M guanidium thiocyanate to expose the PrPSc epitopes and labelled with the Sha31

anti-PrP antibody and with the cytosolic dye HCS CellMask Blue (1:5000) (Invitrogen). The cells were washed and mounted with Aqua-Poly/Mount (Polysciences). Images were acquired with an epifluorescence microscope (Zeiss Axiovert 200M) controlled by Axiovision software. Random mosaics (3 × 3 fields) were obtained using a 63× objective Plan-Apochromat objective [1.4 NA (numerical aperture)]. All *Z*-stacks were acquired with *Z*-steps of 0.4 μm. Representative tiles are presented. For higher-magnification representations of the internalization process, a confocal microscope Andor Revolution Nipkow spinning-disc imaging system was used. The Andor technology was installed on a Zeiss Axiovert 200M microscope, equipped with an Andor EMCCD DV885 camera, three diode-pumped solid-state lasers with excitation at 405, 488 and 560 nm, a piezo mono-objective for fast three-dimensional acquisitions, and a confocal head spinning-disc Yokogawa CSU22. Images were acquired with an oil 63× Plan-Apochromat objective (1.4 NA). All *Z*-stacks were acquired at maximum speed of the microscope with *Z*-steps of 0.250 μm.

For wide-field analysis, cells were fixed with 4% paraformaldehyde for 10 min. Phase-contrast images were then acquired by high-resolution wide-field microscope Marianas (Intelligent Imaging Innovations) using a 63× oil objective. All *Z*-stacks were acquired with *Z*-steps of 0.4 μm.

PK digestion

Prion detection in BMDCs

Following incubation for the times indicated, cells were washed in PBS before lysis in TL1 buffer [50 mM Tris/HCl (pH 7.4), 0.5% sodium deoxycholate and 0.5% Triton X-100]. After a short centrifugation (3000 ***g*** for 5 min), 50 μg of cell lysates were treated with 2 μg of PK for 30 min at 37 °C. Next, the proteins were methanol-precipitated for 1 h at −20 °C before centrifugation at 13000 ***g*** for 30 min. Pellets were resuspended in sample buffer before analysis by SDS/PAGE (12% acrylamide gels) and Western blotting with the Sha31 antibody and secondary anti-mouse antibody coupled to HRP (horseradish peroxidase). Immunoreactivity was visualized by ECL (enhanced chemiluminescence; Amersham).

Prion detection in CGNs

The accumulation of PrPSc was analysed in neuronal cultures at different times post-infection. Neuron lysates performed in TL1 buffer were pre-cleared by centrifugation at 3000 ***g*** for 5 min. Then, 50 μg of cell lysates were treated with 0.5 μg of PK for 30 min at 37 °C before stopping the digestion with 5 mM PMSF. Proteins were methanol-precipitated for 1 h at −20 °C before centrifugation at 13000 ***g*** for 30 min. Pellets were resuspended in sample buffer and denatured before analysis by SDS/PAGE (12% acrylamide gels) and Western blotting with the Sha31 antibody and secondary anti-mouse antibody coupled to HRP. Immunoreactivity was visualized by ECL.

Prion detection in moRK13 cells

The accumulation of PrPSc was analysed in moRK13 cells after 18 h of co-culture. Lysates were performed in TL1 buffer after pre-clearing by centrifugation at 3000 ***g*** for 5 min. Then, 20 or 200 μg of cell lysates were treated with 0.5 μg or 5 μg of PK for 1 h at 37 °C before methanol precipitation (1 h at −20 °C). Proteins were then centrifuged at 13000 ***g*** for 30 min. Pellets were resuspended in sample buffer and denatured before analysis by SDS/PAGE (12% acrylamide gels) and Western blotting with the Sha31 antibody and secondary anti-mouse antibody coupled to HRP. Immunoreactivity was visualized by ECL.

RESULTS

Characterization of prion uptake in BMDCs

We first analysed the rate of internalization of PrPSc by BMDCs after *in vitro* exposure to infected brain homogenate. At 10 days post-plating, BMDCs were exposed to 139A infected brain homogenate for 30 min, 1 h, 2 h or 18 h, fixed, treated with guanidium and labelled with the Sha31 antibody to detect PrPSc. Mosaics of different fields were obtained to analyse the overall spreading and endocytosis of PrPSc aggregates in BMDCs. *Z*-stacks were acquired to encompass all of the homogenate signals (see the Experimental section). In contrast with control BMDCs where no signal was observed after guanidium treatment (Supplementary Figure S1 at http://www.BiochemJ.org/bj/431/bj4310189add.htm), large fields of view show that PrPSc aggregates are well spread and associated with the majority of the BMDCs exposed to the infected brain homogenate (Figure 1A). Whereas most of the aggregates were outside the cells at the early time points (Figure 1A; 30 min–1 h), over time PrPSc aggregates were progressively internalized (Figure 1A; 2–18 h). After 18 h, the PrPSc aggregates were found inside the cells, as shown by the perfect focus of both BMDCs and PrPSc aggregates (Figure 1A; 18 h). Detailed confocal analyses and three-dimensional reconstructions of the PrPSc aggregates associated with BMDCs confirmed their localization at the cell surface and outside the cells after 30 min or 1 h of exposure (Figure 1B; 30 min–1 h, Supplementary Movies S1–S4 at http://www.BiochemJ.org/bj/431/bj4310189add.htm). After 2 h, some PrPSc was still visualized at the level of the plasma membrane, but could also be detected in the cytosol of most cells (Figure 1B; 2 h, Supplementary Movies S5 and S6 at http://www.BiochemJ.org/bj/431/bj4310189add.htm). Finally, after 18 h of exposure, the localization of PrPSc was drastically shifted and entirely restricted to the cytosol of the BMDCs (Figure 1B; 18 h, Supplementary Movies S7 and S8 at http://www.BiochemJ.org/bj/431/bj4310189add.htm). At this time point, no free PrPSc aggregates could be detected outside the BMDCs (Figure 1).

These data demonstrate the rapid uptake of PrPSc homogenate by BMDCs after *in vitro* exposure. The kinetics of internalization observed are in accordance with the results previously described in rat BMDCs and Langherans cells [43,44]. Additionally, biochemical analyses of PrPSc internalization performed on BMDCs derived from KO or PrP-overexpressing mice (tga20) showed a similar increase in PrPSc internalization up to 18 h post-exposure, indicating that prion uptake is independent of the levels of PrPc (Supplementary Figure S2 at http://www.BiochemJ.org/bj/431/bj4310189add.htm).

Characterization of PrPSc degradation in BMDCs

Next, we wanted to investigate the fate of PrPSc once internalized by BMDCs. *In vivo* studies have identified DCs as important candidates during prion spreading from the periphery to the peripheral nervous system [24,43]. However, subsequent *in vitro* experiments have indicated that the rapid uptake of PrPSc is progressively followed by prion degradation in various subsets of DCs [43–46]. Because a rapid degradation of PrPSc would be inconsistent with a role of DCs in prion spreading, we investigated

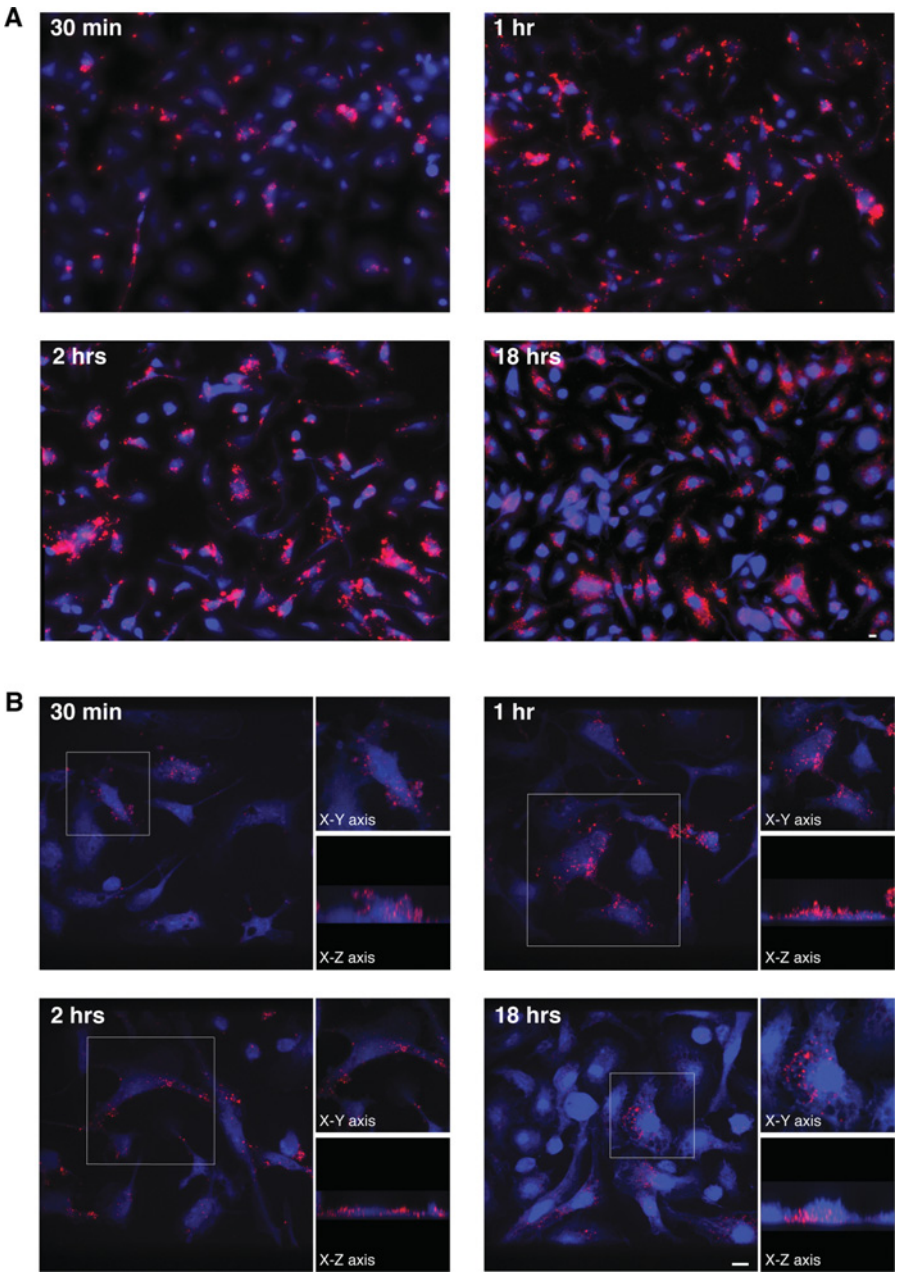

Figure 1 Time course of PrPSc internalization by BMDCs

BMDCs plated on fibronectin-coated Ibidi dishes were loaded with 139A brain homogenate for 30 min, 1 h, 2 h or 18 h. The cells were then washed, fixed, denatured with guanidine hydrochloride and immunolabelled with the Sha31 anti-PrP antibody and Alexa-Fluor®-546-conjugated secondary antibody. HCS CellMask Blue was used to label the cytosol of the BMDCs (blue). The brain homogenate revealed a punctate PrPSc pattern (red). (**A**) Mosaics (3 × 3 fields) were acquired by wide-field microscopy. For the acquisitions, *Z*-stacks (0.4 μm) were taken to visualize all of the PrPSc aggregates. In the early time points (30 min–1 h) PrPSc aggregates are found on top of the cells, as determined by the different focal planes acquired. Over time, the cells come into focus (2 h) as the aggregates start to be internalized. After 18 h, all of the aggregates appear to be inside the cells. (**B**) High-magnification acquisitions using an Andor spinning-disc confocal microscope confirm the internalization of PrPSc aggregates over time (2–18 h). Three-dimensional reconstructions were obtained for selected cells (insets) in both *x*–*y* and *x*–*z* axis planes using OsiriX software. Scale bars represent 10 μm.

whether and how PrPSc was processed in BMDCs by analysing the levels of PrPres over time following the uptake of prion homogenate (Figure 2A).

At 10 days post-plating, 10^6 BMDCs were subjected to 2.5 mg of brain homogenate (obtained from terminally infected mice injected with the 139A scrapie strain) for the duration of the experiment (Figure 2A). Alternatively, the cells were first allowed to internalize PrPSc for 18 h, then washed and replated before performing the PK assay (Supplementary Figure S3 at http://www.BiochemJ.org/bj/431/bj4310189add.htm). Following prion capture, BMDCs isolated from wild-type mice progressively degraded 139A prion aggregates as determined by the decrease in PrPres signal between 24 and 168 h (C57Bl/6) (Figure 2A and Supplementary Figure S3). These results show a progressive clearance of prion aggregates by DCs. However, it is also clear that, following prion uptake, BMDCs are able to carry infectious PrPSc for up to 4 days. This is consistent with a dual role of DCs both in the transfer of prions to other cells and in prion

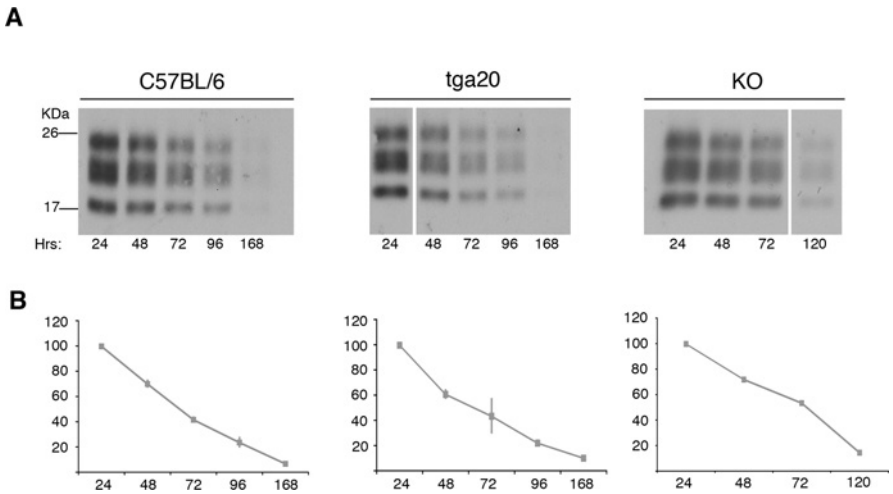

Figure 2 Time course of PrPSc degradation in BMDCs

BMDC cultures were established from C57BL/6 (left-hand panel), tga20 (middle panel) or KO (right-hand panel) mice. At 10 days post-plating, cells were exposed to 139A brain homogenate for the times indicated. (**A**) Cells were lysed and PK-treated before analysis of PrPres expression by immunoblotting using the Sha31 antibody. Western blot analysis indicated a progressive decrease in the PrPres signal between 24 and 96 h of exposure preceding total disappearance of the signal at 168 h. The molecular mass in kDa is indicated on the left-hand side of the blots. (**B**) PrPSc degradation follows similar kinetics in BMDCs isolated from C57BL/6, tga20 or KO mice, suggesting that the PrPSc catabolism we observed is independent of PrPc expression. The relative degradation of PrPSc in prion-loaded BMDCs was quantified from two independent experiments for C57BL/6 and tga20 and from one experiment for KO cells.

clearance over time. This hypothesis is also supported by previous work indicating that, in mice models, DCs with a high content of cytoplasmic PrPSc aggregates could be detected in the lymph nodes from 8 to 16 h post-peripheral inoculation [43].

In order to understand whether PrPc had a role in PrPSc catabolism, we repeated the same experiments using BMDCs isolated either from KO mice or from tga20 mice, which express 10-fold more PrPc than wild-type mice (Figure 2 and Supplementary Figure S3; tga20 and KO). Western blot quantification of the PrPres signal indicated that 139A brain homogenate was catabolized over time with similar kinetics as BMDCs isolated from KO, wild-type or tga20 mice (Figure 2B). Overall, these data indicate that both PrPSc uptake and degradation are independent of PrPc expression.

BMDCs transfer PrPSc to neuronal cells

Having established that BMDCs retained PrPSc for at least 96 h after its uptake (Figure 2A), we further investigated their ability to transfer PrPSc to primary cultures of neurons using *in vitro* co-cultures. To detect PrPSc transfer, BMDCs were loaded with 139A scrapie brain homogenate for 18 h in order to allow complete PrPSc internalization (see Figure 1). Cells were then extensively washed before addition to the CGN primary cultures.

As described previously, co-cultures were established at a 4:1 ratio between neuronal cells and DCs [37]. After overnight incubation, BMDCs were removed from the CGN cultures by extensive washes. The lysates of both removed BMDCs and neuronal cells were analysed for the presence of PrPres by Western blotting after PK treatment. Interestingly, under these conditions PrPres was detected only in the neurons and not in the BMDCs removed from the co-cultures, indicating that a large amount of PrPSc had been transferred from the BMDCs to the neurons (Figure 3A, co-culture). We could exclude prion transfer from membrane-associated PrPSc aggregates, since we demonstrated that at the time of the co-cultures with the primary neurons the PrPSc aggregates were localized exclusively inside the cytosol of BMDCs (see Figure 1, 18 h). Therefore transfer could have occurred either through the secretion of PrPSc in the medium or through direct passage from the cytosol of BMDCs to the cytosol of the neurons, possibly via TNTs as we had suggested previously [37].

To evaluate the possible role of the secretory pathway, and more specifically of exosomal release [47–50], we examined whether prion transfer could occur through filters, which would allow the passage of secretory vesicles and exosomes. Quantification of the PrPres signals demonstrated that the transfer efficiency is reduced by more than 98 % when filters were used to separate the cultures, compared with direct co-cultures (Figure 3A). This suggested that PrPSc secretion was not involved in the transfer (Figure 3A, filter). However, to rule out the possibility that the filters could trap PrPSc aggregates, we analysed whether prion transfer could be mediated by the supernatant of the scrapie-loaded BMDCs. To this aim, neurons were exposed to the supernatant of BMDCs loaded with 139A brain homogenate collected after 24 h. After 18 h of exposure to the conditioned medium, neurons were washed and analysed for PrPres. Similar to the filter conditions, neurons exposed to the supernatant of BMDCs did not contain high PrPres signals as compared with the signal obtained from the direct co-culture experiments (Figure 3B; supernatant), further suggesting that PrPSc secretion was not the main transfer mechanism.

Finally, to ensure that the PrPres signal observed in CGNs were not the result of BMDCs left in the cultures, we analysed, by Western blotting, the presence of BMDCs in the CGN co-cultures using the BMDC-specific MHC class II antibody. BMDCs and non-exposed CGN cell extracts were used as positive and negative controls respectively (Figure 3C). As expected, a very strong signal for MHC class II proteins was detected in BMDC extracts, but not in non-exposed CGNs (NI) or in CGNs exposed to BMDCs through filter (filter) (Figure 3C). Interestingly, only a very faint signal was detected in CGNs directly exposed to BMDCs (co-culture) (Figure 3C), indicating that the BMDCs were efficiently removed from the CGNs. This also excluded the possibility that the PrPres signals detected in CGN post co-cultures could be derived from prion-loaded BMDCs.

