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THE INTERACTION BETWEEN MUTANT PRION PROTEIN AND GLUTAMATE RECEPTORS: A NOVEL MECHANISM FOR NEURONAL DYSFUNCTION IN GENETIC PRION DISEASES

BIO/14

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SOMMARIO

Le malattie genetiche da prioni sono malattie neurodegenerative molto rare ed inesorabilmente fatali causate da mutazioni nel gene *PRNP,* che codifica per la proteina prionica. Queste mutazioni favoriscono la conversione conformazionale della proteina nativa in un'isoforma patologica che si accumula nel sistema nervoso centrale degli individui affetti, causando neurodegenerazione tramite meccanismi ancora sconosciuti.

Esistono numerose indicazioni che la morte neuronale in queste malattie sia preceduta, e probabilmente provocata, da disfunzioni sinaptiche, ma non è ancora stato trovato un nesso convincente tra questi due fenomeni.

Il nostro gruppo ha recentemente dimostrato che una forma mutata della proteina prionica viene trattenuta all'interno della cellula nel reticolo endoplasmico, dove interagisce con la subunità α2-δ1 dei canali del calcio voltaggio-dipendenti. Questa interazione, associata alla ritenzione intracellulare della proteina mutata, ostacola il corretto inserimento in membrana dei canali del calcio, inficiando il processo di neurotrasmissione. Questo fenomeno, però, ancora non identifica le cause della morte neuronale.

D'altra parte è noto che la proteina prionica interagisce anche con altre proteine importanti per la funzionalità sinaptica, come ad esempio i recettori del glutammato. In questo studio abbiamo quindi voluto verificare se la ritenzione la proteina mutata potesse ostacolare anche il trasporto di questi recettori, con effetti deleteri sulla neurotrasmissione postsinaptica e sulla sopravvivenza neuronale.

I nostri risultati, in effetti, dimostrano che la proteina prionica mutata impedisce l'inserimento in membrana dei recettori AMPA e NMDA, causando alterazioni strutturali e funzionali del compartimento postsinaptico e della trasmissione eccitatoria di base. Inoltre, la ritenzione intracellulare della subunità GluA2 dei recettori AMPA fa in modo che vengano esposti in membrana recettori privi di tale subunità e quindi permeabili al calcio, provocando un aumento della permeabilità neuronale a tale ione e, di conseguenza, un aumento della suscettibilità al danno eccitotossico. E' interessante notare come proteine con mutazioni diverse interagiscono in

maniera differente con i recettori glutammatergici, ed abbiano quindi effetti specifici sulla localizzazione e sulla funzione di queste molecole.

I risultati di questo studio descrivono un nuovo meccanismo patologico alla base delle malattie genetiche da prioni che potrebbe portare all'individuazione di interessanti strategie terapeutiche per queste condizioni ad oggi incurabili.

ABSTRACT

Genetic prion diseases are rare, invariably fatal neurodegenerative disorders linked to mutations in the *PRNP* gene encoding the prion protein (PrP). *PRNP* mutations favor the conformational conversion of PrP into a pathogenic, misfolded isoform that accumulates in the central nervous system of affected individuals and kills neurons through an unknown mechanism.

Evidence is emerging that neuronal loss in inherited prion diseases is preceded and possibly caused by synaptic dysfunctions. However, the ultimate link between synaptic dysfunction and neurodegeneration is yet to be found.

We previously demonstrated that mutant PrP is retained in the endoplasmic reticulum where it interacts with the α2-δ1 subunit of voltage-gated calcium channels. This impairs the correct delivery of the channel complex to the cell surface, impacting synaptic transmission. Nevertheless, this phenomenon alone does not account for neurodegeneration. It has been shown that PrP^C engages functional interactions with other proteins that are important for synaptic function, such as glutamate receptors. Here, we aimed to explore whether intracellular retention of mutant PrP affected also the trafficking of glutamate receptors, thereby producing adverse effects on neuronal function and survival.

We found that mutant PrP impairs the membrane delivery of specific AMPA and NMDA receptor subunits, resulting in postsynaptic structural alterations and impaired basal glutamatergic transmission and synaptic plasticity. Moreover, retention of the GluA2 subunit of AMPA receptor results in exposure of GluA2-lacking, calcium-permeable AMPA receptors, leading to increased calcium permeability and enhanced sensitivity to excitotoxic cell death. Interestingly, distinct PrP mutations interact differently with glutamate receptors, altering their localization and function in different ways.

Our findings identify a new pathological mechanism for genetic prion diseases and may lead to novel therapeutic approaches for such incurable conditions.

INTRODUCTION

1. PRION DISEASES

Prion diseases are a group of rare neurodegenerative disorders of humans and mammals, rapidly progressive and invariably fatal.

They are often defined as Transmissible Spongiform Encephalopathies (TSE), since they are characterized by a spongiform degeneration of the Central Nervous System (CNS) and, in most cases, by infectivity which can occur both experimentally and naturally. Common histopathological features are vacuolation of the gray matter, neuronal loss, reactive astrogliosis and deposition of a misfolded, partially protease resistant form of an endogenous protein, the cellular prion protein (PrP^{C}).

Although the precise pathogenesis has not been completely elucidated yet, it is recognized that the abnormal isoform of the PrP^{C}, called scrapie prion protein (PrP^{Sc}), has a central role. The presence of PrP^{Sc} in the nervous tissue of affected individuals is very often observed, and it is also necessary for infectivity. As it was extensively demonstrated, the key etiological event in such diseases is indeed the conversion of PrP^{C} into PrP^{Sc}.

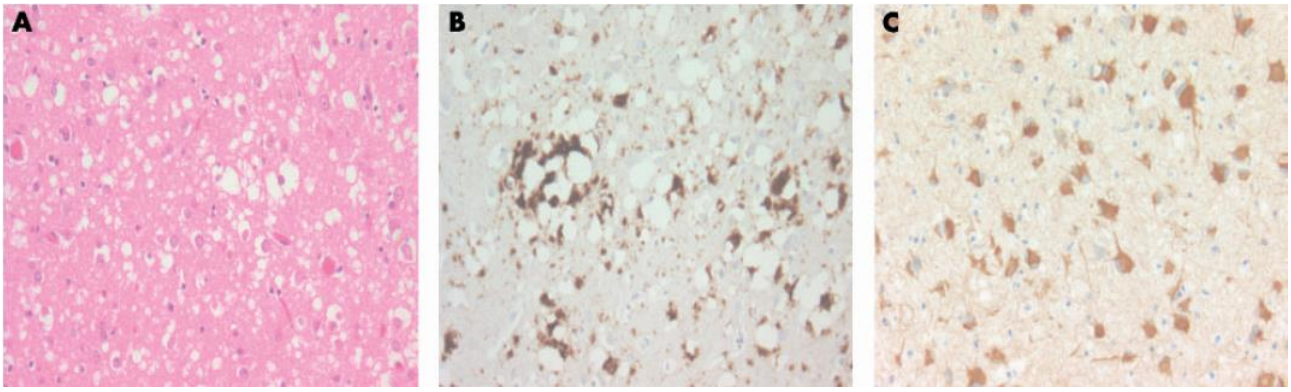


Figure 1- Neuropathological changes in Creutzfeld-Jakob disease (CJD). (A) Spongiform change in the frontal cortex in sporadic CJD. (B) Cerebral PrP^{Sc} accumulation in sporadic CJD. (C) Thalamic gliosis in variant CJD (from Knight and Will, 2004).

1.1 HUMAN PRION DISEASES

Human TSE can be sporadic, genetically inherited or acquired through infection.

Sporadic diseases

Approximately 85% of human prion diseases are sporadic forms of Creutzfeld-Jakob Disease (sCJD) (Aguzzi, Sigurdson et al. 2008). sCJD incidence is about one person per million per year, and it occurs in the absence of any evident exposure to people or animals infected with TSE or mutations in the PrP gene (*PRNP*), although a polymorphism at the codon 129 seems to be involved in the susceptibility. At codon 129 of *PRNP*, an individual may encode for methionine (M) or valine (V). In the normal UK population, the allelic distributions are approximately: MM 40%, VV 10%, MV 50%, whereas in sCJD cases they are: MM 68%, VV 16%, MV 16%. MM homozygosity is therefore a risk factor (Knight and Will 2004).

The disease typically presents with a rapidly progressive dementia and myoclonus; cerebellar ataxia, visual symptoms, pyramidal and extrapyramidal signs, as well as characteristic electroencephalographic alterations can be present, and the progression usually culminates in a terminal akinetic mute state (Knight and Will 2004; Aguzzi, Sigurdson et al. 2008). Onset normally occurs between 45 and 75 years of age, with peak onset between 60 and 65 years. The clinical progression is very rapid, with death occurring often in 2–3 months. Around 70% of patients dies within 6 months (Collinge 2001).

Genetic diseases

Familial CJD (fCJD), Gertsmann-Sträussler-Scheinker (GSS) syndrome and Fatal Familial Insomnia are the most common inherited prion diseases (FFI). They depend on dominant, highly penetrant mutations in *PRNP* and represent the 10-15% of human TSEs.

Hitherto, several point mutations or insertions have been described. Insertions occur in a region encoding four octapeptides and one nonapeptide and consist in octapeptide repeat expansions, the size of which affects the clinical phenotype (Knight and Will 2004). Although each mutation is normally associated to a specific disease, other genetic factors may influence the clinical phenotype. Among these, once again, the polymorphism at codon 129 has remarkable effects. For

example, the D178N mutation gives rise to fCJD when associated to 129V, and to FFI when associated to 129M.

In most cases, the mutations are thought to favor the protein's propensity to convert to PrP^{Sc}. However, there is now considerable evidence that different mutations may have different consequences besides prion generation, including the alteration of the cellular trafficking and localization of the protein (Wadsworth, Asante et al. 2010).

The overall most common mutation is E200K, which is associated to fCJD. The clinical phenotype is similar to that of sCJD, but with a lower age of onset and a shorter duration. Disease clusters are present in Israel, Slovakia and Chile (Knight and Will 2004).

FFI is characterized by sleep disturbances as well as vegetative and focal neurological signs as a result of thalamic lesions (Aguzzi, Sigurdson et al. 2008). It is caused by D178N/129M mutation and affects about 30 families worldwide.