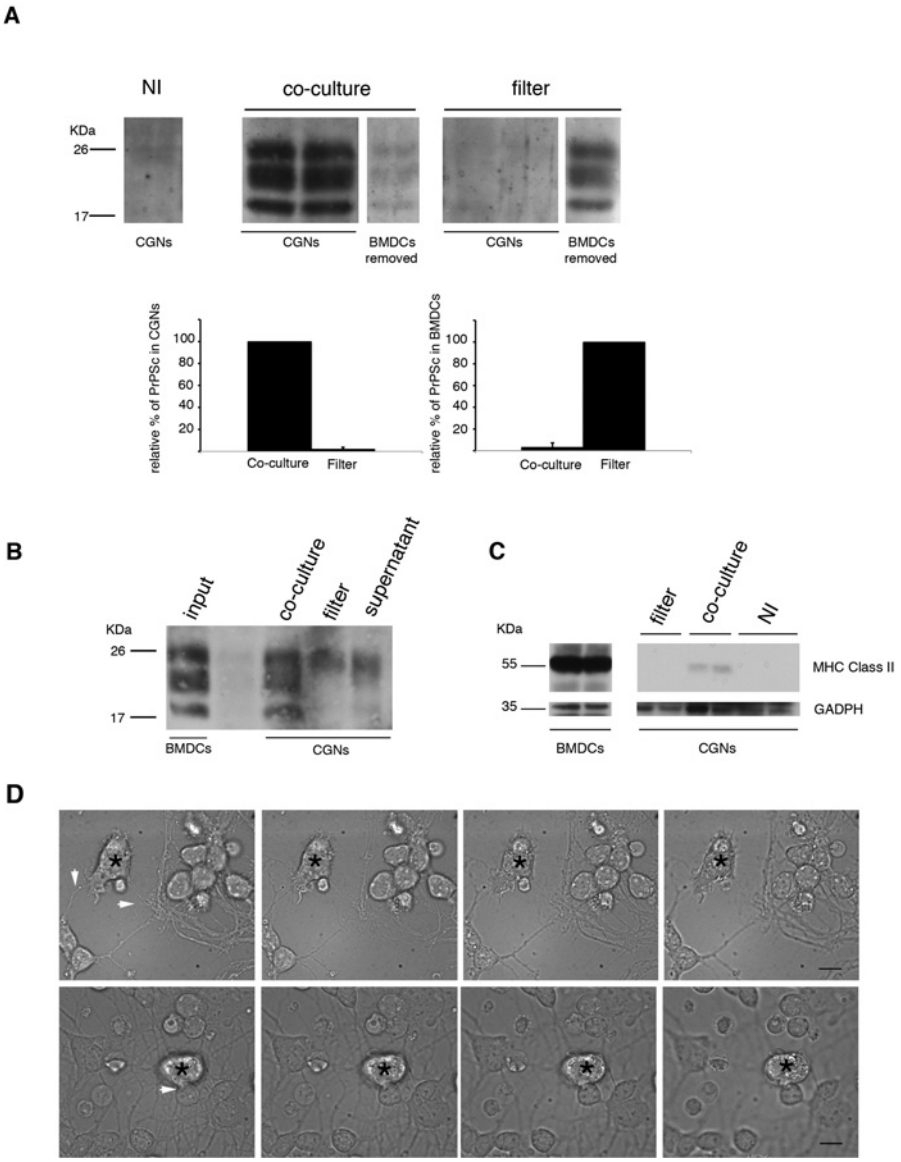

Figure 3 Characterization of PrPSc transfer from prion-loaded BMDCs to CGNs

(**A** and **B**) Cell–cell contact is required for PrPSc transfer from BMDCs to neurons. BMDCs were exposed *in vitro* to 139A brain homogenate for 18 h. (**A**) Prion-loaded BMDCs were co-cultured with neurons directly (co-culture) or through filters (filter). After 18 h BMDCs were removed from the CGNs with extensive washes. The lysates of both the removed BMDCs and of the CGNs were PK-treated to evaluate PrPSc transfer by immunoblotting using the Sha31 antibody. PrPres is only detected in neurons after direct co-culture, suggesting that intercellular prion transfer cannot occur in the absence of cell–cell contact. On the other hand, prion protein is only visualized in BMDCs removed from filters, suggesting that direct contact triggers the prion discharge from BMDCs. The PrPSc signal in CGNs and BMDCs was quantified from Western blot analysis from three different experiments and are presented as relative percentages (lower panels). (**B**) To determine the impact of PrPSc present in the supernatant (e.g. exosomal release, vesicle secretion), prion-loaded BMDCs were co-cultured with neurons directly (co-culture), through filters (filter) or the neurons were exposed to the conditioned medium of loaded BMDCs (supernatant). Similar to what was found in (**A**), PrPres was only detected in neurons after direct co-cultures. (**C**) To evaluate the efficiency of removal of BMDCs in CGN cultures, we evaluated the presence of MHC class II proteins in CGN lysates after co-cultures through filters (filter), direct exposure to BMDCs (co-cultures) or in non-exposed CGNs (NI). Protein (50 μg) from BMDCs or CGNs were analysed by Western blot with MHC class II and GAPDH antibodies for normalization. Whereas a strong MHC class II signal is detected in BMDC cell extracts, a faint signal is observed in CGNs only after direct exposure. (**D**) To assess the types of contact between the two cell populations in our co-cultures, prion-loaded BMDCs were co-cultured with CGNs 2 days post-plating for 18 h before fixation. Wide-field acquisitions were performed using a Marianas microscope (Triplel) and selected frames of two different *Z*-stack acquisitions (0.4 μm steps) are shown. BMDCs (indicated by an asterisk) are in close contact with dendrites (arrow, upper panel) or neuronal cell bodies (arrow, lower panel). The scale bar represents 10 μm. In (**A** and **C**), the molecular mass in kDa is indicated on the left-hand side of the blot.

Overall, these data demonstrate that efficient PrPSc transfer from BMDCs requires cell–cell contact and does not appear to be associated with PrPSc secretion. In order to quantify the amount of PrP discharged by BMDCs, we analysed the amount of PrPres remaining in BMDCs co-cultured directly or through filters with primary neurons (Figure 3A). Interestingly, different levels of PrPres could be detected in an equivalent number of BMDCs from the different co-culture conditions (Figure 3A). Indeed, by normalizing the gel loading to 50 μg of protein of the different BMDC lysates, we found that BMDCs seeded on to filters contained much higher levels of PrPres compared with BMDCs seeded directly on top of CGNs (Figure 3A). Quantification of PrPres signals indicated that direct co-cultures triggered 97 % of PrPSc release as compared with filter conditions

(Figure 3A). These data clearly indicate that the transfer of PrPSc from BMDCs to the primary neurons was triggered by direct cell–cell contact. Similar experiments were performed with epithelial moRK13 cells, which can be infected after prion transfer from BMDCs [37]. As in the case of primary neurons, moRK13 cells contained PrPres after 18 h of direct co-culture with loaded BMDCs, but not if the cells were separated by filters or exposed to the supernatants of BMDCs (Supplementary Figure S4 at http://www.BiochemJ.org/bj/431/bj4310189add.htm). Interestingly, the amount of PrPres found in moRK13 was equivalent to one-quarter of the total amount of PrPres found in loaded BMDCs, and was comparable with the amount left in the BMDCs that were co-cultured through a filter. Therefore these data highlight the important role of direct cell contact in the stimulation of prion discharge by BMDCs.

Furthermore, we observed that, upon co-culture, BMDCs were able to interact with both the dendrites (Figure 3D, upper panels) and the cell bodies (Figure 3D, lower panels) of neuronal cells. These observations are in agreement with *in vivo* results [20,32], which show close contact between DCs and nerve fibres in lymph nodes of scrapie-infected animals.

BMDCs efficiently transfer prion infectivity to neuronal cells

Because we have previously shown that PrPSc transfer from BMDCs could result in *de novo* infection of primary neurons [37], we decided to further characterize the cellular mechanisms involved in the transfer of infectivity.

To this aim, after 12 h of co-culturing with loaded BMDCs, we analysed the evolution of PrPres signals in primary neurons over time, up to 3 weeks of culture. Direct co-cultures established between 139A-loaded BMDCs and CGNs from wild-type C57BL/6 mice gave rise to neuronal infection, as demonstrated by the progressive increase in PrPres signal observed from 7 to 21 days post-co-culture (Figure 4A). On the other hand, similar experiments performed with CGNs derived from PrP KO mice did not show any PrPres signal even after 3 weeks of culture (Figure 4A). Since PrPSc transfer is similar in KO neurons compared with wild-type neurons (results not shown), these data show that the PrPres signal observed in wild-type neurons derives from PrPSc neo-synthesis and is not the result of remnant PrPSc from BMDCs. Next, we analysed whether there was transfer of infectivity from loaded BMDCs to neurons in co-cultures separated through filters. As expected from the observed lack of transfer under these conditions (Figure 3A and Supplementary Figure S4), we were unable to detect newly synthesized PrPSc in primary neurons maintained in culture up to 3 weeks after overnight filter co-culture with loaded BMDCs (Figure 4B). Finally, in order to analyse whether infection was due to an active transfer mechanism, we decided to alter our co-culture experiments in order to inhibit membrane remodelling. To this aim, loaded BMDCs were fixed (with paraformaldehyde/gluteraldehyde solutions) before exposure to neuronal cultures. These treatments strongly inhibited the plasma membrane plasticity, blocking both TNT formation and PrPSc secretion. Similar to the results obtained after co-culture through filters, fixation of BMDCs prior to the co-cultures did not result in neuronal infection, as indicated by the absence of PrPres signals in the co-cultured neurons (Figure 4B). These data indicate that PrPSc infection results from an active process, which cannot occur upon fixation in a short 12 h co-culture and requires cell–cell contact. Because in our experiments we excluded that prion transfer could occur via secretion, all our data are consistent with a role of TNT in intercellular spreading from BMDCs to neurons, as we have proposed previously [37] (see also Supplementary Movie S9 at http://www.BiochemJ.org/bj/431/bj4310189add.htm).

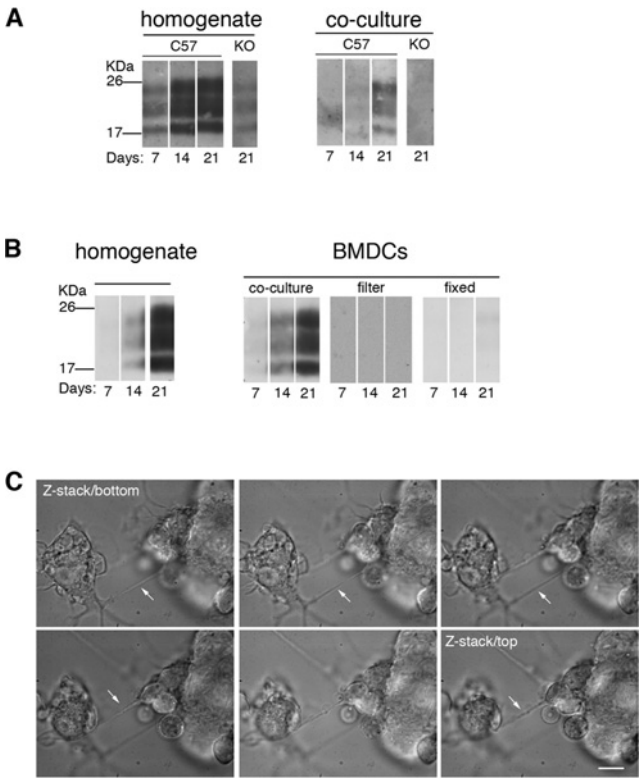

Figure 4 Characterization of the transfer of prion infectivity from BMDCs to CGNs

(**A**) Kinetics of PrPSc accumulation in neuronal cells directly exposed to 139A-loaded BMDCs. C57BL/6 or KO CGNs were directly exposed to prion-loaded BMDCs (co-culture) or to 0.01 % of 139A brain homogenate (homogenate) as a control. The PrPres signal is detected in neuronal cell lysates by immunoblot using the Sha31 antibody. PrPSc amplification is observed in C57 CGNs after exposure to 139A brain homogenate or prion-loaded BMDCs. No PrPres is detected in KO CGNs even after 21 days of culture. (**B**) Co-cultures were performed through filters or after fixation of BMDCs. Under these conditions, PrPSc amplification cannot be observed over time. The molecular mass in kDa is indicated on the left-hand side of the blots. (**C**) Prion-loaded BMDCs were co-cultured with CGNs 2 days post-plating for 18 h before fixation and microscopic observations. Wide-field acquisitions were performed using a Marianas microscope (Triplel). Selected frames of *Z*-stack acquisitions (0.4 μm steps) are shown. BMDCs (indicated with an asterisk) are connected to neurons via TNTs (arrows). The scale bar represents 10 μm.

DISCUSSION

In the present study, we investigated the role of BMDCs in the processing and spreading of prions. To this aim, we developed an *in vitro* approach in which prion-loaded BMDCs were co-cultured with cerebellar primary neurons. First, we characterized the prion uptake by BMDCs exposed to scrapie brain homogenate over time by immunofluorescence analyses and three-dimensional reconstructions (Figure 1 and Supplementary Figure S2). While prion aggregates were mainly associated with the cell surface up to 2 h post-incubation, prion internalization was detected between 2 and 18 h post-exposure resulting in a progressive shift of localization of PrPSc from the plasma membrane to the cytosol.

Following scrapie uptake, we also demonstrated that BMDCs progressively degraded PrPSc between 24 and 72 h post-exposure. After 72 h, we observed higher PrPSc catabolism leading to the rapid disappearance of PrPSc signal between 96 and 168 h,

consistent with what was previously determined in other models [43,44,46,51].

The fact that we have been able to detect consistent amounts of PrPres up to 72 h post-loading indicates that, after uptake, BMDCs could present native prion proteins to other cell types during this time frame, before starting massive protein degradation. Furthermore, complementary experiments performed in BMDCs isolated from KO or PrP-overexpressing mice indicated that neither the uptake, as recently shown [38], nor the degradation of PrPSc was influenced by PrPc expression. Interestingly, similar experiments performed with different prion strains (22L and Me7) showed similar kinetics of uptake and catabolism of PrP (results not shown), suggesting that both mechanisms are not influenced by the different prion strains.

Next, we analysed whether and how BMDCs transferred PrPSc to primary neurons. In these experiments, neurons were co-cultured with BMDCs 2 days post-plating, a stage of differentiation that we found facilitates the establishment of TNTs between neurons and BMDCs. We characterized our co-cultures by microscopic approaches and showed that after overnight co-cultures BMDCs were either in close contact with dendrites or directly linked to neurons via TNTs (Supplementary Movie S9). Recently, similar connections have been observed between BMDCs and peripheral neurons isolated from the dorsal root ganglia [38]. Having established the presence of such cell–cell contacts, we turned our attention to the characterization of intercellular prion transfer mechanisms. According to previous models of prion transfer, prion-loaded BMDCs could transfer prions to neuronal cells by excreting PrPSc in the medium [41,52–54], by secretion of membrane exovesicles [43–46] or by direct cell–cell transfer [37,55].

Direct co-cultures from BMDCs and neurons established for 18 h allowed us to detect prion transfer to neuronal cells. However, when cells were co-cultured through filters, no detectable transfer was observed, arguing against the involvement of secreted PrPSc. To rule out the possibility that prion aggregates were retained on the filters, we also exposed neuronal cultures to medium conditioned by prion-loaded BMDCs. These experiments did not show significant prion transfer, as PrPres signal observed in neurons was much lower compared with the signal observed in the cases of direct cell–cell contact (Figure 3). We also determined the kinetics of prion transfer establishing short time co-cultures (from 30 min to 4 h), and demonstrated that efficient transfer required as little as 4 h of co-culture, which is consistent with the time necessary for the establishment and transfer via TNTs in cell cultures (Supplementary Figure S5 at http://www.BiochemJ.org/bj/431/bj4310189add.htm) [37,56].

Overall, our results indicate that prion-loaded BMDCs are able to transfer PrPSc to neuronal cells upon direct and relatively short cell–cell contact. Although BMDCs could secrete prion-enriched exosomes, we have been unable to show the involvement of the secretory pathway in the PrPSc transfer to neuronal cells under our particular culturing conditions (e.g. short incubation time and one-quarter cell dilution). Thus although we cannot rule out other manners of transfer, our data indicate that the transfer mediated by direct cell–cell contact is very efficient.

Interestingly, no PrPres signal can be detected in BMDCs removed from direct contact with the neuronal cells, whereas PrPres was still present in BMDCs exposed to neurons through filters. These data strongly support the hypothesis that prion transfer from BMDCs to neurons is strongly induced upon cell–cell contact. Furthermore, because at the time of co-cultures (after 18 h of uptake), all of the PrPSc aggregates are in the cytosol of BMDCs (Figure 1) and not at the cell surface, these data suggest a transfer from the cytosol possibly via TNTs, excluding a transfer through plasma membrane to neighbouring cells. Interestingly, the transfer of PrPSc from Me7-loaded BMDCs to dorsal root ganglion neurons has recently been examined [38]. In this study, experimental conditions also suggested prion transfer through TNT-like structures shown to connect BMDCs to dorsal root ganglia and excluded the involvement of PrPSc secretion.

Since we have previously shown that co-cultures with prion-loaded BMDCs results in infection of primary neurons, we next followed up the cultures to determine the requirements for prion infection of the targeted neurons. Consistent with the transfer experiments, we found that prion infection is only detected after direct co-culture conditions and does not occur if cells are separated by filters or when co-cultures were performed with aldehyde-fixed BMDCs. These experiments indicate that the transfer is an active mechanism requiring remodelling of the plasma membrane. Interestingly, a similar experiment performed by Kanu et al. [55] had shown a reduction of 75 % in the efficiency of transfer of infection from fixed scrapie SMB (Scrapie mouse brain) cells co-cultured with targeted HMH (cells expressing a chimaeric mouse and hamster PrPc) cells, as opposed to live cell co-cultures. These data are in agreement with our conclusion that efficient transfer requires an active membrane remodelling, although it is clear that infection can be acquired via different mechanisms in less efficient manners (e.g. long co-cultures with fixed cells) [47–50,55].

In the present study, using a number of restrictive experimental conditions such as short co-culture times, low BMDC/CGN ratios, physical separation and pre-fixation of cells, we were able to show that direct cell–cell transfer of PrPSc between these two cell types occurs in a PrPc-independent manner. Interestingly, having excluded transfer from the cell surface and by secretion, all of our data point towards a role of TNT-like structures in the intercellular transfer of PrPSc from BMDCs to CGNs, similar to what was recently shown with dorsal root ganglion neurons [38]. Finally, our system of co-cultures suggests that DCs could be important players during prion spreading *in vivo* and will allow further characterization of prion spreading from the periphery to the nervous system of different scrapie strains, which could lead to a better understanding of the species barrier phenomenon.

AUTHOR CONTRIBUTION

Chiara Zurzolo conceived the project. Christelle Langevin planned and performed most of the scrapie degradation experiments, co-incubation assays and analysed the data. Karine Gousset and Maddalena Costanzo planned and performed the uptake experiments, and analysed the data. Christelle Langevin and Odile Richard Le Goff prepared the BMDCs. Christelle Langevin, Karine Gousset and Chiara Zurzolo wrote the manuscript. All authors discussed the results and manuscript text.

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multimedia online data

SUPPLEMENTARY ONLINE DATA

Characterization of the role of dendritic cells in prion transfer to primary neurons

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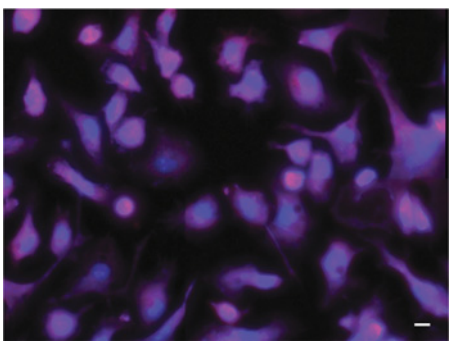

Figure S1 No PrPSc signal is detected in control BMDCs

BMDCs plated on fibronectin-coated Ibidi dishes were washed, fixed and immunolabelled with the Sha31 anti-PrP antibody and Alexa-Fluor®-546-conjugated secondary antibody. HCS CellMask Blue was used to label the cytosol of the BMDCs (blue). No PrPSc punctate (red) can be detected. Mosaics (3×3 fields) were acquired by wide-field microscopy. The scale bar represents 10 μm.

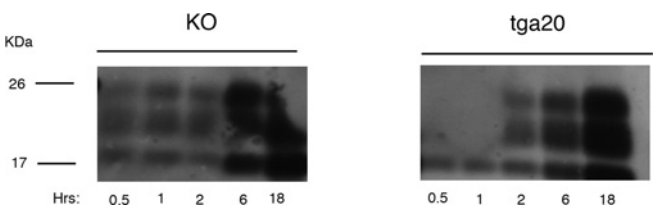

Figure S2 PrPSc endocytosis in BMDCs is independent of PrPc expression

BMDC cultures isolated from KO or tga20 mice, were exposed to 139A brain homogenate for the times indicated. Cells were washed, lysed and 50 μg of proteins were treated with PK prior to immunoblot analysis with the Sha31 antibody. PrPres is progressively internalized by BMDCs with a peak between 6 and 18 h. Identical kinetics of internalization were observed for KO and tga20 mice. The molecular mass in kDa is indicated on the left-hand side of the blots.