GSS syndrome is associated to other point mutations (the most common at codons 102 and 198), and is characterized by chronic progressive ataxia, terminal dementia and a long clinical duration from 2 to 10 years (Aguzzi, Sigurdson et al. 2008).

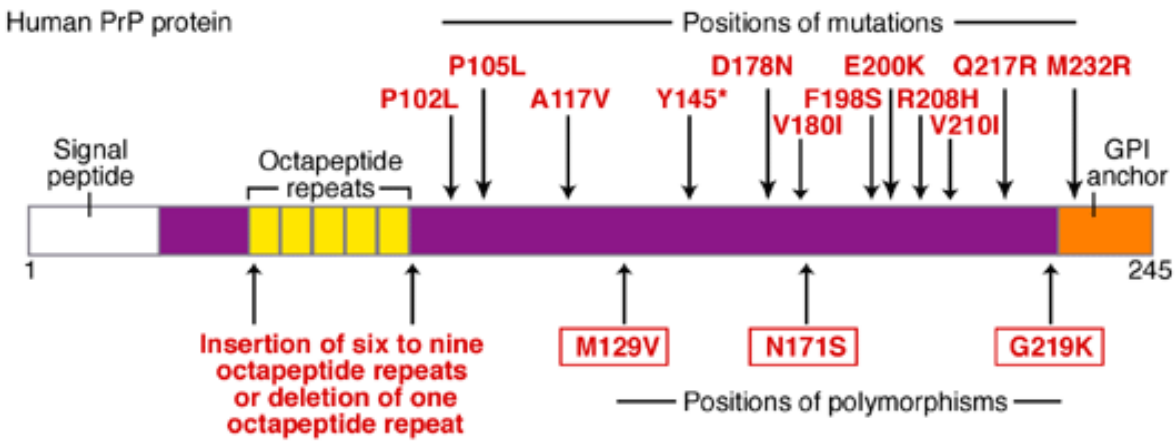


Figure 2- Sequence variability in human PrP. Main mutations, polymorphisms, insertions and deletions that have been found in the human PrP gene. The positions of the mutations (listed above the protein) and polymorphisms (listed below the protein) are indicated. The asterisk indicates a stop codon and therefore this mutation results in a truncated protein (from Manson and Tuzi, 2001).

Acquired diseases

Transmissibility is one of the crucial traits of prion diseases (Aguzzi, Sigurdson et al. 2008). Although the definition refers to experimental transmission in animals through inoculation of infected brains extracts, many well documented cases of animal and human diseases acquired through infection do exist.

Kuru is surely one of the most famous examples. It is a slowly progressive neurodegenerative disease that reached epidemic proportions within a population of Papua New Guinea between the beginning and the half of the 20th century. It propagated especially in women and children in consequence of oral exposure to infected nervous tissue occurring during cannibalistic rituals.

Another example is iatrogenic CJD. Over the past 30 years about 300 cases of CJD have been caused by the transmission of infection from person to person during medical or surgical treatments (Knight and Will 2004), owing to the use of improperly sterilized neurosurgical instruments or in consequence of infected dura mater and corneal transplants or pituitary-derived growth hormone administration. Interestingly, cases arising from intracerebral or optic inoculation manifest clinically as classical CJD, with a rapidly progressive dementia, whereas those resulting from peripheral inoculation frequently present with a slowly progressive cerebellar ataxia, reminiscent of kuru. The incubation periods range from months in case of intracerebral exposure (19–46 months for dura mater grafts) to as many as 40 years (with an average of approximately 12 years) after peripheral exposure (Collinge 2001).

However, the most famous acquired human prion disease is certainly variant CJD (vCJD). This variant has been described for the first time in 1996 in some cases of CJD with atypical presentation. Compared to the classical form, vCJD primarily affects young adults, with a mean age of onset of 29 years and a longer duration, on average 14 months. It initially presents as a neuropsychiatric disorder with pronounced behavioral signs and sensory phenomena. Ataxia, dementia and involuntary movements progressively follow. CJD's typical electroencephalographic alterations are absent and neuropathological features are different. Interestingly, vCJD is the sole human prion disease in which PrP^{Sc} depositions are detectable in tonsils and other lymphoreticular tissues in preclinical disease. Although no mutations in the *PRNP* gene were detected, all primary vCJD cases so far were found to carry the MM genotype at codon 129.

The initial occurrence of these patients in the UK some years after a Bovine Spongiform Encephalopathy (BSE) epidemic, indicated a possible association between the diseases. Subsequent studies showed indeed a great similarity in terms of neuropathological lesion distributions in mice, PrP^{Sc} glycoform gel banding patterns and neuropathology after transmission to monkeys. On these basis, vCJD is now thought to represent a spread of BSE from cattle to humans (Aguzzi, Sigurdson et al. 2008).

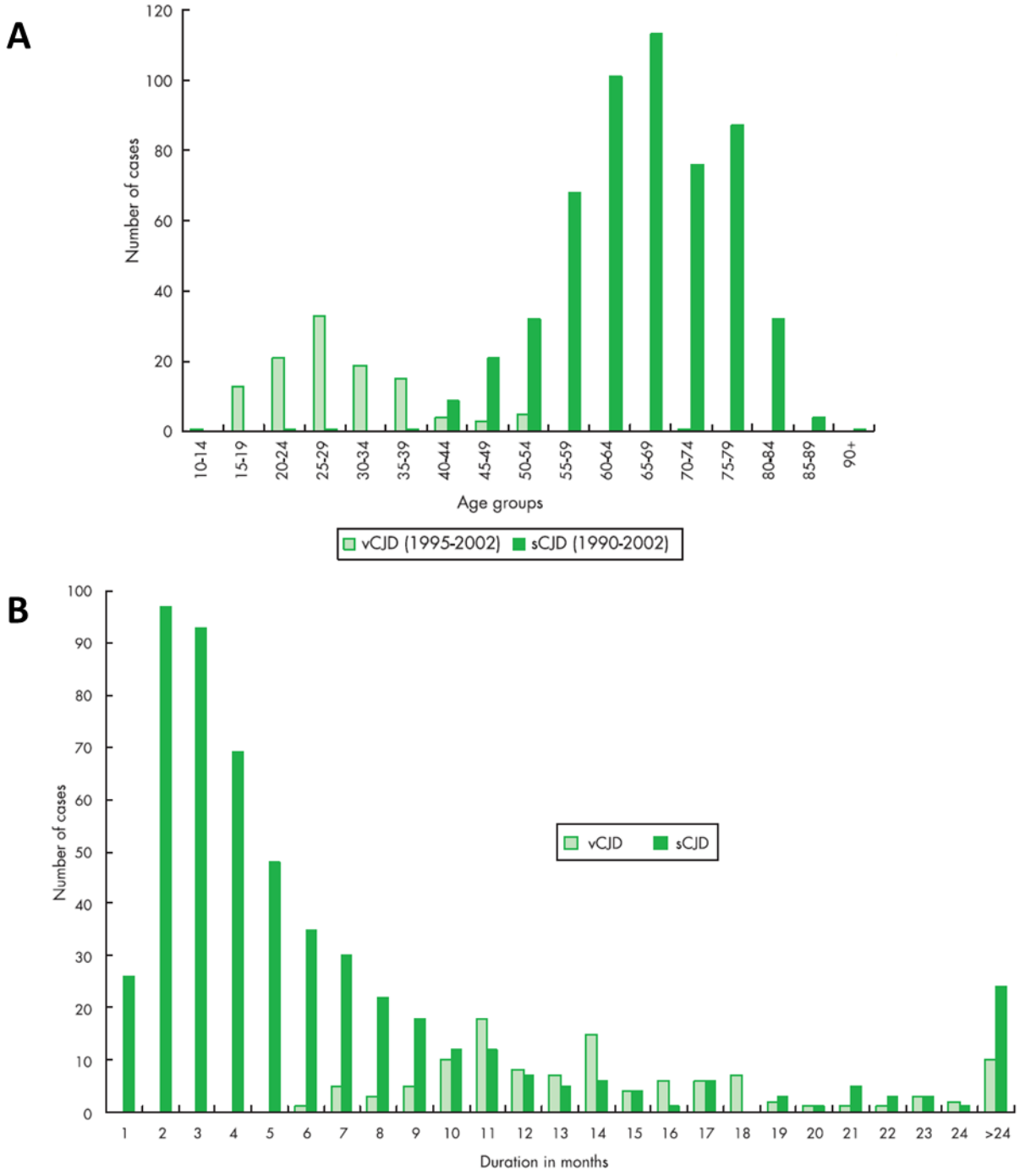


Figure 3- Epidemiology of CJD. (A) Age at death and (B) duration of the disease (months) in variant and sporadic CJD in the UK (from Knight and Will, 2004).

Although there have been serious concerns about the possible epidemic proportions that vCJD could reach, only approximately 200 cases have been reported to date, and the incidence of the disease is now decreasing. It should be kept in mind, however, that other types of prion diseases, such as Chronic Wasting Disease of deer, are easily transmissible, and that horizontal spread of

prions can be mediated by body fluids such as saliva, milk and urine. Moreover, since millions of Europeans were most likely exposed to BSE, it is possible that some individuals might be asymptomatic carriers able to transmit the infection, for instance, via blood transfusion, as there is evidence that such a route of transmission of vCJD may have occurred (http://www.who.int/mediacentre/factsheets/fs180/en/).

To date, validated diagnostic tests in living animals or humans and proven treatments for prion diseases are not available. For these reasons, prion diseases represent an issue of great importance for public health, requiring a strict adherence to guidelines for surveillance and prevention, and further extensive studies for a better understanding of such a peculiar phenomenon, and for the development of effective diagnosis and therapy.

1.2 THE PROTEIN-ONLY HYPOTHESIS

The unusual characteristics of TSEs have challenged some of the most established dogmas in life sciences, leading to one of the most debated theories in biology: the protein-only hypothesis. The first evidence of the peculiar nature of TSEs emerged in 1937 when sheep scrapie was transmitted through a vaccine prepared from sheep brains treated with formalin, which normally abolished any known form of infectivity. Later on, the infectious nature of other animal and human TSEs was confirmed in both naturally-occurring and experimental cases of transmission. Because of the long incubation period, the agent responsible for the diseases was initially thought to be a slow virus, but this hypothesis was soon called into question. The unknown agent, in fact, appeared to be extremely resistant to treatments that normally destroy nucleic acids, such as UV and ionizing radiations. Moreover, the minimum molecular weight necessary for infectivity was found to be of about 200 kDa, lower than the mass of any known virus. Eventually, although certain scientists remain skeptical about the “protein-only hypothesis”, in 30 years of investigations no conventional pathogen convincingly associated to TSEs was ever found.