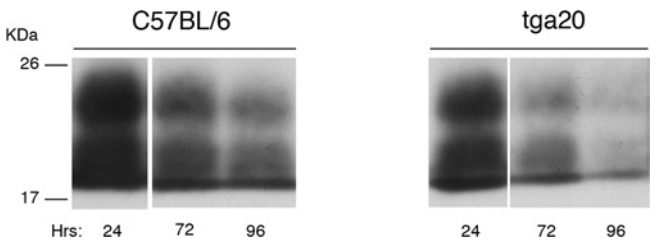

Figure S3 Time course of PrPSc degradation in BMDCs

BMDC cultures were established from C57BL/6 (left-hand panel) or tga20 (right-hand panel) mice. At 10 days post-plating, cells were exposed to 139A brain homogenate for 18 h. The cells were washed three times by centrifugation and plated for the times indicated. Cells were lysed and PK-treated before analysis of PrPres expression by immunoblotting using the Sha31 antibody. Western blot analysis indicates a progressive decrease in the PrPres signal between 24 and 96 h of exposure. The molecular mass in kDa is indicated on the left-hand side.

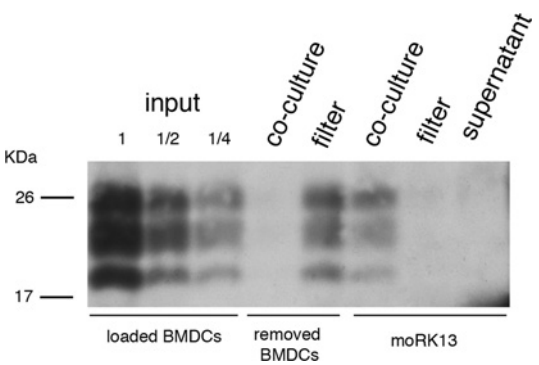

Figure S4 Cell–cell contact is required for PrPSc transfer from BMDCs to moRK13

Similar to CGNs, prion-loaded BMDCs were co-cultured with moRK13 cells directly (co-culture), through filters (filter) or moRK13 cells were exposed to the conditioned medium of BMDCs (supernatant). After 18 h BMDCs were removed and the moRK13 cells were extensively washed. Then a PK assay was performed on BMDCs 24 h post-loading (input). Prion transfer was evaluated by detection of PrPres in moRK13 cells and prion-loaded BMDCs after the co-cultures. Similar to CGNs (Figure 3A of the main text), PrPres is only detected in moRK13 cells after direct co-culture, whereas it stays in BMDC cultures separated by a filter, confirming that cell–cell contact is a prerequisite for the discharge of BMDCs and prion transfer to the recipient cells. Several dilutions of the original input show that one-quarter of the original PrPSc is discharged from BMDCs to the recipient cells. The molecular mass in kDa is indicated on the left-hand side of the blot.

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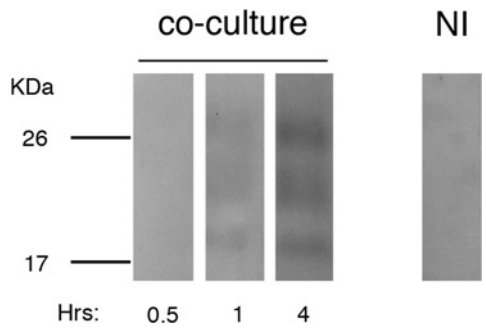

Figure S5 Kinetics of PrPSc transfer from prion-loaded BMDCs to neurons

Following BMDC exposure to 139A brain homogenate, co-cultures were established with CGNs for the times indicated. BMDCs were removed and neuronal cell extracts were analysed to detect PrPres by immunoblot using the Sha31 antibody. PrPres was detected as early as 4 h of co-culture, suggesting a rapid mechanism of transfer. The molecular mass in kDa is indicated on the left-hand side.

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RESULT 2:

Characterization of the mechanisms of polyglutamine aggregates transfer in neuronal cells and primary neurons

2.1 Objectives

a. To investigate whether spontaneous cell-to-cell transfer of polyQ aggregates occurs in co-cultured neuronal cells and primary neurons.
b. To characterize the mechanism of intercellular transfer and evaluate a possible role for Tunneling nanotubes (TNTs).
c. To examine whether cell-to-cell transfer of polyQ Htt induces nucleation of endogenous wild-type Htt (e.g. seeding).

2.2 Summary of the results and discussion

Huntington's disease (HD) is a dominant inherited neurodegenerative disorder caused by the expansion of a CAG repeat in the exon 1 of the huntingtin gene, resulting in an expanded polyglutamine (polyQ) tract in the N-terminal part of the encoded protein. PolyQ stretches above a threshold of 35-40 Q cause misfolding and aggregation of huntingtin (or of a fragment of the protein) (Davies et al., 1997; Di Figlia et al., 1997)
The hallmark of HD is the presence of inclusion bodies both in the cytoplasm and nucleus of affected cells (Gutekunst et al., 1999). HD is mainly characterized by the selective loss of medium spiny projection neurons in the striatum (Vonsattel and DiFiglia, 1998), although the pathology spreads progressively to other areas of the brain, following topologically predictable patterns (Walker, 2007; Rosas et al., 2008). Yet, it is unknown whether and how Htt misfolding progresses through the brain, contributing to the anatomical spreading of the pathology.
In this part of my PhD work, I explored the occurrence and the mechanisms of polyQ Htt transfer between neuronal cells and primary neurons.

First, I directly investigated the capacity of intracellular aggregates of a mutant Htt fragment of transferring between neuronal cells (CAD cell) and between primary neurons (CGN).
I set up an in vitro approach that allowed distinguishing donor cells, transfected with a GFP-tagged mutant Htt fragment (GFP-HttQ68) from acceptor cells expressing a cytosolic mCherry construct.
Interestingly, using flow cytometry and microscopy analysis, I found that the transfer of polyQ aggregates was triggered by direct cell–cell contact, as it occurred when GFP-HttQ68 cells (donor) were co-cultured with acceptor cells both in the case of neuronal cells and primary CGN. As a consequence, no transfer to mCherry labeled cells was observed in CAD cells when a filter separated the two cultures or when acceptor cells were exposed to the conditioned medium of GFP-HttQ68 cultures. These data exclude the involvement of polyQ aggregate secretion as an efficient transfer mechanism in our condition. Similarly no transfer was observed in primary neurons when they were only allowed to share the medium in the absence of cell-cell contact between donor and acceptor cells. Additionally, we could not detect an increased rate of cell death in neuronal cells upon transfection with GFP-HttQ68 (up to 48 h post transfection), suggesting that in our system aggregate transfer is an active mechanism that does not rely on passive release upon cell death. In addition, when I characterized CAD co-cultures by microscopy I found that a relevant percentage of mCherry cells containing multiple aggregates were either in close contact or directly linked to GFP-HttQ68 donor cells via TNTs. By performing three-dimensional reconstruction, I demonstrated the presence of GFP-HttQ68 inside TNTs connecting the two cell populations. Because polyQ aggregates are either cytosolic or nuclear and are not at the cell surface, these data suggest a transfer from the cytosol possibly via TNTs, excluding a transfer through plasma membrane to neighbouring cells. Of interest, TNT-like structures have been shown to efficiently transfer PrPSc in neuronal cells (Gousset et al., 2009) and between BMDCs and primary neurons (Langevin et al., 2010; Dorban et al., 2010). Recently, Aβ particles have also been shown to transfer via TNTs between primary neurons and astrocytes (Wang et al., 2011). Interestingly, I also reported the formation of aggregates of endogenous huntingtin within neurites of mCherry labeled primary neurons upon direct co-culture with GFP-HttQ68 expressing neurons, suggesting that, following

transfer, polyQ aggregates are able to seed misfolding of endogenous wild-type Htt.
In conclusion, using a number of restrictive experimental conditions, I provided the first direct demonstration (to my knowledge) that polyQ aggregates formed within a cell (and not exogenously added to the cell culture) can efficiently transfer to neighbouring cells, possibly via TNTs and can seed misfolding of endogenous protein. This can contribute to the early stage of HD pathogenesis and to the progression of the disease in the brain.

This work is now ready for submission and is appended as manuscript below.

ARTICLE 2

Mechanism of cell-to-cell transfer of polyglutamine aggregates in neuronal cells and primary neurons

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Running Head: Intercellular spreading of polyglutamine aggregates
Keywords: protein misfolding, HTT, intercellular transfer

Abstract

Huntington's disease (HD) is a dominantly inherited neurodegenerative disease caused by expansion of a CAG tract in the exon 1 of the huntingtin gene. The mutant gene encodes a variant of the huntingtin (Htt) protein containing a homopolymeric tract of polyglutamine (polyQ) in excess of the pathogenic threshold of ~35Q.

Despite the rapidly accumulating evidence supporting a role for intercellular transmission of protein aggregates in the pathological spread of neurodegeneration in animal and cell culture disease models, little is known about whether and how huntingtin misfolding progresses through the brain. Uptake of externally applied synthetic polyQ peptides and recombinant fragments of mutant huntingtin has been reported in cell cultures as well as the ability of the internalized aggregates to seed polymerization of soluble huntingtin. In the present study we directly investigated the capacity of intracellular polyQ aggregates to transfer from one cell to another in neuronal cells and primary neurons. Using both flow cytometry and microscopy analysis, we determined that Htt aggregates spontaneously transfer between neuronal cells and in primary neurons. We demonstrated that in both neuronal cells and primary neurons it requires cell-cell contact and does occur upon aggregates secretion in the culture medium. Furthermore, we show that Tunneling nanotubes (TNTs) provide an efficient transfer mechanism in neuronal cells. Finally, we reported aggregation of wild-type Htt in primary neurons upon direct co-culture with mutant Htt expressing neurons, suggesting that, following transfer, polyQ aggregates possibly stimulates further misfolding of the endogenous protein. To our knowledge, this study represents the first direct demonstration that polyQ aggregates formed within a cell (and not exogenously added to the cell culture) can efficiently transfer to neighbouring cells, possibly via TNT structures and seed misfolding of the wild-type protein.

Introduction

Huntington's disease (HD) is a dominant inherited neurodegenerative disorder caused by the expansion of a CAG repeat in the exon 1 of the huntingtin gene, resulting in an expanded polyglutamine (polyQ) tract in the N-terminal part of the encoded protein. PolyQ stretches above a threshold of 35-40 Q cause misfolding and aggregation of huntingtin (or of a fragment of the protein) (DiFiglia et al., 1997; Davies et al., 1997) with a positive correlation between the length of the CAG repeat and the amount of the aggregates (Scherzinger et al., 1999). Within the expanded range, longer repeats cause early onset (Andrew et al., 1993), consistent with the hypothesis that aggregation of the protein is related to pathogenesis. Indeed, HD belongs to the group of "protein conformational disorders" that include, among systemic and organ-specific amyloidosis, Alzeimer's disease, Parkinson's disease and prion disorders (Carrell and Lomas, 1997). Although the disease-associated proteins are very diverse in their primary sequence, when it cames to aggregation, all of them form insoluble, fibrillar, β-sheet rich aggregates, termed amyloid, that accumulate either in the cytoplasmic or extracellular space (Ross and Poirier, 2004).

The hallmark of HD is the presence of inclusion bodies both in the cytoplasm and nucleus of affected cells (Gutekunst et al., 1999). HD is mainly characterized by the selective loss of medium spiny projection neurons in the striatum (Vonsattel and DiFiglia, 1998), although the pathology spreads progressively to other areas of the brain (Walker, 2007). Brain imaging studies showed that cortical degeneration in HD follows a topologically predictable pattern (Rosas et al., 2008) and precedes degeneration in the striatum (Vonsattel and DiFiglia, 1998; Brundin, Melki, et al., 2010) . Inclusions containing huntingtin are present in the regions of the brain that degenerate, although the presence of visible aggregate does not always correlate with cell death (Kuemmerle et al., 1999).

Yet, it is unknown whether and how Htt misfolding progresses through the brain. Of interest, many protein conformational neurodegenerative disorders have been shown to begin in a specific area of the brain and extend along predictable anathomical paths (Brundin, Melki, et al., 2010). A growing amount of literature, has suggested that disease-associated protein aggregates can transfer between cells contributing to the anatomical spreading of the underlying pathology, a prerogative that, until

recently, was confined to infectious prions (Brundin, Melki, et al., 2010; Lee et al., 2010). Furthermore, prion-like transmission mechanisms may be responsible for propagation of protein misfolding in non-prion neurodegenerative disorders, suggesting the existence of a general pathogenic principle in neurodegenerative proteinopathies (Frost and Diamond, 2010). In the specific case of HD, cell culture experiments have shown that synthetic polyQ peptides or recombinant fragments of mutant Htt when applied externally to cultured cells are readily taken up (Yang et al., 2002; Ren et al., 2009) and gain access to the cytoplasm where they can seed polymerization of a soluble 25Q huntingtin reporter (Ren et al., 2009). Moreover, these assemblies persist for over 80 generation in prolonged cell culture, despite their dilution in dividing cells, suggesting a self-sustaining seeding and fragmentation process similar to prion replication (Caughey and Lansbury, 2003; Ren et al., 2009). The relevance of these observations in HD pathogenesis is unclear. Indeed, fetal grafts of striatal tissue in HD patients' brains have shown, upon autopsy, to be susceptible of disease-like neurodegeneration, but abnormal huntintingtin aggregation was not observed within a decade from the trasnsplant (Cicchetti et al., 2009). Thus, it is not clear how the pathogenesis is transmitted to the grafted cells and whether this is linked to the spreading of oligomeric form of the disease protein or of the toxic signal. This appear to be different from the case of cytoplasmic a-synuclein rich Lewy bodies that were found in grafted cells in the brain of Parkinson's disease patients, suggesting that prion-like transmission had possibly occurred from the diseased brain to the healthy grafted tissue (Allan et al., 2010; Li et al., 2008). In addition, Ren and colleagues reported that natural cell-to cell transmission of Htt, measured indirectly from the seeded-polymerization of a cytoplasmic 25Q huntintin reporter, was rather inefficient in co-cultured HEK293 cells, and could be drastically increased by selective lysis of donor cell (Ren et al., 2009). Deep-etch electron microscopy of polyQ aggregates in the cytosol revealed no evidence of surrounding membranous structure, suggesting the absence of a vesicular uptake (Ren et al., 2009).

The question whether and how aggregated misfolded protein transfer between cells and lead to the spreading of the pathology is central to many neurodegenerative disorders. Recently, seeding of intracellular protein aggregates by external amyloid fribrils has been reported to occur in a cell culture model for tau aggregation (Frost et al., 2009). Importantly, in this case, spontaneously formed aggregates were also able

to naturally transfer between cells (Frost et al., 2009). Moreover, uptake of extracellualar aggregates containing tau (Frost et al., 2009; Kfoury et al., 2012) and a-synuclein (Desplats et al., 2009; Lee et al., 2008) is reported to occur through phagocytic or endocytic processes that results in delivery to the endoplasmic compartment, from which they must escape to nucleate aggregation of endogenous cytoplasmic proteins. Finally, it has been shown that prions and amyloid-β transfer between cells via Tunelling Nanotubes (TNTs), thin actin-rich membrane bridges connecting the cytoplasm of distant cells and allowing the exchange of cell components and pathogens (Gousset et al., 2009; Abounit and Zurzolo, 2012; Marzo et al., 2012). Therefore, it is possible that TNTs act as transport conduits also for other prion-like protein aggregates as recently showed for amyloid-β particles (Wang et al., 2011).

In the present study we investigated directly the capacity of intracellular aggregates of a mutant Htt fragment of transfer between co-cultured neuronal cells as well as in primary neurons. Using both flow cytometry and microscopy analysis, we found that, Htt aggregates formed within donor cells are spontaneously transferred to receiving cells and characterized the mechanism of transfer. We demonstrate that Htt aggregates transfer is an active mechanism that does not rely on their passive release from dying cells as a result of mutant Htt induced toxicity. Moreover, transfer requires cell-to-cell contact and does not occur through the supernatant. We show that TNTs provide an efficient mechanism of transfer of polyQ aggregates between neuronal cells and seeded-polymerization of soluble endogenous Htt in primary neurons.

Materials and methods

Cell lines, mouse lines, primary cell cultures

CAD cells (mouse catecholaminergic neuronal cell line, Cath.a-Differentiated) were a gift of Dr. Laude H. (Institut National de la Recherche Agronomique, Jouy-en-Josas, France) and were cultured in Opti-MEM (Gibco) with the addition of 10% FBS (fetal bovine serum). Primary cultures were established from C57BL/6J mice provided by Charles River Laboratories. All experiments were performed according to national guidelines.

Primary cultures of CGNs (cerebellar granule neurons) were established as previously described (Langevin et al., 2010; Cronier et al., 2004). CGNs were cultured for the indicated time on poly-D-lysine (10 ug/ml; Sigma) pre-coated coverslips at a density of 400 000 cells/coverslip in DMEM (Dulbecco's modified essential medium; Gibco) supplemented with 10% (v/v) FBS, 20 mM KCl, penicillin (50 units/ml), streptomycin (50 μg/ml; Gibco) and complemented with B27 and N2 supplement (Gibco).

All cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Plasmids and transfection procedures

GFP-HttQ68 and GFP-HttQ17 were a kind gift of Dr. Humbert S. (Institut Curie - UMR 146 du CNRS, Centre Universitaire Orsay, France). mCherry vector was from Clontech.

CAD cells were transfected at 50% confluence with the indicated construct using Lipofectamine 2000 (Invitrogen), according to the producer's protocol.

CGNs were transfected with the appropriate construct in suspension immediately after isolation using the Amaxa nucleofector system and the amaxa electroporation transfection reagent VPD-1005 (Lonza) according to the manufacturer's procedure.

Western Blots

CAD cells were seeded 1.000.000 in 25 cm flasks. The following day, cells were transfected with 4 μg of GFP-HttQ68 or GFP-HttQ17 as described above. After 48 h, cells were washed in D-PBS and lysed in 0.5% Triton X-100, 0,5% sodium deoxycholate, 100 mM NaCl, 10mM Tris-HCl (pH 8). After a short centrifugation

(3000*g* for 5min), 40 μg of cell lysate were resolved by SDS-PAGE either on a 7,5% acrylamide gel and Western blot with MAB2166 anti-huntingtin antibody (1:5000) or on a 12% acrylamide gel and probed with antibodies against cleaved caspase 3 [(Asp175) (5A1E); Millipore] and cleaved PARP [(Asp214) (7C9); Millipore], as markers of apoptosis. Blots were stripped and re-probed with mouse anti-tubulin (mouse monoclonal antibody, 1:5000) (Sigma). HRP-conjugated secondary antibodies and ECL TM reagents from Amersham (GE Healthcare) were used for detection.

Flow Cytometry

CAD cells were transfected separately with GFP-HttQ_{68}, GFP-HttQ_{17}, and mCherry constructs in 25 cm flasks as descrided above.

For co-culture experiments, 1-day after transfection, mCherry-expressing CAD cells were co-cultured with cells expressing either GFP-HttQ_{68} or GFP-HttQ_{17} at a ratio 1:1 in 35 mm dishes. After 24h co-cultures, cells were scraped in D-PBS plus 1% FBS, passed through 40 μm nylon cell strainers and fixed in 2% paraformaldehyde overnight prior to flow cytometry analysis (BD Biosciences LSRFortessa cell analyzer). Each experiment was performed in triplicate and repeated three times. 10.000 cells were counted each time.

GFP-HttQ_{68} or GFP-HttQ_{17} expressing cells were also plated on 0.4 μm filters (Costar) placed on top of mCherry expressing cells in order to inhibit cell-cell contact. After 24h co-cultures, the filters were removed and the mCherry expressing cells were analyzed by flow cytometry as described above.

In order to test supernatant involvement in transfer, CAD cells were transfected separately with GFP-HttQ68 and GFP-HttQ17. After 24h, cells were gently washed with D-PBS and fresh medium was added for additional 24h. Then, GFP-HttQ_{68} or GFP-HttQ_{17} CADs medium was used to culture mCherry expressing CAD (transfected the day before). After 24h incubation, mCherry expressing cells were analyzed by flow cytometry as described above.

CGN co-cultures

For co-culture experiments, CGNs transfected with mCherry construct were mixed with GFP-HttQ68 transfected neurons at a ratio 1,5:1 immedialely after nucleofection and plated on coverslips as described above.

Immunofluorescence

At the indicated times post-transfection, cells were washed in D-PBS (Dulbecco's Phosphate Buffered Saline; Gibco) and fixed in 4% paraformaldehyde (Electron Microscopy Sciences). The cells were permeabilized with 0.1% Triton X-100 and labeled with mouse anti-huntingtin antibody (1:300, for 18 h at 4°C) (MAB2166; Millipore). The Alexa Fluor® 633 secondary antibody was purchased from Invitrogen. When indicated, CAD cells were stained with HCS *CellMask*™ Blue (1:10 000, for 20 minutes at R.T.) (Invitrogen), Wheat Germ Agglutinin (WGA)-rhodamine or WGA-Alexa Fluor® 350 conjugate (1:300, for 20 minutes at R.T) (Invitrogen). CGNs were also stained with DAPI (1:5000) (Sigma). The cells were washed and mounted with Aqua-Poly/Mount (Polysciences).

Images were acquired with a wide-field microscope (Zeiss Axiovert 200M) controlled by Axiovision software. All Z-stacks were acquired with Z-steps of 0.4 μm. For CAD cells, the HCS *CellMask*™ staining was used to set the autofocus module, providing single focal plane images. When indicated, random mosaics of (3 × 3 fields) were obtained using a 63× objective Plan-Apochromat objective [1.4 NA (numerical aperture)]. Representative tiles are presented.

Images of CAD cells used for 3D reconstruction and TNTs (tunneling nanotubes) detection were acquired with an optimal Z-step of 0.25 μm covering the whole cellular volume.

TNTs (Tunneling nanotubes) detection

CAD cells were transfected with the indicated constructs in 25 cm flasks. The following day or 12 h post-transfection, cells were plated on μ-Dish$^{35\ mm,\ high}$ (Ibidi ®) and fixed at the indicated time with a solution of 2% paraformaldehyde, 0.05% gluteraldehyde and 0.2 M Hepes in D-PBS for 20 min, followed by a second 20 min fixation with 4% paraformaldehyde and 0.2 M Hepes in D-PBS. Then cells were gently washed in D-PBS and stained as indicated.

Image processing and quantification

Raw data were processed with Axiovision 4.8 software. The auto-scaling (min/max) of signal detection was applied to all images. When indicated, images were deconvolved using 3D Huygens Deconvolution software and three-dimensional reconstructions were performed with Imaris software.

To quantify the percentage of CAD cells with huntingtin aggregates and to evaluate the number of TNT-connected cells, a manual analysis was performed as previously shown (Gousset et al., 2009). Experiments were made in triplicate and repeated three times.

FACS raw data were analyzed by Kaluza® Flow Cytometry software (Beckman Coulter, Inc.).

Image Analysis using Acapella™ software

In order to evaluate and quantify the transfer of polyQ aggregates from donor (GFP-HttQ68 transfected) to acceptor (mCherry transfected) CGN in co-culture experiments, we used the Acapella™ image analysis software (version 2.3 - Perkin Elmer Technologies) provided by the Plate-forme Imagerie Dynamique (Institut Pasteur) that allowed detecting in an automated manner Htt aggregates (GFP-tagged) in mCherry labeled neurons.

The script is subdivided in four object segmentation subroutines and required the setting of several input parameters:

- Segmentation of the nuclei in the channel 305 (DAPI staining) (nuclei_detection)
- Automated detection of the cell body of acceptor cells (mCherry labeled neurons) in the channels 305 (nuclei, DAPI staining) and 546 (mCherry signal) by applying a mask that allowed to select only the cell bodies labeled in both channels (DAPI/mCherry overlap).
- Neurite detection. Starting from the selected cell bodies, the application of a specific module of the Acapella software (neurite_detection) allowed to automatically draw the neuritic arborization corresponding to each cell body that, at this stage, appeared as “lines” in the 546 channel (mCherry signal). Then, to gain the thickness, a dilatation filter (radius = 3) was applied to the neuritic arbors.

- Spot and small object detection. In order to detect Htt aggregates two different algorithms were applied: spots and small object detection in both 488 (GFP-HttQ68 signal) and 633 (anti-Htt MAB2166) channels. While the spot detection is based on a local intensity analysis with each spot corresponding to a local intensity maximum, the small object detection takes in to account not only the global intensity but also shape and size. Spot and small objects were scored as "within neurite" only in presence of a shape overlap with the neurite of at least 70%. We consider only spot and small object that were positive in both 488 (GFP-HttQ68 signal) and 633 (anti-Htt MAB2166) channels (based on a shape overlap) and we reported the presence within neurites of spots and small object detected only in the 633 channel (not GFP positive), thus indicating aggregation of the endogenous protein.

The input parameters were optimized with feasibility studies in collaboration with image analysis experts at Plate-forme Imagerie Dynamique (Institut Pasteur). Different versions of the script corresponding to parameter adjustment were validated and included the use of GFP vector transfected neurons (versus GFP-HttQ68) as negative control.

Results

Intracellular mutant Htt aggregates transfer between co-cultured CAD cells

The first 480 amino acids of huntingtin with either 17Q (wild-type Htt) or 68Q repeats (mutant Htt) fused to green fluorescent protein (GFP-Htt17Q and GFP-Htt68Q respectively) were expressed in CAD neuronal cells (Figure 1A) and the fraction of cells with aggregates was quantified. At 48h post-transfection about 20% of the cells expressing GFP-Htt68Q contained aggregates, differently from GFP-Htt17Q that showed a diffuse nucleocytoplasmic fluorescence with GFP puncta in less then 5% of cells (Figure 1B).

In order to understand whether intracellular Htt aggregates transfer between co-cultured cells, we set up a flow cytometry assay. To this aim, CAD cells were transfected with either GFP-Htt68Q (donor population) or with mCherry-vector (a red fluorescent protein variant, to label the acceptor population). At 1-day post-tranfection, the two cell population were co-cultured at a ratio 1:1 for 24h prior to flow cytometry (see Materials and Methods). As control for background signal, donor and acceptor cells were cultured separately for 24h and mixed immediately before flow cytometry. In co-cultures with GFP-Htt68Q, 3.7% of cells scored as GFP/mCherry double positive versus 0,5% of cells mixed prior to flow cytometry (i.e. background, mix) (Figure 2A-B).

To visualize the transfer of aggregates, we co-cultured donor and acceptor cell populations for 24h on ready-to use supports for microscopy (µ-Dish35 mm, high, Ibidi ®). By fluorescence microscopy, we observed the presence of GFP-Htt68Q aggregates in mCherry acceptor cells (Figure 2C).

Because GFP-Htt68Q has been shown to induce cell death (Bjorkoy et al., 2005), the transfer of aggregates to recipient cells could derive from the internalization of Htt aggregates released in the medium from dying cells. To test this possibility, we measured cell death in GFP-Htt68Q cells at the time of the co-culture experiments (48h post-transfection) compared with cells transfected with GFP-Htt17Q. We evaluated both active caspase3 and cleaved PARP levels, as markers of apoptosis, by Western Blot (Figure 3) and we assessed DNA fragmentation by TUNEL (TdT-mediated dUTP nick end labeling), using fluorescence microscopy (data not shown). With these combined approaches, we could not detect any induction of cell death in

CAD cells expressing either mutant or wild-type Htt at 48h post-transfection. Therefore, these data support the hypothesis that aggregates formed within a cell are able to translocate to acceptor cells through a process that is independent of aggregate release following cell death.

Intercellular transfer of Htt aggregates requires cell-cell contact.

The transfer of Htt aggregates could have occurred through either cell-cell contact or secretion. To evaluate the possible impact of secretion and of exosomal release, we examined whether Htt aggregates transfer could occur through filters, which would allow the passage of secretory vesicles and exosomes but would not allow cell-to-cell contact. To this aim, 1-day post-transfection, donor cells expressing mutant Htt were plated on filters positioned in a plate on top of a layer of mCherry-labeled acceptor cells (see Materials and Methods). After 24h incubation, the acceptor cells were analyzed by flow cytometry and only 0,18% of cells were scored as double positive (Fig. 4). Thus, the transfer efficiency was reduced by more than 95% (to the background levels) when filters were used to separate the two populations compared to direct co-cultures.

This result suggested that a secretion mechanism is not involved in the transfer of aggregates in our system. However, to rule out the possibility that filters could trap Htt aggregates, we analyzed whether transfer could be mediated by the supernatant of cells transfected with GFP-Htt68Q. To this aim at 1-day post-transfection the medium of mCherry CAD cells was replaced with the supernatant of GFP-Htt68Q cells collected for 24h post-transfection. Then, after 24h exposure to the conditioned medium, mCherry cells were analyzed by flow cytometry and scored for the presence of Htt aggregates. Similar to the filter experiments, only 0,04% of cells scored as double positive, further confirming that secretion into the medium was not involved in the transfer of Htt aggregates or that this mechanism was not very efficient (Figure 4).

Tunneling Nanotubes mediate intercellular transfer of Htt aggregates in CAD cells

Overall the above data indicated that cell-to cell contact is required for intercellular transfer of Htt aggregates. One attractive possibility is that Htt aggregates formed within a cell might access the cytoplasm of neighboring uninfected cells via Tunneling Nanotubes (TNTs) (Abounit and Zurzolo, 2012; Marzo et al., 2012), as it

was previously shown for PrPSc (Gousset et al., 2009) and amyloid-β (Wang et al., 2011) particles. To evaluate this possibility, 24h after transfection CAD cells expressing either GFP-HttQ68 or mCherry were co-cultured on plastic bottom dishes ready for imaging (μ-Dish35 mm, high, Ibidi ®) in a well-spaced manner to favor the formation of TNTs, as previously shown (Gousset et al., 2009). After 24h co-culture (48h post-transfection, same as the flow cytometry analyses) cells were fixed, stained with WGA-Alexa Fluor® 350 conjugate to label TNT membranes and observed by fluorescence microscopy (Zeiss Axiovert 200M). At this time after co-culture, as expected from the flow cytometry data, we could visualize a relevant percentage of mCherry cells containing multiple aggregates connected through TNTs to GFP-HttQ68 cells (data not shown). However, we could not detect GFP-Htt aggregates inside these structures. This could indicate that transfer of aggregates through TNTs had already occurred. In order to test this hypothesis, we repeated the same experiment by co-culturing the two cell populations for shorter time (18 h, 36 h post-transfection). In these conditions, we could detect GFP-Htt aggregates within TNTs connecting distant cells (Figure 6A) and between the two different cell populations (GFP-HttQ68/mCherry cell pairs) (Figure 6B). Furthermore, we found aggregates in the lumen of TNT-paired mCherry cells, suggesting that transfer of Htt aggregates through TNTs had taken place. These data indicate that TNTs provide an efficient mode of transfer for Htt aggregates between CAD cells.

Next, we evaluated the effect of mutant Htt on the number of TNT-connected cells. In order to assess this, we transfected CAD cells with either GFP-Htt68Q or GFP-Htt17Q. After 12h, cells were detached and plated as described above. Cells were fixed 12 or 24h after plating (corresponding to 24 and 48h post-transfection) and stained with Wheat Germ Agglutinin (WGA)-rhodamine and HCS CellMask™ Blue stain in order to detect TNT structures and cell bodies respectively. Then, by fluorescence microscopy, we quantified the number of transfected cells connected by TNTs. GFP-vector transfected cells were used as control. Interestingly, we found that at 48h post-transfection over-expression of mutant but not wild-type Htt increased the number of TNT-connected cells by about 20% compared to the control cells (Figure 5). Since this corresponds to the total time of the co-culture experiments analyzed by flow cytometry, it possible that mutant Htt on one hand induces an increase of TNT connections and on the other hand hijacks this enhanced network of TNT connections to optimize its transfer between cells.

Cell-to-cell transfer of mutant Htt aggregates occurs in primary neurons and requires intercellular contact.

Having demonstrated that transfer of Htt aggregates occurs in CAD cells, we further investigated the ability of Htt aggregates to transfer between primary neurons. To this aim, we established in vitro co-cultures of primary Cerebellar Granule Neurons (CGNs) (See Materials and Methods). Specifically, GFP-HttQ68 and mCherry transfected CGNs were plated on coverslips at a ratio 1: 1.5 respectively and incubated for 140 hours before fixation. Mosaics of different fields were obtained by wide-field microscopy (Zeiss Axiovert 200M) to analyze the overall neuronal network. We could detect aggregates of GFP-HttQ68 in mCherry neurons both in neuritis and in the cell body around the nucleus (Figure 7). Quantification of the transfer events was made by using a dedicated version of the Acapella™ software (Perkin- Elmer) (see Materials and methods) and revealed that about 4% of mCherry neurons contained GFP-Htt aggregates.

To our knowledge, this is the first evidence that Htt aggregates formed within one neuron can transfer to non-transfected cells in primary neuronal cultures.

In primary neurons cell-to-cell transmission of cytosolic aggregates, such as the one formed by mutant Htt, could occur either through their release to the extracellular space (endo/exocytosis, exosomes, trans-synaptic transmission at axonal terminals) or through direct passage from the cytosol of one neuron to the other, possibly via TNTs (Marzo et al., 2012; Moreno-Gonzalez and Soto, 2011). To evaluate the possible role of the secretory pathway, we plated GFP-HttQ68 and mCherry transfected CGNs on separated coverslips in the same dish, thus, impairing intercellular contact but allowing exchange between the two different populations through the medium. After 140h of incubation, neurons were washed, fixed and analyzed by wide-field microscopy. In this condition, we were not able to detect Htt aggregates in mCherry labeled neurons, thus suggesting that secretion was not the main transfer mechanism for mutant Htt aggregates in primary neuronal cultures.

Overall, these data demonstrate that the transfer of Htt aggregates does not appear to be associated with aggregates secretion in primary CGN (similar to CAD cells) and requires cell- cell contact

Cell-to-cell transfer of Htt aggregates induces nucleation of endogenous huntingtin in primary CGNs.

One of the major recent findings in the pathogenesis of HD is the ability of externally applied polyQ aggregates to be internalized by cells in culture and to induce misfolding and aggregation of endogenous and otherwise soluble wild-type Htt, a process also known as seeding (Ren et al., 2009). Therefore we evaluated whether the transfer of Htt aggregates produced in the donor (GFP-HttQ68 transfected cells) resulted in aggregation of wild-type Htt in the acceptor (mCherry transfected) neurons following TNT mediated transfer. To this aim, we co-cultured GFP-HttQ68 and mCherry transfected CGNs on coverslips as described above. After 140h, cells were fixed and immunolabelled with anti-Htt antibody MAB2166, that recognizes both endogenous Htt and the mutant transfected fragment. In these conditions we detected aggregates labeled with the anti-Htt antibody in mCherry neurons (acceptor neurons) that were not positive for GFP, thus indicating aggregation of endogenous Htt. By using the Acapella software, we quantified the number of mCherry neurons with aggregates that were positive for MAB2166 antibody but not for GFP fluorescence (the analysis is on going for the quantitation).

Discussion

In the present study, we have tested the hypothesis that intracellular mutant huntingtin aggregates transfer between neuronal cells. To this aim we developed an in vitro approach in which CAD (mouse catecholaminergic neuronal cell line, Cath.a-Differentiated) cells expressing an expanded-polyglutamine fragment of huntingtin (GFP-HttQ68) prone to aggregation were co-cultured with acceptor cells expressing a cytosolic mCherry and analyzed by flow cytometry the number of acceptor cells containing Htt aggregates. Remarkably, we detected 3.7% of double fluorescent cells (GFP-HttQ68/mCherry). This was confirmed by microscopy analysis which showed that after 24 h co-culture, mCherry labeled cells contained multiple GFP-positive aggregates and were in close proximity of GFP-HttQ68 expressing cells.

To our knowledge, this is the first direct demonstration that polyQ aggregates formed within a neuronal cell can efficiently transfer to neighbouring cells. This data are in agreement with a previous very nice report that documented the change in the state of a soluble huntingtin reporter (from diffuse fluorescence to puncta) in non neuronal cells (HEK293) when co-cultured with cells expressing an expanded-polyQ fragment. The precise colocalization of the reporter puncta with expanded-polyQ aggregates strongly suggested cell-to-cell transfer of protein aggregates (leading to the seeded polymerization of the soluble reporter), but no direct evidence was provided in this sense (Ren et al., 2009). Moreover, in the same study it was suggested that spontaneous transfer of polyQ aggregates was rather inefficient since the number of reporter cells with puncta could be markedly increase only upon selective lysis of the donor cells compared to control (reporter cells cultured alone) suggesting that aggregates were internalized upon their passive release from dead or dying cells (Ren et al., 2009). The fact that in our conditions (e.g. 48 h post transfection) we could not detect activation of apoptotic pathways or increased TUNEL staining (data not shown) upon GFP-HttQ68 expression indicates that in our system aggregates transfer between neuronal cells is an active mechanism and occurs efficiently between intact, viable neuronal cells.

Having established that transfer of aggregated intracellular huntingtin occurs between intact CAD cells, we began to characterize the transfer mechanisms. We

distinguished between direct cell-cell transfer which needs cell-to-cell contact and transfer through the cell medium (e.g following secretion of the aggregates). When cells were co-cultured through filters, the transfer efficiency was reduced to background noise (more than 95%) compared to direct co-culture, arguing against secretion. Furthermore, to rule out the possibility that aggregates where retained on the filters, we exposed acceptor cells to the conditioned medium of GFP-HttQ68 cells. Also these experiments did not show significant aggregates transfer.

Since in the pathogenesis of HD the target cells are post mitotic neurons, next we characterized whether and how expanded-polyQ aggregates transferred between primary neurons. Consistently with the findings in CAD cells, we could detect cell-to-cell transfer of aggregates from primary neurons expressing GFP-HttQ68 to mCherry expressing neurons when they were co-cultured on the same coverslips, but not when the two different populations only shared the medium.

Overall, these results indicate that in both neuronal cell cultures and primary neurons direct cell-cell contact is required for efficient transfer of GFP-HttQ68 aggregates and no transfer occurrs through release/secretion of the aggregates in the medium in our culturing condition (e.g. 24 h co-culture; 1:1 cell ratio). Furthermore, as Htt aggregates are either cytosolic or nuclear, transfer to neighbouring cells through the plasma membrane upon cell surface contact is not likely to occur.

By fluorescence microscopy and three-dimensional reconstruction we found that GFP-HttQ68 aggregates were inside TNTs connecting two neuronal cells similarly to what was previously reported for infectious prions (Gousset et al., 2009) and recently for β-amyloid (Wang et al., 2011). Interestingly Htt aggregates were found in TNTs upon 12 h co-culture (corresponding to 36h post transfection) whereas after 24 h co-culture (48 h transfection) we could visualize donor/acceptor cell pairs connected via TNT structures, with acceptor cells containing multiple Htt aggregates inside the cell but not in TNTs. Of interest, at 36 h post transfection acceptor cells contained less aggregates then at 48 h (data not shown). This indicates that transfer of aggregates occurs quite early after the co-culture is established and continues to occur between 36 and 48h post transfection. Furthermore, as previously reported (Shin et al., 2005) we observed that the size of the aggregates in cultured cells increases upon time (result not shown), possibly due to the progressive nucleation of Htt molecules, therefore this might influence the ability of this structures to hijack TNTs after prolonged time in co-cultures. In addition, upon overexpression of GFP-HttQ68 (but

not of the wild-type fragment GFP-HttQ17), we detected an increase in the number of TNT structures between, CAD cells (Figure 5). Remarkably, this increase was occurring between 24 and 48 h post transfection, which is consistent with the timing of aggregatete detection in TNT structures (36 h after transfection) (Figure 6)

Overall, our results indicate that Htt aggregates hijack TNT structures and that aggregation-prone polyQ huntingtin itself increases TNT formation, thus optimizing aggregate transfer, similar to what has been recently shown for HIV particles spreading (Eugenin et al., 2009). Because polyQ aggregates are cytosolic (or nuclear) and do not appear to be associated with membrane vesicle upon internalization (Ren et al., 2009) a cytosolic passage as aggresomes through TNTs can be envisaged. In addition, huntingtin can interact with acidic phospholipidis enriched on the cytoplasmic leaflet of the plasmamembrane (Kegel et al., 2005, 2009) suggesting a possible surfing process along the inside of TNT membrane (Marzo et al., 2012). Further, exploration of the mechanisms by which cells are induced to form TNTs and how the transfer of material is regulated within these structures will be essential for a better understanding of the mechanisms of aggregate spreading.