On the basis of these findings, a revolutionary hypothesis was formulated in 1967 by J.S.Griffith: the agent responsible for TSE transmission might be a protein endowed with the unprecedented ability to replicate in the body. The term “prion” was subsequently coined by S. Prusiner in 1982 to

define this new **pro**teinaceous **in**fectious particle.
The first support to the theory came with the isolation of a specific protein, called prion protein (PrP), from infectious material. This protein was associated to infectivity, and infectivity was reducible by proteolytic treatments, although PrP showed to be rather protease-resistant.

Purification of the protein permitted then to identify the endogenous PrP encoding gene, which was present also in the uninfected individuals and particularly expressed in the brain. Further studies associated all the familial TSEs to mutations in the same gene.

Later, it became clear that PrP can exist in two isoforms: the normal PrP^{C} and the pathological PrP^{Sc}. The two versions differ in terms of chemical and physical properties, and PrP^{Sc} possesses one unique characteristic: the ability to self-propagate by converting the cellular isoform into the aberrant one.

However, one of the biggest challenges for this theory has been to explain the existence of different prion strains. Prion strains are defined as infectious isolates that confer a specific phenotype, different from that of other isolates, when experimentally transmitted. In other words, individuals affected by prion diseases may develop different pathologies, the clinical, neuropathological and biochemical characteristics of which are maintained through serial transmission to rodents. In analogy to other infectious agents, these variants have been termed strains. A classical definition of strain refers to a genetic variant or subtype of the infectious agent responsible for the disease, but this concept, valid in virology, cannot be extended to prions, because the phenomenon is independent of nucleic acid sequence variations (Morales, Abid et al. 2007).

Prion strains differ in terms of incubation periods, clinical signs, distribution of brain lesions, tissue tropism and host range. For instance, when FFI prions are inoculated into mice, PrP^{Sc} deposition is confined to the thalamus, as occurs in the parental disease. In contrast, inoculation of fCJD prions produces a widespread deposition of PrP^{Sc} within the cortex and the deep nuclei (Prusiner 1998).

To conciliate the strain phenomenon with the hypothesis of a protein-only infectious agent with no variations in its primary structure, it has been proposed that prion strains arise from conformational variability, that is, PrP can assume several different, self-propagating conformations, each encoding a distinct prion strain (Colby and Prusiner 2011).

As a matter of fact, it is well known that proteins may exist in different conformers with different functions (Prusiner 1998). Under this respect, it should not be surprising that a single protein may show so many different behaviors within the network of genetic, cellular and environmental combinations that characterize every single individual or system.

Although difficult to accept, the prion hypothesis has resisted to all the attempts to disprove it, and recent reports demonstrating the generation of highly infectious prions completely *in vitro* have provided the ultimate proof for the protein-only hypothesis to be true (Diaz-Espinoza and Soto 2010).

2. THE PRION PROTEIN

PrP is encoded by an autosomal gene, *PRNP*, that in humans is located on the short arm of chromosome 20 (position 20p13). The gene contains two or three exons depending on the species, but the open reading frame (ORF) is entirely contained in one. Although some inter- and intraspecies variability exists, the sequence is highly conserved (Colby and Prusiner 2011).

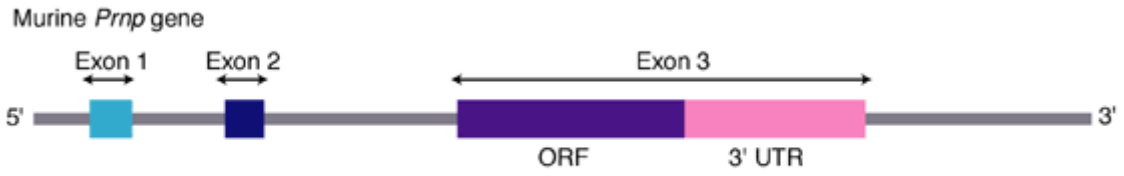


Figure 4- Murine PrP Gene. The murine PrP gene possesses three exons; exon 3 contains the whole open reading frame (ORF) and 3' untranslated region (3' UTR). Conversely, the human PrP gene (not shown) possesses two known and one putative exon; the equivalent of murine exon 2 has not been found in human RNA transcripts to date (from Manson and Tuzi, 2001).

The protein is present in mammals and in other classes as marsupials, birds, reptiles and fish, and may be present in all the vertebrates (Collinge 2001; La Mendola, Pietropaolo et al. 2008). It is expressed since the first phases of embryogenesis and is found in many adult tissues, showing the highest expression levels in the CNS (Collinge 2001). Here, it is present in both astrocytes and neurons, where is particularly enriched in the synaptic membranes (Aguzzi, Sigurdson et al. 2008).

Human PrP is translated as a 253 amino acids peptide. During the maturation process, PrP undergoes the cleavage of a 22 amino acid, amino-terminal (N-terminal) signal, and a 23 amino acid carboxy-terminal (C-terminal) peptide which drives the addition of a glycosylphosphatidylinositol (GPI) anchor (Colby and Prusiner 2011). The mature protein has an unstructured N-terminal domain of about 100 amino acids and a globular C-terminal domain. This contains 3 α-helices and a two-stranded antiparallel β-sheet, and it is stabilized by a disulphide bond between helices 2 and 3. PrP is also glycosylated on two asparagine residues (Collinge 2001).

The unfolded N-terminal contains two important conserved regions. The first is a segment of five repeats of an octameric amino acid sequence (octapeptide repeat region) which contains a copper binding site and which, owing to its sequence characteristics, is prone to expansions resulting in prion diseases (Collinge 2001; Aguzzi, Sigurdson et al. 2008). The second is a hydrophobic domain which is thought to have an important functional role in preventing PrP^C cytotoxicity: small deletions in this region, in fact, render PrP a potent neurotoxin (Baumann, Tolnay et al. 2007; Aguzzi, Sigurdson et al. 2008) .

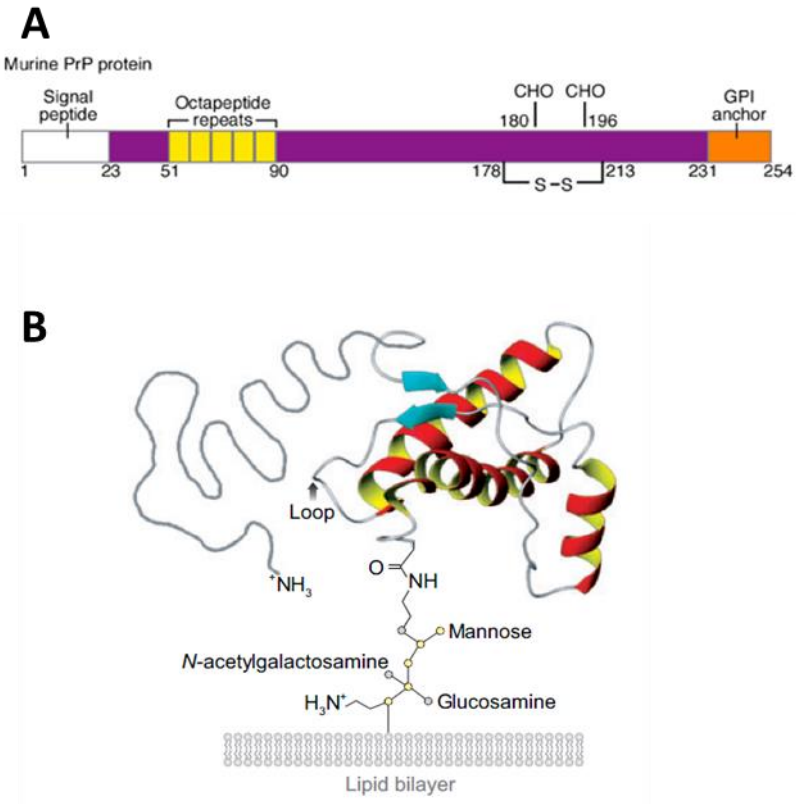


Figure 5- Structural features of PrP. (A) Primary structure of the murine PrP. The murine protein consists of 254 amino acid residues. Features include a 22 amino acid signal peptide, five octapeptide repeats, one disulphide bond (S–S) between cysteine residues 178 and 213, and two sites for N-linked glycosylation (CHO) at residues 180 and 196. A glycosylphosphatidylinositol (GPI) anchor is attached to the C-terminus of PrP from residues 231 to 254. Human PrP (not shown) has the same structural features, but is 253 amino acids in length (from Manson and Tuzi, 2001). (B) Tertiary structure of PrP, inserted into a lipid bilayer, including the unstructured N-terminal tail (gray) and the GPI anchor. The α-helices are indicated in red; the antiparallel β-sheets are shown in blue. The small circles represent sugar residues (from Aguzzi and Heikenwalder, 2006).

2.1 PrP FUNCTION

Despite a great interest in PrP developed after the discovery of its involvement in the pathogenesis of TSEs, the physiological role of the protein remains unclear. Whereas its developmentally regulated expression in most tissues suggests a widespread function, as a matter of fact, PrP knock-out mice do not show any dramatic phenotype, nor the protein seems to be determinant for viability (McLennan, Brennan et al. 2004; Aguzzi, Sigurdson et al. 2008).

PrP Knock-out models

The generation of knock-out models has been one of the first steps in the attempt to unravel the biological role of PrP. Because of its high conservation among mammals, there was great expectation that the ablation of PrP would reveal the function of the gene (Steele, Lindquist et al. 2007). However, results have been disappointing. No alterations in development, behavior and life expectancy were found in PrP knock-out mice, and the only evident phenotype was their complete resistance to prion infection (Carleton, Tremblay et al. 2001; Collinge 2001; Rangel, Burgaya et al. 2007).

Many different PrP-deficient mice have been generated with a wealth of strategies. For example, early models (*Zurich* and *Edimburgh* strains) were obtained by disrupting modifications confined to the ORF, and in this case, no obvious phenotype was observed (Carleton, Tremblay et al. 2001; Steele, Lindquist et al. 2007).