The finding that infectious prions, polyQ aggregates and possibly β-amyloid (Wang et al., 2011) transfer from cell-to-cell through TNTs makes tempting to speculate that they might constitute a general mechanism for the spreding of different β-sheet rich proteinaceous aggregates (Marzo et al., 2012). Further studies are needed to confirm this hypothesis and the identification of specific markers and of these structures in vivo is critical to confirm their role in the progression of protein misfolding throughout the brain in prion, in Huntington's disease and possibly other neurodegenerative disorders (Marzo et al., 2012). Of interest, it has been recently shown that stress induced by hydrogen peroxide (H2O2) treatment lead to an increase in TNT formation in both astrocytes and neurons (Wang et al., 2011). Remarkably, increased levels of oxidative stress in disease brains accompany different neurodegenerative disorder (Jomova et al., 2010; Abounit and Zurzolo, 2012).

It has been reported before that both synthetic and recombinant mutant huntingtin fragments can promote the fibrillogenesis of wild-type htt fragments resulting in the formation of co-aggreagtes (Busch et al., 2003; Ren et al., 2009). However, to satisfy the requirement of "infectivity" in analogy to prion replication breakage of stable oligomers (e.g co-aggregates of exogenous and newly misfolded protein) has to

occur thus resulting in an increased number of aggregation nuclei (e.g. newly aggregated endogenous protein alone). Here, we reported the formation of aggregates of endogenous huntingtin within neurites of acceptor primary neurons upon direct co-culture with GFP-HttQ68 expressing neurons. These results suggest that, following transfer, polyQ aggregates stimulates further misfolding of endogenous wild-type Htt. Remarkably, we detected aggregation of endogenous Htt (with the use of an N-terminal anti-huntingtin antibody) in the recipient cells and not the change in the soluble state of a fluorescent overexpressed reporter, as previously shown (Ren et al., 2009). Our findings strongly argue in favor of a self-sustaining seeding and fragmentation process (similar to prion replication) as it has been suggested before by the persistence of assemblies of a wild type htt reporter fragment in prolonged cultures of dividing cells (Ren et al., 2009).

Of interest, aggregates of full-length tau protein alone (above the threshold of spontaneous aggregation upon overexpression) have been recently reported after exposure and internalization of extracellular aggregates of a tau fragment, together with the presence of co-aggregates of the two proteins (Frost et al., 2009).

In the present study by using a number of restrictive experimental conditions we were able to show that direct cell-cell transfer of Htt aggregates occurs between dividing neuronal cells and in cultures of post-mitotic neurons. To our knowledge, this is the first direct demonstration that polyQ aggregates formed within a cell (and not exogenously added to the cell culture) can efficiently transfer to neighbouring cells. In HD deposition of protein aggregates is an early event in the pathogenic cascade and precedes neurodegeneration. Here we demonstrate that aggregate transfer occurs between intact, viable cells and therefore, possibly can contribute to the early stage of HD pathologenesis and to the progression of the disease in the brain. Consistently, we reported the aggregation of endogenous huntingtin in new aggregation nuclei, suggesting that cell-to-cell transfer of protein aggregates can lead to further propagation of protein misfolding in receiving cells. We also provide a plausible cellular mechanism for transfer. Having excluded secretion, and since cell surface transfer is not likely to occur due to the nature of Htt aggregates, all our data points towards a role of TNT-like structures in the intercellular transfer of Htt aggregates, similar to what has been shown for PrPSc (Gousset et al., 2009) and proposed in the case of β-amyloid (Wang et al., 2011), suggesting the possibility of a general pathogenic mechanism in different neurodegenerative disorders (Brundin,

Melki, et al., 2010; Frost and Diamond, 2010). In later stages, spreading of the aggregates upon their passive release from dead or dying cells, as suggested before (Ren et al., 2009; Brundin, Melki, et al., 2010), can also be envisaged and might further contribute to the progression of the disease. Large amounts of data, along with our present study, suggest that both cell autonomous and non-cell autonomous processes might have a role in the pathogenic cascade of HD. Progressive accumulation of protein misfolding can be the result of events occurring separately in single cells and/or prion-like transmission mechanisms may contribute in this sense leading to altered cell-cell communication and degeneration of vulnerable cells, besides the accumulation of visible protein aggregates. More studies in vivo will be required to understand how, to which extend and at which stage of the disease the cell autonomous and non cell autonomous mechanisms contribute to the disease progression.

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Figure legends

Figure 1. GFP-HttQ68 overexpression in CAD cells leads to aggregates formation. (A) 48 hours after transfection with GFP-HttQ17 or GFP-HttQ68 constructs, CAD cells were stained with HCS *CellMask*™ Blue to label the cytosol. Pictures are representative of three independent experiments. Scale bars, 10 μm. (B) Quantitation of the number of fluorescent aggregates based on manual counting in transfected cells after 48 hours.≈ 4.9% of cells have spontaneous aggregation of GFP-HttQ17 versus ≈ 23% of GFP-HttQ68 expressing cells (n = 3, 100 transfected cells counted per experiment). Mean ± s.e.m are shown.

Figure 2. GFP-HttQ68 aggregates transfer between co-cultured CAD cells. (A) Cells were separately transfected with either GFP-HttQ68 (donor) or mCherry (acceptor) cronstructs for 24 hours. The two cell populations were either mixed immediately prior to analysis (mix) or co-cultured for additional 24 hours (co-culture). Flow cytometry was used to quantify the percentage of acceptor cells containg aggregates. Representative cell plot are shown. (B) Quantitation of flow cytometry experiment revealed that 0.5% of cell scored positive for both GFP and mCherry fluorescence (upper right quadrant of the cell plot) when they were mixed just before analysis whereas 3.7% of cells scored double positive when co-cocultured for 24 h prior the analysis indicating transfer of GFP-HttQ68 aggregates (n = 3, 10,000 cells recorded per condition in each experiment). (C) 1-day post transfection GFP-HttQ68 and mCherry cells were co-cultured on Ibidi ® dishes for 24 h. Cells were then fixed and stained HCS *CellMask*™ to label the cytosol. Multiple GFP-HttQ68 aggregates (insets, white arrowheads) are visible within mCherry cells, confirming transfer of GFP-HttQ68 aggregates. *, labels GFP-HttQ68 transfected cell with aggregates. Scale bars, 10 μm.

Figure 3. GFP-HttQ68 transfected CAD cells do not undergo apoptosis. (A) GFP-HttQ17 and GFP-HttQ68 CAD cells were lysed 48 h after transfection. Whole-cell extracts were prepared and separated by SDS-PAGE gel and revatled by Western blot using antibodies against cleaved PARP and cleaved caspase-3. The second and the third blot are from the same gel. The third blot was stripped and re-blotted with

anti-tubulin to show equal amount of loading. Results are representative of three independent experiments. Activation of apoptosis in GFP-HttQ68 cells was not detected compared to control GFP-HttQ17 cells.

Figure 4. Cell to cell contact is required for GFP-HttQ68 aggregates transfer in CAD cells. To determine the impact of GFP-HttQ68 aggregates present in the supernatant (e.g exosomal release, vesicle secretion), cells were separately transfected with either GFP-HttQ68 or mCherry constructs. The day after, mCherry cells were co-cultured with GFP-HttQ68 cells directly (co-culture) or through filters (filter) or exposed to the 24-hours-conditioned medium of GFP-HttQ68 cells (supernatant) for additional 24 hours. Flow cytometry was used to quantify double positive cells. Representative cell plot are shown. (B) Quantitation of flow cytometry experiments revealed only 0.18% and 0.04% of cells scored positive for both GFP and mCherry fluorescence in filter and supernatant condition respectively. These data indicate that an efficient transfer (3.7% of GFP/mCherry double positive cells) is occurring only when direct cell to cell contact is allowed (mean ± s.e.m, n = 3, 10,000 cells recorded per condition in each experiment).

Figure 5. Over-expression in CAD cells of GFP-HttQ68 but not GFP-HttQ17 increases TNTs number. (A) CAD cells were transfected with GFP-vector, GFP-HttQ17 or GFP-HttQ68. To ensure the optimal cell density for TNT formation, after 12h cells were detached and plated on Ibidi ® dishes. The cells were then fixed 12 or 24 h after plating (corresponding to 24 and 48 h post-transfection respectively) and labelled with WGA-rhodamine (in red) and HCS cell mask (in blue) in order to detect both TNT structures and cell body. Scale bar 10 μm. (B) The relative percentage of TNT-connected cells upon GFP-HttQ17 or GFP-HttQ68 over-expression compared to GFP-vector transfected cells is shown. (mean ± s.e.m, n = 3, 100 transfected cells per experiment). Note that the effect of GFP-HttQ68 over-expression is at 48 h post-transfection that corrisponds to the total time of the co-culture experiments.

Figure 6. Transfer of GFP-HttQ68 aggregates occurs through TNTs in co-cultured CAD cells. 1-day post transfection, GFP-HttQ68 transfected cells (donor,

green) and mCherry transfected cells (acceptor, red) were co-cultured on Ibidi ® dishes and fixed after 12 h (36 h post-transfection). Cells were stained with WGA-Alexa Fluor® 350 conjugate to label TNTs (white). (A) GFP-HttQ68 aggregates were found inside TNTs connecting distant cells (white arrowheads top panel and insets a., b.). Three-dimensional reconstructions were obtained for the selected cells using Imaris software (second middle panel and insets). Insets a. and b. represents magnification of the boxed areas. (B) GFP-HttQ68 aggregates were found inside TNTs connecting GFP-HttQ68/mCherry cell pairs (white arrowheads top and bottom panels), as well as in the cytoplasm of the mCherry transfected cell (white asterisk) suggesting TNT-mediated transfer of GFP-HttQ68 aggregates between the two cell populations. Middle second and third pictures of top and bottom panels represent magnification of the boxed areas. In the third pictures the 305 channel (in white) corresponding to the TNT structures has been removed to better visualize the GFP-HttQ68 aggregates (white arrowheads and asterics). Images are representative of three independent experiments. Scale bars, 10 μm.

Figure 7. GFP-HttQ68 aggregates transfer between primary CGN co-culture. Immediately after isolation, CGNs were transfected with either GFP-HttQ68 (donor) or mCherry (acceptor) constructs and co-cultured at a ratio 1:1.5 on coverslips for 140 hours. Cells were then washed fixed and labeled with DAPI. Mosaics (3 × 3 fields) were acquired by wide-field microscopy to visualize the neuronal network. For acquisition, Z stacks (0.4 μm) were taken. GFP-HttQ68 aggregates (white arrowheads) were found both in the cell body (A) and in the neurites (B) of mCherry labeled neurons, indicating cell-to-cell transfer of GFP-HttQ68 aggregates in primary neuronal co-cultures. * marks one GFP-HttQ68 transfected cell with aggregates. Insets in (A) represent magnification of the boxed areas (first top and third image). In the insets the brightness of the green spot was equally increased. Representative tiles of three independent experiments are shown. Scale bars, 10 μm.

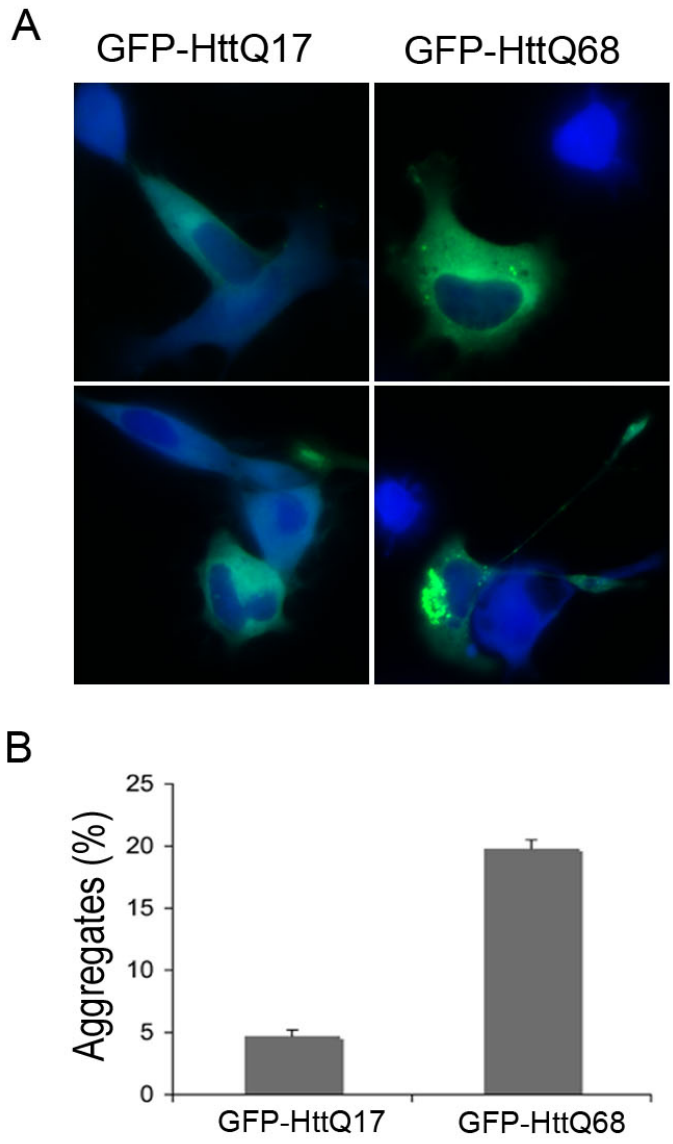
A
GFP-HttQ17
GFP-HttQ68
B
Aggregates (%)
25
20
15
10
5
0
GFP-HttQ17
GFP-HttQ68

Figure 1

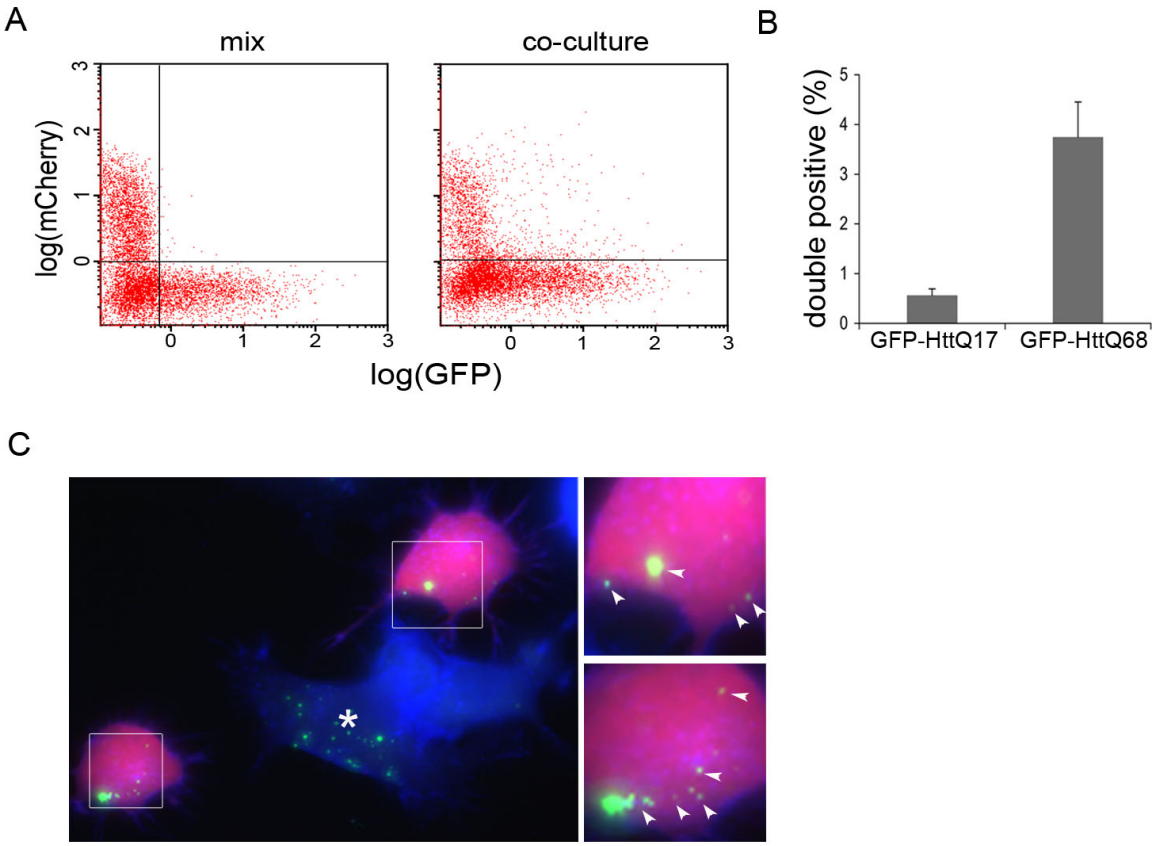
A
mix
co-culture
log(mCherry)
log(GFP)
B
double positive (%)
GFP-HttQ17
GFP-HttQ68
C

Figure 2

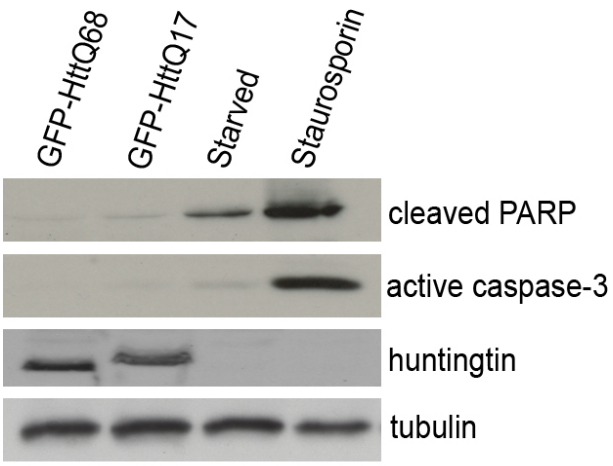
GFP-HttQ68
GFP-HttQ17
Starved
Staurosporin
cleaved PARP
active caspase-3
huntingtin
tubulin

Figure 3

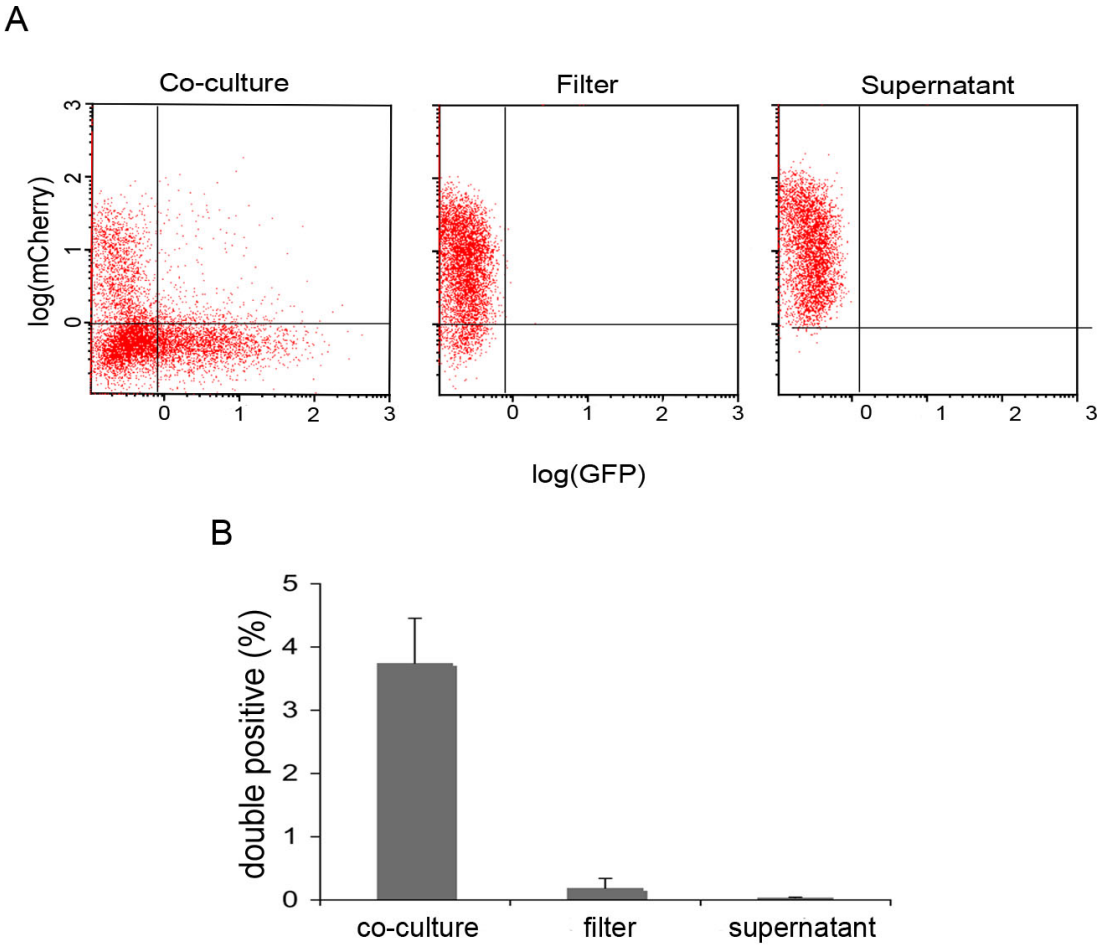
A
Co-culture
Filter
Supernatant
log(mCherry)
log(GFP)
B
double positive (%)
co-culture
filter
supernatant

Figure 4

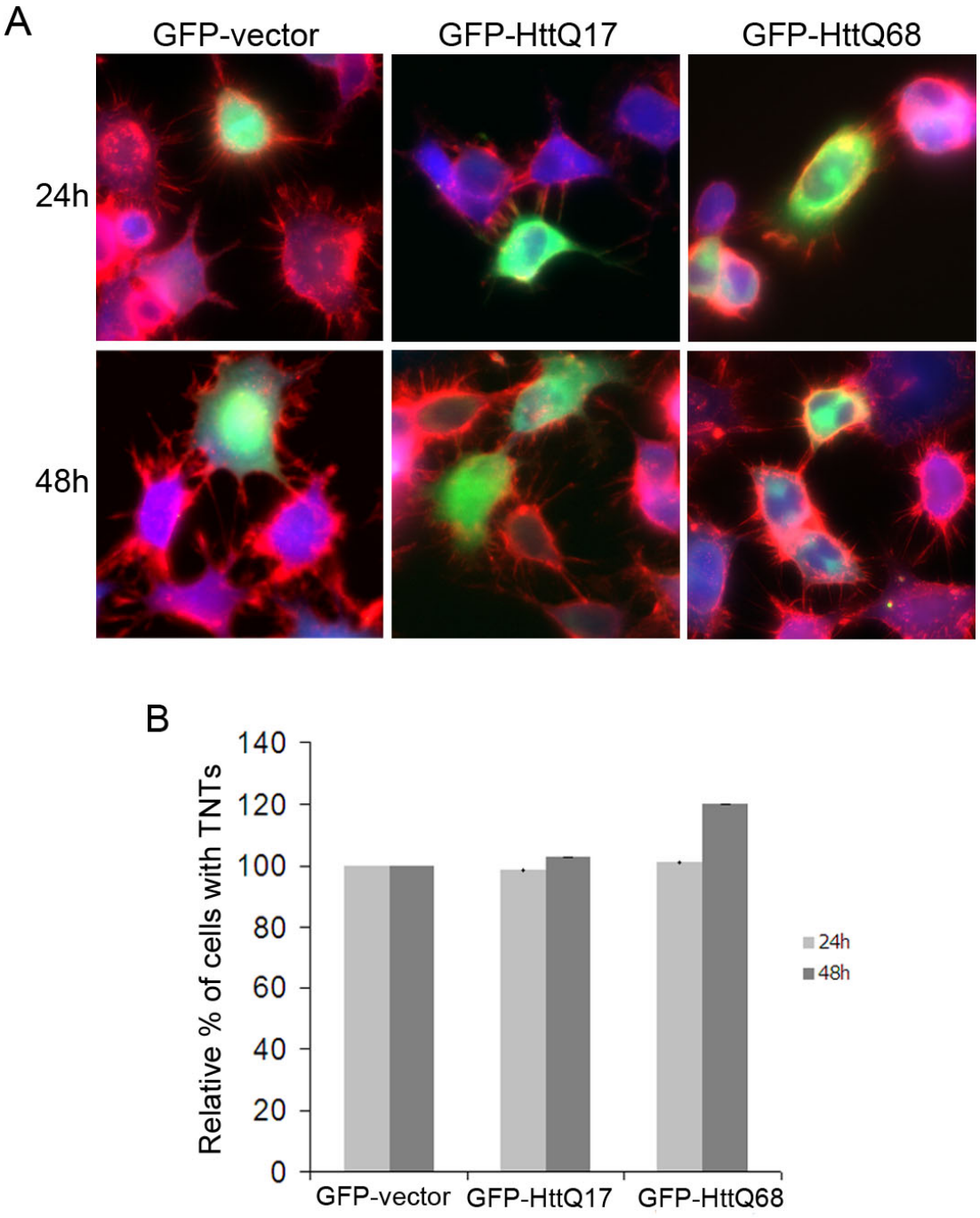
A
GFP-vector
GFP-HttQ17
GFP-HttQ68
24h
48h
B
Relative % of cells with TNTs
140
120
100
80
60
40
20
0
GFP-vector
GFP-HttQ17
GFP-HttQ68
24h
48h

Figure 5

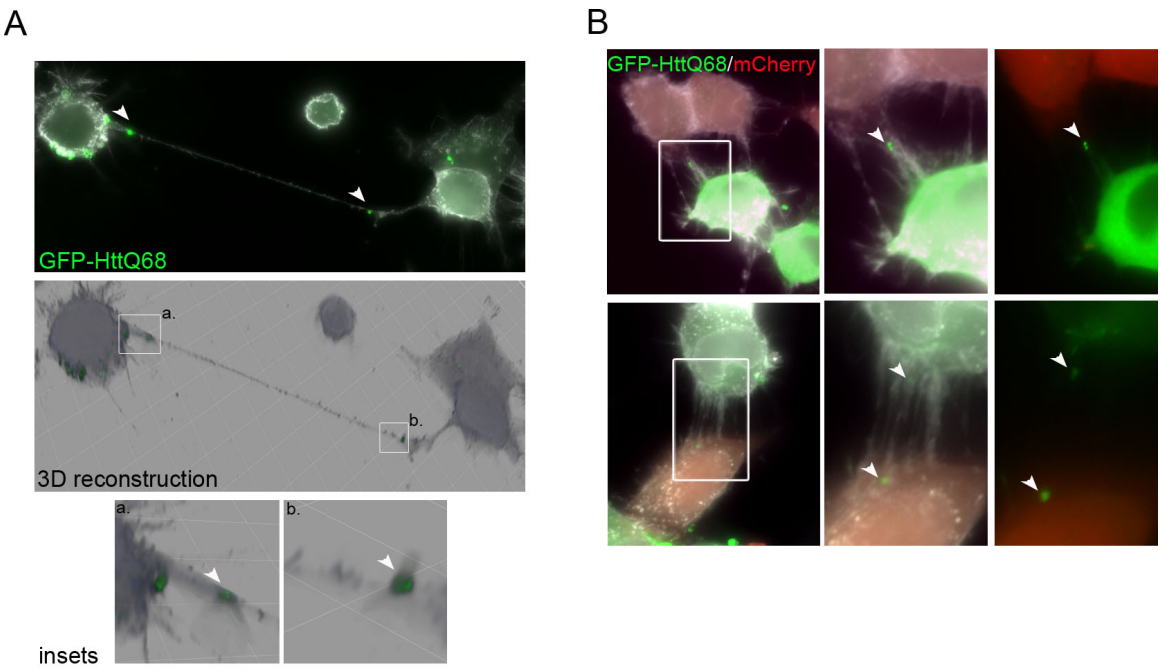
A
GFP-HttQ68
a.
b.
3D reconstruction
a.
b.
insets
B
GFP-HttQ68/mCherry

Figure 6

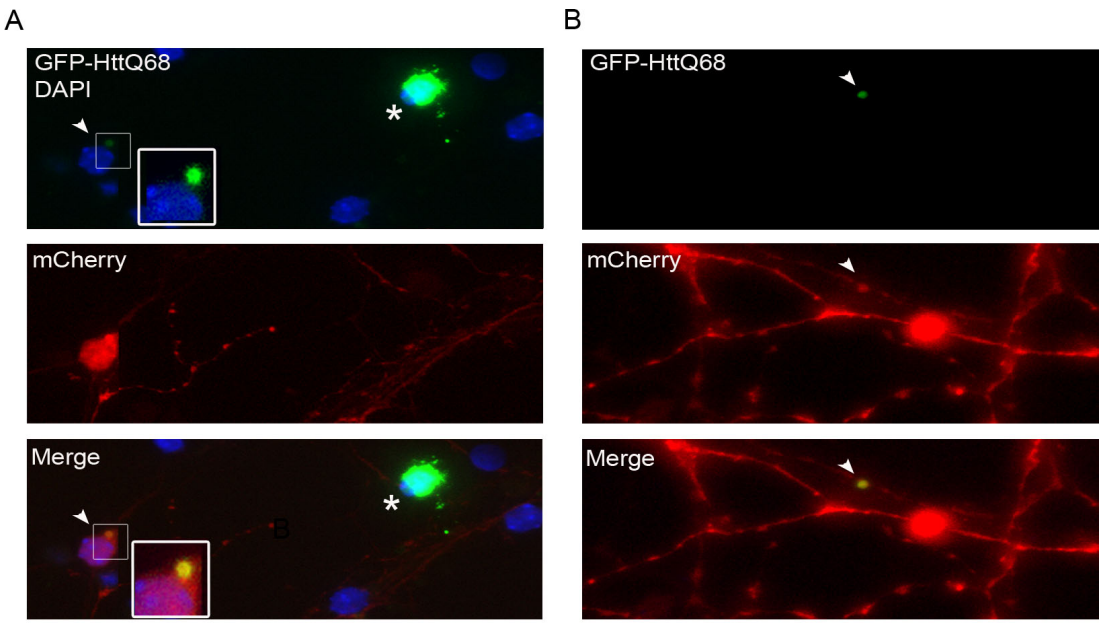

Figure 7

RESULT 3:

Role of the cellular prion protein in the pathogenic pathways of Huntington's disease

3.1 Objectives

a. To evaluate the effect of PrPC on polyQ-huntingtin aggregation and cell-to-cell transfer
b. To evaluate the effect of PrPC on the toxicity induced by mutant huntingtin

3.2 Specific background

As already described (see introduction, chapter 5) Huntington disease (HD) is caused by a CAG repeat expansion in the exon 1 of the huntingtin gene, which encodes an abnormally long polyglutamine (polyQ) stretch in the N-terminal part of the huntintin protein (Htt) (The Huntington's Disease Collaborative Research Group, 1993). HD is monogenic, fully penetrant and differently from other neurodegenerative diseases, there are no sporadic cases involving the wild-type form of the Htt gene. The disease is inherited in an autosomal dominant manner with age-dependent penetrance. The pathological hallmark of HD consists of intranuclear inclusion bodies (IBs), which are larger aggregates of the mutant protein. Aggregates also arise in cytoplasm, dendrites and axonal terminals (DiFiglia et al., 1993; Davies et al., 1997; Scherzinger et al., 1997).
In the first part of my PhD work, I demonstrated that intracellular expanded-polyQ huntingtin aggregates transfer between neuronal cells and primary neurons. I also demonstrated that cell-to-cell contact is required and that Tunneling nanotubes (TNTs) provide an efficient mechanism of transfer, as previously shown in the case of PrPSc (Gousset el al., 2009) and more recently of amyloid-β spreading (Wang et al., 2011). Furthermore, consistently with a previous report (Ren et al., 2009) our study strongly argues that, following transfer to naïve cells, polyQ aggregates stimulates further misfolding of endogenous wild-type

Htt (Costanzo et al., Results 2, manuscript). These findings are in agreement with the growing amount of evidence present in the literature suggesting that intercellular prion-like transmission mechanisms may be responsible for propagation of protein misfolding in non-prion neurodegenerative disorders including Alzheimer's, Parkinson's, Huntington's disease and taupathies (Brundin et al., 2010; Jucker and Walker, 2011; Soto et al., 2006). It is possible that huntingtin misfolding progresses through the brain, thus explaining the topologically predictable progression of HD along defined anatomical pathways. Yet, whether and how the propagation of protein aggregates is linked to neurotoxicity is not completely understood (Brundin et al., 2010; Ross and Poirier, 2004). Indeed, as described before (see chapter 6 of the introduction), in the case of HD as well as in other neurodegenerative disorders, the correlation between the brain regions that degenerate and protein aggregate deposition is weak, as neurons with inclusions do not correspond exactly to the neurons that degenerate (reviewed in Ross and Poirier, 2004). In addition, in the case of HD, post-mortem autopsy of transplanted HD patients revealed that fetal grafts of striatal tissue were susceptible to neurodegeneration displaying increased caspase-3 activation, vacuolization and decreased structural integrity in the absence of abnormal huntingtin aggregation (Cicchetti et al., 2009). Thus, it appears that IBs formation is dissociated from the vulnerability of different neuronal types and affected regions of HD brains (Arrasate and Finkbeiner, 2012; Ross and Poirier, 2004). Different studies supported the hypothesis that small aggregates or even aberrantly folded monomeric forms of mutant huntingtin are toxic to cells (Arrasate et al., 2004; Bennett et al., 2007; Ravikumar et al., 2004). Accordingly, IBs may be a beneficial coping response of the affected cells that reduces the levels of toxic misfolded proteins in the soluble fraction by sequestering them in insoluble aggregates. (Arrasate and Finkbeiner, 2005; Finkbeiner et al., 2006). This is consistent with the hypothesis that selective neuronal toxicity is the result of both cell autonomous and non-cell autonomous mechanisms involving soluble oligomeric species rather then large aggregates (see introduction, paragraph 6.3). Events occurring independently at single cell levels and/or due to the intercellular spreading of toxic oligomers may cause dysfunction and damage in other cell-types by altering cell-to-cell interactions, thus resulting in the distinct

patterns of neurodegeneration that characterize a given disorder (Brundin et al., 2010; Garden and La Spada, 2012)
Understanding how soluble oligomeric species (and possibly their cell-to-cell propagation) lead to neurodegeneration following neurotoxicity is still an open question in the field (Aguzzi and Falsig, 2012).
Interestingly, recent studies provided evidence that PrPC could mediate the toxicity of amyloid-β oligomers and other β-sheet-rich protein conformers by acting as a receptor for soluble ligands (Biasini et al., 2011; Bate and Williams, 2011; Resenberger et al. 2011). Therefore it is tempting to speculate the existence of a common mechanism for toxicity with PrPC functioning as a "danger sensor" (Barton and Caughey, 2011; Aguzzi and Falsig, 2012). It is possible that oligomeric forms of several different neurotoxic proteins could exert their effects by blocking, enhancing or subverting the normal function of PrPC as its pathogenic counterpart, PrPSc, does. Thus, binding of either oligomeric Aβ or PrPSc or other pathogenic aggregates to cell-surface PrPC may initiate toxic signals that lead to neuronal loss and/or synaptic dysfunction (Aguzzi and Falsig, 2012)
Contrasting data are present in the literature on the possible role of PrPC in HD pathogenesis. In 2001, it has been reported that "phenocopies" of HD (HD-like disease in the absence of CAG expansion in the Htt gene) can be due to a 192-nucleotide insertion within the coding region of the prion protein gene (*PRNP*), which encodes an expanded PrP with eight extra octapeptide repeats (Moore et al., 2001). PrP repeat expansions are well characterized and provoke early-onset, slowly progressive atypical prion diseases with an autosomal dominant pattern of inheritance and a remarkable range of clinical features, many of which overlap with those of HD (Moore et all, 2001). To examine the potential of PrP neuroprotective or neurotoxic properties in the context of HD, PrP was deleted from two transgenic models of HD. Deletion of PrP, in the R6/2 mouse model, modestly slowed motor deterioration as measured on an accelerating rotarod (a behavioral test) but otherwise did not alter other major features of the disease. Also transgenic overexpression of PrP did not exacerbate the Huntington motor phenotype (Steele et al., 2009). On the other hand, another report has suggested that PrPC has a protective effect on both HTT aggregation and toxicity in several neuronal cell lines (Lee et al., 2007). A connection between the cellular prion protein and the toxicity of expanded-polyQ aggregates has been made in

different systems including yeast (Saccharomyces cerevisiae) and Drosophila. For exemple, studies extending the HD model in yeast showed that cytoplasmic aggregation and toxicity of Htt fragments containing expanded-polyQ strech, is facilitated by the presence of the endogenous yeast prions in the amyloid conformation, [PIN+] and/or [PSI+] (Meriin et al., 2002; Gokhale et al., 2005; Duennwald et al., 2006) The prions [PIN+] and [PSI+] are self-perpetuating aggregates of the endogenous yeast proteins Rnq1 (unknown function) and Sup35 (translation termination, or release factor, also called eRF1), respectively (for review, see Wickner et al., 2007). Also, it has been reported that the expression of normal prion protein enhances the neurotoxicity of pathogenic polyQ proteins during eye development in Drosophila (Park et al., 2011).
Considering all these contrasting data, in the second part of my PhD I have analyzed the effect of PrPC on the expression and toxicity of mutant Htt using primary neurons from PrPC overexpressing and PrP knockout mice (tga20 (mouse *Prnp*a allele) and PrP0/0 (Zurich I) respectively)

3.3 Results

Effect of PrPC on polyQ-huntingtin aggregation

In order to evaluate the effect of the cellular prion protein, PrPC, on polyQ huntingtin aggregation, I expressed the first 480 amino acids of huntingtin with either 17Q (wild-type Htt) or 68Q repeats (mutant Htt) fused to green fluorescent protein (GFP) (GFP-Htt17Q and GFP-Htt68Q respectively) (see results Costanzo et al., Annex 2) in primary Cerebellar Granule Neurons (CGNs) obtained from PrP knockout and PrP overexpressing mice (see Materials and Methods). After fixation at different time points (from 72 h to 140 h post transfection), random mosaics of different fields were obtained by wide-field microscopy in order to analyze the presence of Htt aggregates in the overall neuronal network. Then, the fraction of cells with aggregates was quantified by blind manual counting discriminating between nuclear and cytoplasmic (in perinuclear position and/or within neurites) aggregates. Surprisingly and opposite to what has been reported before in neuronal cell lines (Lee et al., 2007), we found that at 140 h post transfection in PrP overexpressing neurons (from tga20 mice) the percentage of cells with cytoplasmic

aggregates was significantly higher compared to PrP knockout neurons, while there was no difference in the number of cells with nuclear aggregates (Figure 35A and B). As expected, independently of PrPC expression, overexpression of GFP-Htt17Q in primary CGN resulted in a diffuse cytoplasmic pattern of fluorescence with no significant aggregation detectable at different times post transfection (results not shown).