A second knock-out strategy involved the deletion of both the coding region and its flanking sequences. With ageing, these mice developed a severe progressive ataxia and Purkinje cell degeneration. This phenotype was rescued by the reintroduction of functional PrP, and for this reason it was initially considered a primary consequence of the absence of the protein. Further studies, though, revealed that such effects were due to a knock-out artifact rather than to the lack of PrP. The deletion, in fact, resulted in an intergenic splicing mutation leading to the up-regulation of a downstream protein named Doppel. This PrP-related protein shares about 25% homology with PrP and it is expressed in many tissues but not in the adult brain. Therefore, the degenerative phenotype showed by these PrP-deficient line was likely caused by the ectopic expression of Doppel in the brain (Carleton, Tremblay et al. 2001; Weissmann and Flechsig 2003).

However, despite the absence of a major phenotype, many studies identified several more subtle alterations both *in vivo* and *in vitro*.

First, mice devoid of PrP showed alterations in sleep and circadian rhythms. This effect interestingly correlates with some features found in different prion diseases. Sleep disruption is in fact the main symptom in FFI, but is also increasingly recognized in sporadic and familial CJD (Tobler, Gaus et al. 1996; Dossena, Imeri et al. 2008).

Other aberrations in synaptic activity, cognition and olfactory behavior were reported (see below).

By an anatomical point of view, PrP-deficient mice showed a collateral and terminal sprouting in mossy fiber comparable to that observed in epilepsy. These data were complemented by others demonstrating an increased sensitivity to kainate-induced seizures and cell death (Rangel, Burgaya et al. 2007).

Eventually, PrP knock-out neurons appeared to be more susceptible to apoptosis induced by both oxidative stress and serum deprivation (Kuwahara, Takeuchi et al. 1999; Vassallo and Herms 2003).

However, most of the described phenotypes are quite controversial: some of them have been contested, some others are very subtle or manifest only upon physiological challenge. One point of concern is that the detection of many of these phenotypes seems to depend on which strain of mice is used (Steele, Lindquist et al. 2007), and it is therefore very likely that all the observations made so far are contaminated by the confounding effects of different genetic backgrounds.

All in all, after 30 years of extensive investigation the only straightforward characteristic of the knock-out mice is their resistance to prion infection, and the physiological role of PrP remains more mysterious than ever.

However, based on the analysis of the mild phenotypic traits that develop in PrP knock-out mice and on cell culture studies, PrP^{C} has been assigned numerous roles, the most relevant of which are listed below.

Metal uptake and antioxidant activity

PrP has been involved in metal uptake, and in particular in copper and zinc metabolism.

PrP is a copper (II) binding protein, having the major copper binding site located in the octapeptide region with a dissociation constant (K_d) of 5 μM. This K_d is compatible with the copper concentration released in the synaptic cleft during vesicle release. In fact, the presence of copper binding activity in one of the most conserved regions of the protein, the localization of the protein at the presynaptic membrane and the ability to bind copper at physiological concentrations argue for an involvement in the metabolism of this metal.

PrP is thought to buffer copper (II) in a pH-dependent manner during vesicle exocytosis and to return it to the cytosol, either by transferring ions to copper transport proteins or, in case of higher copper concentrations, by copper-mediated PrP endocytosis (Vassallo and Herms 2003).

Not only copper, but also zinc can bind the octapeptide region, although with a lower affinity (about 200 μM).

Zinc is the most abundant trace metal in the CNS, and just like copper is contained in synaptic vesicles and released in the synaptic cleft during the process of neurotransmission, where it reaches concentrations in the millimolar range. Also in this case, therefore, the binding affinity is compatible with a role in binding or transporting zinc ions under physiological conditions (Watt, Griffiths et al., 2013).

Moreover, PrP was evolutionary linked to Zrt/Irt-like protein (ZIP) family of transmembrane zinc transporters (Schmitt-Ulms, Ehsani et al. 2009). Unlike other ZIP proteins, however, PrP does not possess a transmembrane channel domain for direct zinc uptake. Therefore, other mechanisms have been proposed. For example, PrP could contribute to zinc homeostasis via endocytosis, for it has been shown that zinc biding triggers the internalization of the protein (Perera and Hooper 2001). Another possibility is that PrP is a zinc sensor which mediates the uptake of the metal by means of other proteins. And indeed, it was found that PrP-dependent zinc uptake can be mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) (Watt, Griffiths et al., 2013).

Regulating free metal ion levels is extremely important to protect the cell from oxidative stress. In the presence of hydrogen peroxide, ions like iron and copper take part to Fenton redox reactions that generate powerful radicals such as superoxide and hydroxyl ions. Metal buffering may thus cooperate to prevent the generation of excessive levels of reactive oxygen species.

PrP antioxidant activity has been proposed based on several observations obtained from knock-down models, such as a higher sensitivity to oxidative stress and lower activity of the main antioxidant enzymes like glutathione reductase and superoxide dismutase (SOD) compared to wild type animals.

An actual copper-dependent SOD activity has been detected in PrP, but many observations oppose to this hypothesis. First, PrP copper affinity is much higher than the average affinity that is found in this kind of metal binding proteins, and the pH dependence of the binding between the protein and its catalytic ion is also unusual for a dismutase. Furthermore, PrP has been demonstrated to contribute only to a minor percentage of total SOD activity in the brain (Vassallo and Herms 2003).