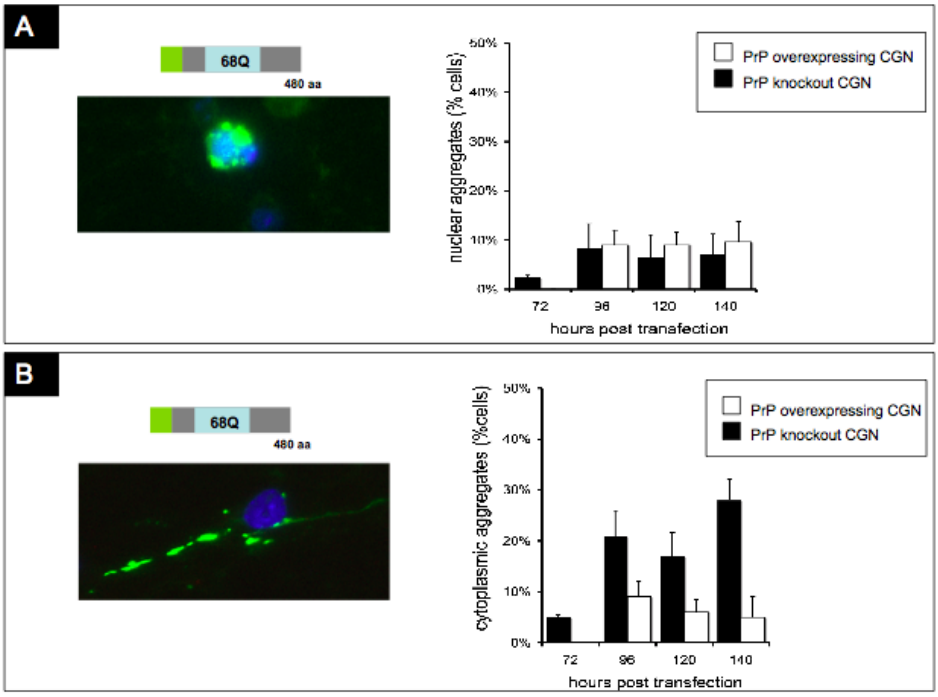

Figure 35 Effect of PrPC expression on GFP-HttQ68 aggregation in primary CGN. PrP overexpressing (from tga20 mice) or PrP knockout (from *Prnp*0/0 mice) CGN were transfected with GFP-HttQ68 cronstruct and cultured up to 140 h post transfection. Cell were fixed and labelled with DAPI. (A) For each genotype, the percentage of CGN with nuclear aggregates was quantified at different time points by blind manual counting. Representative neuron with nuclear aggregates is shown. (B) Quantification of the percentage of CGN with cytoplasmic aggregates (in perinuclear position and or within neurites) at the different time points for both genotypes . Representative neuron with cytoplasmic aggregates is shown. Black bars, PrP overexpressing neurons; white bars, PrP knockout neurons. (mean ± s.e.m, n = 3)

Because I have demonstrated that cell-to-cell transfer of polyQ aggregates occurs in primary CGN in the same condition (e.g. 140 h post transfection when direct cell-cell contact is allowed) (see Results 2, appended manuscript), one attractive possibility to explain the finding that high levels of PrPC cause an increase in the number of primary neurons with perinuclear and neuritic Htt aggregates, is that PrPC might influence the cell-to-cell transfer of GFP-Htt68Q aggregates. To this aim, PrP knockout or PrP overexpressing neurons were transfected either with GFP-Htt68Q

(donor cells) or a cytosolic mCherry construct (acceptor cells) immediately after isolation and then plated on the same coverslips to allow transfer. The two populations were incubated up to 140 h post transfection (Figure 36A). Then cells were fixed and stained with a N-terminal anti-huntingtin antibody (MAB2166) and DAPI. Random mosaics of different fields were obtained as described above. We quantified the the number of mCherry labeled neurons containing GFP-Htt68Q aggregates, referred to as transfer events, in PrP knock out and PrP overexpressing co-cultures by using the same automated software developed in collaboration with the imaging platform at the Institut Pasteur (see section Materials and methods and project 1, appended manuscript). Strikingly, quantitation of the transfer events showed that after 140 h co-culture (and transfection), 3,4% of mCherry neurons contained GFP-Htt68Q aggregates in the case of PrP overexpressing neurons co-culture compared to 0.5% of co-cultures of PrP knockout neurons (Figure 36B).

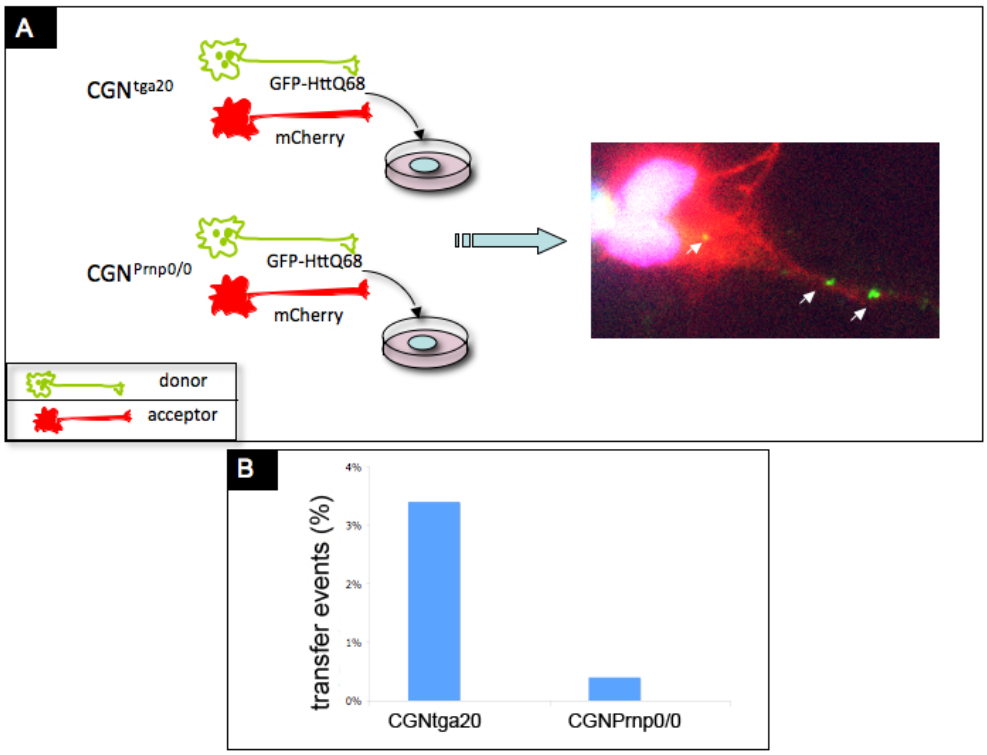

Figure 36 PrPC increases the transfer of GFP-HttQ68 aggregates between co-cultured CGN. (A) Schematic of the in vitro co-culture allowing to distinguish donor (transfected with GFP-HttQ68) and acceptor neurons (transfected with mCherry construct). The experiment were performed in both PrP overexpressing neurons (CGNtga20) and PrP knockout neurons (CGN *Prnp*0/0). Representative image of acceptor cells (mCherry labeled) containing GFP-HttQ68 aggregates (arrows). (B) Quantitation of the percentage of mCherry labeled neurons containing GFP-HttQ68 aggregates (transfer events) in PrP overexpressing and PrP Knockout co-cultures.

These data suggest that in the absence of the prion protein, cell-to-cell transmission of HTT aggregates is negligible. How PrPC increases the transfer of aggregates is not clear. Crucial experiments in which exchange of media was allowed between donor and acceptor neurons but not intercellular contact, excluded that the presence of PrPC increases the transfer of Htt by a secretion mechanism as already demonstrated in wild-type CGN (see Article 2). However, whether this is linked to a role of PrPC in increasing the number of aggregates in the periphery of the cell body and in the neurites is not clear. In addition, further experiments are required to address whether this is linked to an increase in the number of TNT-like structure similar to what we have shown in CAD cells (see Article 2 and below paragraph 3.4).

Effect of PrPC on the toxicity induced by mutant huntingtin

Recent studies have raised the unexpected possibility that PrPC, may also mediate the toxicity of amyloid-β oligomers that are associated with Alzheimer's disease (Lauren et al, 2009) and of other β-sheet-rich oligomers (Resenberger et al., 2011) independent of infectious PrPSc propagation, thus, suggesting a pathophysiological role of the prion protein beyond prion diseases (Biasini et al., 2011; Aguzzi and Falsig 2012).
Given that the presence of PrPC increased the number of primary neurons with perinuclear and neuritic polyQ aggregates as well as their cell-to-cell transfer, I next evaluated whether PrPC had also an effect on the toxicity triggered by mutant Htt. To this aim, I transfected GFP-Htt68Q and GFP-Htt17Q constructs in PrP knockout and PrP overexpressing CGN and analyzed caspase-3 activation, as a marker of apoptosis. At different time points, from 72 h up to 140 h after transfection, cells were fixed and stained using an antibody that specifically recognizes the cleaved and therefore active form of caspase-3. Random mosaics of different fields were acquired by wide-field microscopy as previously described. Surprisingly, in PrP over expressing CGN at 140 h post transfection, caspase-3 was active not only in the neurons with GFP-Htt68Q detectable aggregates but also in the neighbouring non-GFP-Htt68Q transfected neurons (Figure 37A). In contrast, in PrP knockout neurons, after 140 h transfection only the cells expressing GFP-Htt68Q with "visible" aggregates were positive for active caspase-3 and not the neighbouring non transfected neurons (Figure 37A). These data were confirmed by

western blot analysis showing a higher level of active caspase-3 in PrP overexpressing neurons compared to PrP knockout neurons (Figure 37B). As expected, in the cells transfected with the wild-type Htt fragment, GFP-Htt17Q, there was no activation of caspase-3 independently of PrPC expression (results not shown).

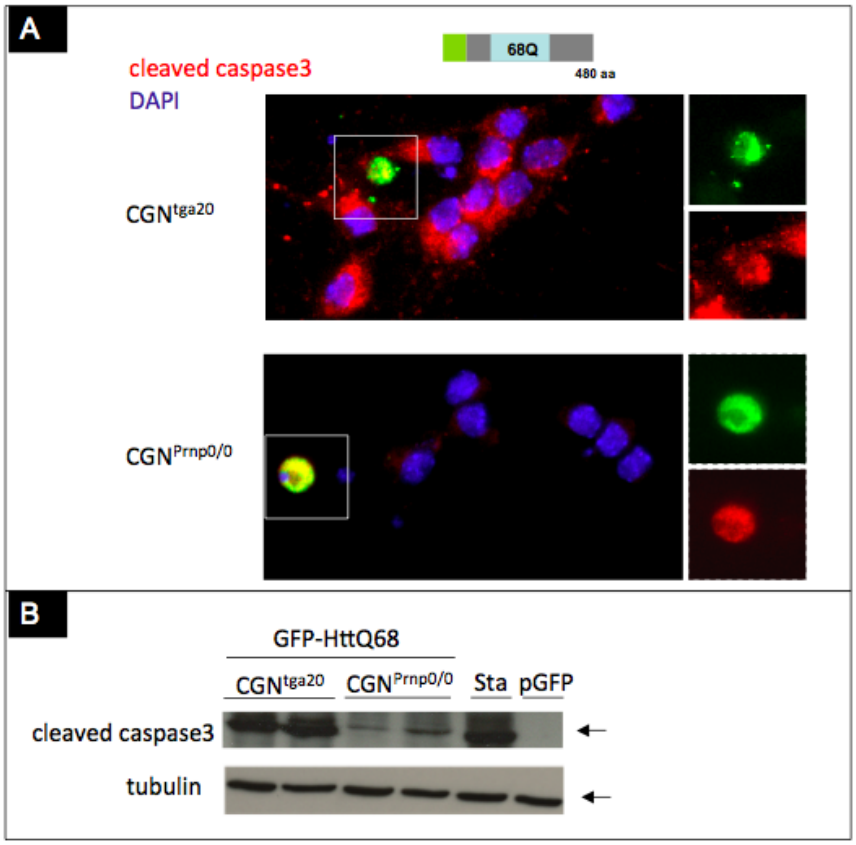

Figure 37 Effect of PrPC on caspase-3 activation in GFP-HttQ68 transfected CGN. (A) PrP overexpressing (tga20) and PrP knokout (*Prnp*0/0) CGN transfected with GFP-HttQ68 were fixed and stained with cleaved caspase-3 (red) and DAPI (blue). At 140 h post transfection in presence of PrPC cleaved caspase-3 staining was present not only in the neurons with GFP-HttQ68 detectable aggregates but also in the neighbouring non GFP-HttQ68 transfected neurons. In contrast, in PrP Knockout neurons only neurons with aggregates were positive for cleaved caspase.(B) Wester blot with anti cleaved caspase-3 on CGN whole-cell extract obtained after 140 h transfection with GFP-HttQ68 confirmed the immunoflurescence result represented in (A). CGN treated with staurosporin were used as positive control for caspase-3 activation. PrP overexpressing CGN upon 140 h transfection with a GFP-vector were used as negative control. Results are representative of three independent experiment.

These data indicate that activation of caspase-3 is mediated by the presence of Htt aggregates in the cell population. However, they also show that in the presence of PrPC (high levels) activation of caspase-3 occurs independently of the presence of detectable aggregates in the same cell, suggesting a role of PrPC in cell-cell communication (see discussion below).

In order to assess if the activation of caspase-3 in neighbouring neurons was mediated by soluble factors we plated PrP overexpressing CGN transfected either with GFP-Htt68Q (donor population) or a nucleo-cytoplasmic pGFP construct (acceptor population) on separated coverslips in the same dish, thus, impairing intercellular contact but allowing exchange of soluble factors between the two different populations through the medium. After 140 h incubation, we could not detect caspase-3 activation in pGFP neurons plated on different coverslip. In contrast, a widespread signal of cleaved caspase-3 was detected among the acceptor population (GFP-Htt68Q transfected and non transfected neurons plated on the same coverslips) (Figure 38A). These results indicated that intercellular contact is needed in order to allow widespread activation of caspase-3 between neurons. This suggests that in our system, PrPC is not acting as receptor for soluble factors (or aggregates present in the extracellular medium), as it has been proposed in the case of Alzheimer's disease.

Next we investigated whether PrPC was required in both GFP-Htt68Q transfected neurons and in the neighboring neurons (acceptor neurons) which did not express mutant Htt, or was dispensable in one of the two cell populations.

To this aim, we co-cultured PrP overexpressing neurons transfected with GFP-Htt68Q (donor population) with PrP knockout neurons transfected with pGFP vector (acceptor population) on the same coverslip, thus, allowing intercellular contact to occur. The nuclear and cytosolic diffuse staining of the pGFP construct allowed us to distinguish between the two populations. In a complementary experiment, we transfected GFP-Htt68Q in PrP knockout neurons (donor population) and co-cultured them with PrP overexpressing neurons transfected with pGFP construct (acceptor population) on the same coverslip. In this condition, after 140 h co-culture, we evaluated the presence of active caspase-3 as described above (Figure 38B and C).

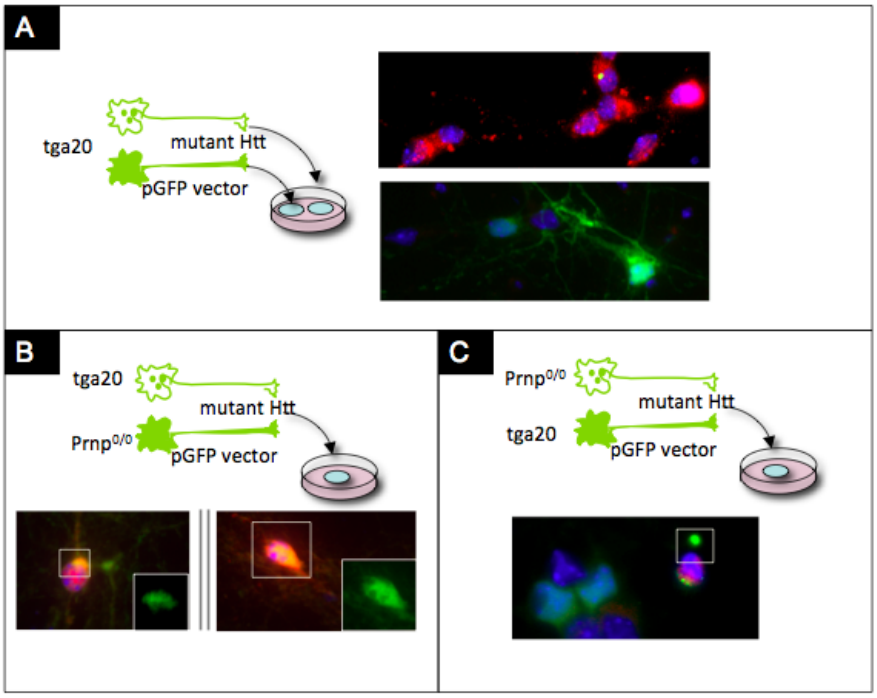

Figure 38 Cell-cell contact and expression of PrP in GFP-HttQ68 transfected neurons are required for activation of caspase-3 in cell non expressing mutant Htt. (A) PrP overexpressing CGN (tga20) were transfected either with GFP-HttQ68 or with GFP vector and plated on different coverslips in the same dish and therefore shared the same culture medium (in the absence of cell-cell contact). In these condition we could not detect cleaved caspase-3 (red) in GFP neurons (bottom image). (B) PrP overexpressing CGN were transfected with GFP-HttQ68 and PrP knockout neurons (*Prnp*0/0) with pGFP. The two cell population were plated on the same coverrslips, thus allowing intercellular contact. In this condition, we could detect cleaved caspase-3 staining in GFP CGN. (C) PrP overexpressing CGN were transfected with GFP vector and PrP knockout neurons (*Prnp*0/0) with GFP-HttQ68. The two cell population were plated on the same coverrslips, thus allowing intercellular contact. In this condition, we could not detect cleaved caspase-3 staining in GFP CGN.

Interestingly, we could detect active caspase-3 in the acceptor neurons regardless of the genotype (overexpressing or knockout for PrP) when GFP-Htt68Q was expressed in neurons containing PrPC. Conversely, we could not detect staining for cleaved caspase-3 in acceptor neurons when polyQ Htt was expressed in PrP knockout neurons. Overall, these results indicate that expression of PrPC is required in the donor neurons (containing Htt aggregates) while it is dispensable in the receiving neurons in order to determine a widespread activation of caspase-3 in the cell population (independently of the presence of the aggregates in the single cells).

3.4 Disussion

Huntington Disease (HD) is an autosomal dominant neurodegenerative disorder characterized by progressive deterioration of cognitive, motor, and psychiatric function. Expression of mutant huntingtin is ubiquitous throughout the brain yet, the striatum is the primarily affected brain region in HD. The pathological hallmark of the disease is the deposition of aggregates of mutant Htt (inclusion bodies) in selected brain regions. Different studies supported the hypothesis that small aggregates or even aberrantly folded monomeric forms of mutant huntingtin are toxic to cells (Arrasate et al., 2004; Bennett et al., 2007; Ravikumar et al., 2004). Accordingly, inclusion bodies may be a beneficial coping response of the affected cells that reduces the levels of toxic misfolded proteins in the soluble fraction by sequestering them in insoluble aggregates (Arrasate and Finkbeiner, 2005; Finkbeiner et al., 2006). Furthermore, recent evidence suggests that selective neuronal toxicity in different neurodegenerative diseases is the result of both cell autonomous and non-cell autonomous mechanisms involving soluble oligomeric species rather then large aggregates (see introduction, paragraph 6.3). Events occurring independently at single cell levels and/or due to the intercellular spreading of toxic oligomers may cause dysfunction and damage in other cell-types by altering cell-to-cell interactions, thus resulting in the distinct patterns of neurodegeneration that characterize a given disorder (Brundin et al., 2010; Garden and La Spada, 2012). Understanding how soluble oligomeric species (and possibly their cell-to-cell propagation) lead to neurodegeneration following neurotoxicity is still an open question in the field. Recently a role for the cellular prion protein in the toxicity of amyloid-β oligomers and other β-conformers has been proposed (Biasini et al., 2011; Bate and Williams, 2011; Resenberger et al., 2011).

In this context, in the second part of my PhD, I focused on evaluating whether PrPC is a player in the pathogenic pathways of HD. Expecially, I examined the effect of PrPC on both the aggregation and toxicity of a mutant fragment of Htt in primary neurons.

To this aim, I expressed N-terminal fragments of Htt containing an expanded or a wild-type polyQ tract as GFP-fusion proteins (GFP-Htt68Q and GFP-Htt17Q respectively) in primary cerebellar granule neurons (CGN) obtained from PrP knockout mice and

from transgenic mice overexpressing PrPC (PrP0/0 (Zurich I) and tga20 (mouse *Prnp*a allele) respectively). The polyQ-expanded N-terminal region of Htt is sufficient for reproducing the characteristics of polyQ aggregation and toxicity as it was shown to aggregate and produce disease-like neurodegeneration in primate, rodents and invertebrate models of HD, it is clear that this region (reviewed in Rubinsztein 2002). Furthermore, although the cortical-striatal network is the most affected in HD, a recent study in a mouse model of HD have revealed that other components of the motor circuit, such as the cerebellum, are possibly involved in motor symptom development in HD (Dougherty et al., 2012) as the cerebellum forms disynaptic connection with the basal ganglia through the thalamus (Bostan et al., 2010; Bostan and Strick, 2010). The output neurons of the cerebellar cortex are the GABAergic Purkinje cells (PCs). Intranuclear inclusions have been found in PCs in postmortem tissue from HD patients and in knock-in animal models of HD (Adachi et al., 2001) and there is evidence suggesting a reduced density of PCs in HD patients (Rosas et al., 2003; Fennema-Notestine et al., 2004; Jeste et al., 1984). The cerebellum is commonly affected in juvenile HD as exhibited by a loss in overall cerebellar volume (Fennema-Notestine et al., 2004; Nicolas et al., 2011; Ruocco et al., 2006; Sakazume et al., 2009). Recently, in the R6/2 mouse model of HD that carries transgenic expression of exon 1 of the human huntingtin gene with an expanded CAG repeat (Mangiarini et al., 1996) it was reported a significant reduction in PC number by end-stage but no change in presymptomatic animals (4 weeks of age) (Dougherty et al., 2012). Analysis of cellular function prior to cell loss and symptom onset revealed a striking deficit in spontaneous PC firing in asymptomatic R6/2 mice in the absence of “visible” huntingtin inclusions (that were observed only at end-stage) indicating that soluble huntingtin and/or abnormalities in other cell types (e.g. dysfunction in the molecular layer of interneurons where Htt aggregates accumulate at early stage) may contribute to PC dysfunction. Intriguingly, although aggregate deposition was detected at early stage, the interneurons were preserved through the disease time course supporting the hypothesis that the intranuclear inclusions may be protective and not causative in cell death (Dougherty et al., 2012; Arrasate et al., 2004; Kuemmerle et al., 1999). Taken together these studies suggest the possibility that the cerebellum and PCs play an important role in HD. In agreement with this hypothesis, in the first part of my

thesis (see section Results 2, article 2), I have shown that cerebellar neurons transfected with GFP-HttQ68 make aggregates suggesting that they are a good in vitro model for the disease.
In order to evaluate the role of PrPC in HD, I used PrP knockout [PrP0/0 (Zurich I) (Bueler et al., 1992)] and PrP overexpressing transgenic mice [tga20 (mouse *Prnp*a allele) (Fischer et al., 1996)] to obtain primary cultures of cerebellar granule neurons (CGN) as the marked difference in PrP expression (on an otherwise identical genetic background) could provide better evidence on a possible role of the cellular prion protein in the disease. Indeed, tga20 mice express PrPC 5 to 20 folds more compared to wild-type mice (Fischer et al., 1996).
I evaluated first, the aggregation of GFP-Htt68Q in time course experiments and then compared the fraction of cells with aggregates in the two genotypes at different time post transfection. Strikingly, in primary CGN high level of PrPC resulted in a consistent increase in the number of cells with perinuclear and/or neuritic aggregates at 140 h post transfection (20% more compared to PrP knockout) while there was no difference in the percentage of cells containing nuclear aggregates.
Although nuclear localization appears necessary for pathogenesis, extranuclear localization of Htt may also contribute to the disease through disruption of vesicle trafficking, BDNF transport, microtubule structure, NMDA receptor (NMDAR) and synaptic activity and organelle morphology (for review see Zuccato et al., 2011).

These findings could be explained by two possibilities: either PrPC is increasing the number of cytosolic aggregates within single cells (e.g. by enhancing their formation) and/or is it facilitating the cell-to-cell transfer of polyQ aggregates. Since PrPC is anchored to the external leaflet of the plasmamembrane by mean of a GPI-anchor (Prusiner 1998) while polyQ htt accumulates in intracellular location (either cytosolic or nuclear) (Di Figlia et al., 1995; Sharp et al., 1995; Velier et al., 1998) the two proteins, under most circumstances, would not come in to contact (Barton and Caughey, 2011). In addition, I did not observe an overall variation in the intracellular distribution of PrPC in CGN with polyQ aggregates, when compared to control neuron (result not shown). However, it should be considered that huntingtin can interact with acidic phospholipidis on the cytoplasmic leaflet of the plasma membrane and polyQ expansion increases its insertion

in to lipid bilayers (Kegel et al., 2005; Kegel et al., 2009). Moreover, the first 17 N-terminal amino acids before the polyQ stretch do form an amphipatic a-helical membrane-binding domain that has been shown to regulate Htt subcellular localization, to enhance aggregate formation and also to promote disregulation of calcium homeostasis (Atwal et al., 2007; Rockabrand et al., 2007). Therefore, it is possible to speculate that Htt, (also considering its role as scaffold protein (reviewed in Zuccato et al., 2011)), might associate with the polypeptide chain of cell-surface PrPC via a putative transmembrane linker proteins as previously hypothesized for other cytoplasmic putative PrP interactors (reviewed in Biasini et al., 2011). However, how this interaction can possibly influence the process of aggregates formation is not clear. Degradation pathways for Htt include endosomal-lysosomal and autophagic pathways and may require targeting to membranes to initiate clearence (kegel et al., 2000; Qin et al., 2003).

To explore the possibility that PrPC overexpression might influence the process of aggregate formation it may be possible to monitor the intracellular change of soluble mutant huntingtin to the aggregated state by live imaging in several neurons in presence or absence of PrPC. A similar approach was used by Finkbeiner and colleagues to monitor over time the fate of different individual primary neurons expressing a GFP-tagged exon 1 fragment of huntingtin (Arrasate and Finkbeiner, 2005; Finkbeineret al., 2006).

By using a co-culture system that allowed us to evaluate cell-to cell transfer of polyQ aggregates from donor (GFP-Htt68Q transfected) to acceptor primary CGN (expressing a cytosolic mCherry) (see annex 2 appended manuscript), I showed that in presence of PrPC (after 140 h co-culture) the percentage of acceptor cells with aggregates was three times higher compared to PrP knockout (3,5% versus 0,5%), suggesting a correlation between aggregates transfer and PrPC expression. Since we excluded transfer mechanism through the culture media through uptake of GFP-Htt68Q aggregates following secretion, one attractive possibility is that PrPC may promote cell-to-cell interaction thereby enhancing aggregate transfer. Interestingly, PrPC itself has been recently involved in cytoskeleton dynamic and remodeling and in cell-to-cell adhesion (Malaga-Trillo 2009; Chiesa and Harris 2009; Schrock et al 2009). For example, it has been shown that in zebrafish, PrP-1, an homologue of the mammalian PrPC, might directly mediate homophilic interactions

or indirectly regulate the trafficking of E-cadherins and β-catenin to the plasma membrane, thus promoting adherents junctions (Malaga- Trillo, 2009). This is in agreement with previous observations made for mammalian PrPC in which a role for this protein in neurite outgrowth and cell-cell interaction, respectively in hippocampal neurons and neuroblastoma cells has been reported (Monge et al 2002; Santuccione et al 2005). Interestingly, recent data from our laboratory have disclosed a possible role of PrP in enhancing tunneling nanotube (TNT) formation (Marzo and Zurzolo, unpublished results). Indeed, overexpression of full-length GFP-PrP in neuronal CAD cells causes an increase in the number of TNT structures suggesting that PrP might have a structural role driving the anchoring on the nascent nanotube (e.g an actin-driven protusion) to the membrane of the target cell. In the first part of my thesis, I have demonstrated that intracellular polyQ aggregates transfer between neuronal cells by hijacking TNTs, similar to what has already been shown for infectious prions (Gousset et al., 2009) and intracellular amyloid-β particles (Wang et al., 2011). Different studies have shown the occurrence of TNT-like structure between astrocytes and primary neurons (for review see abounit and Zurzolo, 2012; Marzo et al., 2012) and in the lab I have shown that TNT-like structures can be found in co-culture of astrocytes and primary CGN (Costanzo and Zurzolo, unpublished data). Thus, one possibility would be that high level of PrPC might increase the number of TNT structures in primary CGN, as in neuronal CAD cells (Marzo and Zurzolo unpublished data) leading to an increase transfer of polyQ aggregates. Further experiments will be required to evaluate this hypothesis. A limiting step in primary neurons is the low transfection efficiency, therefore we are currently producing lentiviral particles of both GFP-Htt68Q and GFP-Htt17Q constructs in order to establish more favorable condition for aggregate detection in TNT-like structures in primary CGN. On the other hand, since we hypothesize that PrP can be involved in the transfer of polyQ aggregates by participating to the anchoring of the nascent TNT and/or by the establishment of cell-to-cell contact with a neighboring cell (thus interacting with different partners) one important experiment will be to evaluate the occurrence of aggregate transfer in presence of different anti-PrP antibodies (directed against different epitops).

Overall, my data indicate that in presence of high levels of PrPC there is an increased number of primary neurons with perinuclear

and neuritic aggregates as well as an increased cell-to-cell transfer of polyQ aggregates. Further investigation will be required to link PrP expression to TNT formation and polyQ aggregate transfer. If confirmed in primary neurons, it is likely that prion disease (Gousset et al., 2009) Alzheimer's (Wang et al., 2011) and Huntington disease exploit a common spreading mechanism. The identification of specific TNTs marker will be required to evalute whether this common mechanism may contribute to disease progression in vivo.

Stimulated by the recent findings that PrPC is able to mediate the toxicity triggered by different β-sheet-rich oligomeric species including amyloid-β oligomers (Biasini et al., 2011; Bate and Williams, 2011; Resenberger et al. 2011), I have analyzed the role of PrPC in mediating mutant Htt toxicity. Strikingly, I found that in presence of high levels of PrPC, caspase-3 was active not only in CGN with GFP-Htt68Q detectable aggregates but also in the neighbouring non-GFP-Htt68Q transfected neurons. Conversely, in the absence of PrP, I could detect active caspase-3 only in CGN transfected with GFP-Htt68Q in presence of aggregates while the neighbouring non-transfected neurons were unaffected. Activation of caspase-3 in neighbouring neurons occurred only in co-culture where cell-cell contact was allowed and could not be detected in condition that allowed only sharing of the media (but not cell-to-cell contact) between donor (GFP-Htt68Q transfected) and acceptor (pGFP transfected) neurons. This suggested that it was not mediated by a soluble (i.e secreted) factor and that in our system, PrPC is not acting as receptor for soluble factors (or aggregates present in the extracellular medium), differently to what been proposed in the case of Alzheimer's disease.

Overall, these data show that activation of caspase-3 is mediated by the presence of Htt aggregates in the cell population. However, in the presence of PrPC activaction of caspase-3 occurs independently of the presence of detectable aggregates in the single cells. One possibility is that this is mediated by soluble toxic oligomers that spread between cells when cell-cell contact is allowed.

Explaining a role of PrPC in mediating Htt toxicity becomes more complicated then for other disease-associated aggregates. Indeed, for amyloid-β oligomers and others β-conformers a direct ligand-receptor interaction via the N-terminal domain of cell-surface PrPC has been proposed, leading to the transduction of pro-apoptotic signals as measured by caspase-3 activation

(Resemberg et al., 2011; Lauren et al., 2009; Chen et al., 2010). As mentioned above, in the case of polyQ Htt a direct interaction with PrPC is not likely to occur and caspase-3 activation in neghbouring non-transfected neurons, although requiring PrPC, is not mediated by soluble factors. Regarding the toxicity of Htt both soluble and aggregated Htt have the potential to be toxic (Weiss et al., 2012; Lajoie and Snapp, 2010). Given that PrPC increases cell-to cell transfer of polyQ aggregates, it is tempting to speculate that PrPC might also increase the transfer of toxic soluble polyQ oligomers, thus leading to caspase-3 activation in the receiving neurons in the absence of detectable aggregates. Also in this context, one important experiment will be to evaluate the occurrence of widespread caspase-3 activation in presence of different anti-PrP antibodies, directed against different epitops of the protein. Another possibility is that PrPC is mediating the transfer of active caspase 3 in neigbouring cells. In agreement with this hypothesis is the spreading of death signals by tunneling nanotubes recently discovered in Jurkat and primary T cell (Arkwright et al., 2010). The existence of a similar mechanism is, therefore, possible in our system. Indeed, in primary astrocytes I could observe active caspase-3 in TNTs connecting neighbouring cells (result not shown). I recently established the condition to visualize TNTs in CGN cultures by plating them on a feeder layer of astrocytes (neurons plated directly on poly-D-lysine coated coverslips develop an intricate neurites network that does not allow visualizing TNTs on the basis of their typical morphology since specific TNT markers are still missing). Therefore, one possible experiment will be to evaluate the presence of active caspase-3 in TNT-like structure bridging primary neurons upon GFP-Htt68Q transfection. Interestingly, I demonstrated that for the activation of caspase-3 in the neighbouring cells, independently of the presence of “visible” aggregates, PrPC expression is required in the neurons expressing polyQ Htt (donor population) while it is dispensable in the receiving neurons (transfected with a GFP-vector).

Whether this is linked to an interaction between PrPC and polyQ Htt in the donor neurons needs to be further explored.

Furthermore, these findings lead to some speculations and open up a series of new questions that could be the basis for further investigations. Recently, the effect of stress on TNTs formation have been analyzed in different cell types (Wang et al., 2011). In particular, stress induced by hydrogen peroxide (H2O2) leads to an increase in TNT formation in both astrocytes and neurons

(Wang et al., 2011) and in neuronal CAD cells (Gousset and Zurzolo, unpublished data). Moreover, It has been shown that in vitro and in vivo aggregation of a N-terminal fragment of huntingtin directly causes free radical production (Hands et al., 2011). Interestingly, for both astrocytes and neurons it is always the cells undergoing stress that developes TNTs and transfers cellular materials in a unidirectional fashion to the non-activated cells (reviewed in Marzo et al., 2012). Therefore, it is tempting to speculate that the cells undergoing oxidative injuries upon expression of a polyQ fragment of huntingtin, might spread toxic signal in a PrPC-dependent manner, possibly through an increase in cell-to-cell connections, with a possible role for TNT structures.
Overall, these results indicate that PrP has a role in both cell-to-cell transfer of polyQ Htt (detectable aggregates and possibly toxic soluble species) as well as in the toxicity mediated by caspase-3 activation. Further investigations are required to confirm that in our system caspase-3 activation is driving acute neuronal cell death (e.g TUNEL staining, detection of cleaved PARP by wester blot on cell lysates), since it has been reported that activation of caspase3 triggers early synaptic disfunction independently from apoptosis in mouse model of Alzheimer's disease (D'Amelio et al., 2010; Spires-Jones et al., 2008)
These results are also consistent with the finding that caspase-3 activation occurs in the absence of abnormal huntingtin aggregation in fetal grafts of striatal tissues in transplanted HD patients (Cicchetti et al., 2009) and that functional abnormalities in cerebellar neurons (purkinjie cells) occur at early stage in a mouse model of HD in the absence of visible aggregates deposition. They are also in agreement with several line of evidence that point to a role of the cellular prion protein in the toxicity of amyloid beta and other beta-sheet-rich conformers, although possibly with different mechanism.
Finally, these findings are in agreement with the growing amount of evidences in literature indicating that soluble huntingtin and/or abnormalities in other cell types may account for toxicity in vulnerable neurons, thus indicating that the pathogenesis of this disease is a result of both cell autonomous and non-cell autonomous mechanisms and open the path to new investigation and novel therapeutical approaches.

CONCLUSION AND PERSPECTIVES

Conclusion and perspectives

- In the first part of my PhD work, I investigated the role of DCs (dendritic cells) in the spreading of prion infection to neuronal cells in order to better clarify whether and how they play a role in prion spreading in vivo from the periphery to the nervous system. This study was performed by using in vitro cultures of primary BMDCs (bone-marrow-derived dendritic cells) and cerebellar neurons and by the combined use of microscopy and biochemical approaches.

In these experiment, in collaboration with Dr Langevin, I found that

a) direct cell-cell transfer of PrPSc occurs between BMDCs and primary CGN in a PrPC-independent manner and results in the transfer of infectivity to neurons;
b) the secretory pathway and transfer from the cell surface of prion-loaded BMDCs are not involved in PrPSc intercellular spreading in our culturing condition;
c) TNT-like structures occur between co-cultured BMDCs and CGNs. Therfore having excluded transfer from the cell surface and by secretion, we concluded that TNTs could be an efficient mechanism to mediate the transfer from the cytosol of PrPSc from BMDCs to CGNs.

Although BMDCs have been shown to secrete prion-enriched exosomes, using a number of restrictive experimental conditions such as short co-culture times, low BMDC/CGN ratios, physical separation and pre-fixation of cells, we were not able to show the involvement of the secretory pathway in the transfer of prions from BMDCs to CGNs. On the other end, the transfer mediated by direct cell-cell contact was very efficient and all of our data point towards a role of TNT-like structures in the intercellular transfer of PrPSc from BMDCs to CGNs, similar to what was recently shown with dorsal root ganglion neurons.
Overall, these results highlight the importance of DCs as potentially important candidate in mediating prion transfer to nerve cells, possibly through TNT-like structures that might exist in vivo. Our system of co-cultures will allow further characterization of prion spreading from the periphery to the nervous system of different scrapie strains, which could lead to a better understanding of the species barrier phenomenon. In this

view, additional experiments are needed to assess the role of DCs during prion spreading in vivo. For exemple it would be possible to load dendritic cells with fluorescent Alexa-labeled PrPSc (Gousset et al., 2009) and then evaluate their ability to contact different cells and transfer prion in exposed limph nodes by intravital imaging.

In the second part of my thesis I focused on Huntington's disease and carried on two strictly related projects.

- In the first project I explored the occurrence and the mechanisms of polyQ Htt transfer in neuronal cells and primary neurons.

By using fluorescence microscopy and flow cytomery techniques combined with restrictive experimental co-culture conditions, I could show that:

a) spontaneous transfer of intracellular Htt aggregates occurs between dividing neuronal cells and between post-mitotic primary neurons.
b) aggregate transfer is an active mechanism that does not rely on their passive release upon cell death and requires cell-to-cell contact
c) in our culturing conditions secretion is not involved in the transfer of Htt aggregates
d) Htt aggregates hijack TNTs formed between neuronal cells that provide an efficient transfer mechanism.
e) overexpression of mutant Htt increases by itself TNT-formation.
f) aggregation of endogenous wild-type Htt occurs in acceptor neurons upon direct co-cultures with mutant Htt expressing neurons indicating occurrence of seeding.

In conclusion, my results support the possibility that also in the case of Huntington's disease prion-like transmission of protein misfolding contributes both to the early stage pathogenesis and to the progression of the disease in the brain. These data reinforce the hypothesis that Huntington's disease and other neurodegenerative proteinopathies are non cell autonomous diseases and that the spreading of intracellular aggregates as well as toxic species (results 3 and below) contributes to the pathogenesis. Furthermore my data extend the role of TNTs from

prion diseases to other pathological conditions associated with the deposition of different aggregate proteins (e.g., β-Amyloid, mutant Htt). They support the role of TNTs as general means for the spreading of signals (e.g., death signals) and pathogens (e.g., HIV virus, bacteria) between different cells. Therefore a more accurate characterization of this type of long distance form of intercellular communication together with a better understanding of their physiological role is an intriguing and challenging still open question in biology.
Finally, these data also highlight the fact that a multidisciplinary synergistic research merging different fields is required to allow the development of new approaches and new way of looking at diseases and possibly allowing the development of new strategies to fight them.

In the last part of my PhD, I focused my study on evaluating whether the cellular prion protein, PrPC, could have a role in the pathogenesis on Huntington's disease

- By using fluorescence microscopy techniques combined with restrictive experimental co-culture conditions, I found that:

    a) PrP overexpressing neurons (from tga20 mice) transfected with a mutant Htt fragment (GFP-HttQ68) have a higher percentage of cells with cytoplasmic aggregates compared to PrP knockout neurons while there is no difference in the number of cells with nuclear aggregates.
    b) high levels of PrPC results in an increase transfer of polyQ aggregates between primary CGN
    c) transfer requires cell-cell contact and is not mediated by secretion of polyQ aggregates in the medium (as shown above)
    d) in presence of PrPC there is a widespread activaction of caspase-3 in the cell population independently of the presence of detectable aggregates in the single neurons while in PrP knockout neurons caspase-3 is active only in the neurons containing aggregates.

Overall, my results indicate that PrPC has a role in both cell-to-cell transfer of polyQ Htt (detectable aggregates and possibly toxic soluble species) as well as in the toxicity mediated by caspase-3 activation. They are also in agreement with several line

of evidence that points towards a role of the cellular prion protein in the toxicity of amyloid-β and other beta-sheet-rich conformers. However, considering the different nature of the proteinaceous aggregates, it is possible that the mechanism of PrPC involvement could be different in HD and in other neurodegenerative disorders. Further studies will be required to understand the mechanisms by which PrPC might mediate these different processes which lead to neuronal dysfunction and neurodegeneration as this could have an enormous importance in understanding and fighting protein conformational diseases.

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