Cell adhesion and neurite outgrowth

As a GPI-anchored cell surface glycoprotein, another reasonable hypothesis is that PrP may be involved in cell adhesion (Collinge 2001). Indeed, many of the numerous PrP-interacting partners that have been reported are adhesion or recognition molecules, such as the Neural Cell Adhesion Molecule (NCAM), the laminin receptor, the laminin receptor precursor and the extracellular matrix protein laminin itself. Notably, all these molecules are involved in a series of signal transduction pathways that regulate neurite outgrowth and neural survival and differentiation.

And indeed, PrP is directly involved in a neuritogenesis-promoting signaling pathway. NCAM interacts with PrP, which is localized in lipid-enriched membrane domains. This enables NCAM to interact with other lipid rafts proteins to activate the downstream signaling pathways which are involved in neurite outgrowth and synaptic development (Chen, Mange et al. 2003; Santuccione, Sytnyk et al. 2005; Aguzzi, Sigurdson et al. 2008; Babus, Little et al. 2011). More details about PrP signaling activity are provided below.

Myelin maintenance

Several observations suggest that PrP may also cooperate to myelin maintenance. It was initially noted that some knock-out mice lines developed a late-onset peripheral neuropathy, and later PrP was found to be enriched in purified myelin fractions. Moreover, myelin alterations have been documented in some cases of GSS syndrome and in other experimental TSE models (Baumann, Tolnay et al. 2007; Aguzzi, Sigurdson et al. 2008; Bremer, Baumann et al. 2010).

Other studies found that the classical features of demyelinating neuropathies in PrP-deficient nerves could be abolished by rescuing PrP expression in neurons but not in Schwann cells. This suggests that PrP may exert an indirect protective action on myelin, taking part in the axonal signals which regulate Schwann cells trophism. Interestingly, the hydrophobic domain of the protein seems to be determinant for this role, for deletions in this region give rise to a lethal myelin damage in mice (Baumann, Tolnay et al. 2007; Aguzzi, Sigurdson et al. 2008; Bremer, Baumann et al. 2010).

Stem cell regulation and neurodevelopment

PrP is highly expressed during embryogenesis throughout the whole organism (Manson, West et al. 1992). This suggests that it may participate in developmental processes, and indeed, PrP knock-out embryos show prominent alterations in many genes involved in development (Khalife, Young et al. 2011).

In particular, PrP is involved in different aspects of stem cell biology.

First, it regulates the self-renewal capacity of stem and progenitor cells. In the CNS, its expression levels correlate with the proliferation rate of both fetal and adult progenitors of the subventricular zone and the dentate gyrus (Steele, Emsley et al. 2006; Santos, Silva et al. 2011; Prodromidou, Papastefanaki et al. 2014).

In addition, PrP expression increases as the differentiation of neural precursors proceeds, and maturation is delayed in the absence of the protein, suggesting a role in driving stem cell fate.

However, neither the total number of surviving neurons nor the gross morphology of the hippocampus are altered in PrP knock-out mice. This indicates that proliferation rates are not the sole determinants of the total number of neurons in adult brain and PrP is likely to be one minor factor in the complex process of neurodevelopment (Steele, Emsley et al. 2006).

Interestingly, neural stem cells are not the only lineage regulated by PrP. For example, the protein is highly expressed in hematopoietic stem cells (HSCs), and HSCs derived from PrP knock-out mice lack the ability of long-term reconstituting the hematopoietic system of lethally irradiated mice (Zhang, Steele et al. 2006). More recently, PrP was further shown to promote the expansion and engraftment of human mesenchimal stem cells (Mohanty, Cairney et al. 2012).

Cell survival

Many observations support a role of PrP in cell survival which may be exerted at multiple levels.

For example, it may be a consequence of its antioxidant activity. In this regard, it has been demonstrated that PrP deficient cells display enhanced sensitivity to oxidative stress-dependent cell death (Wong, Pan et al. 2000; Chiarini, Freitas et al. 2002).

To confirm this hypothesis, many studies made use of focused deletions in specific regions of the protein to assess the effects on cell viability. Interestingly, it emerged that also some of the deletions outside the copper binding octapeptide region were associated to neurodegeneration. On the other hand, not all the ones within the octapeptide region resulted in cell death, suggesting that other factors besides copper-binding activity are associated with resistance to neurodegeneration (Chiarini, Freitas et al. 2002).

For example, the stress-induced protein 1 (ST-1) was proposed as a ligand capable of transducing neuroprotective signals mediated by cAMP, protein kinase A (PKA) and Erk upon binding to PrP, suggesting that PrP may function as a neurotropic receptor (Chiarini, Freitas et al. 2002).

A putative neuroprotective activity was also hypothesized after observing that PrP is upregulated in the penumbra region in hypoxic brain injuries, and that ischemic lesions are larger in PrP-deficient than in wild type mice (McLennan, Brennan et al. 2004).

Different molecular mechanisms mediate neuronal cell death after focal cerebral ischemia in vivo. In addition to excitotoxicity and neuroinflammation, both oxidative stress and apoptosis contribute to neuronal loss. Given the antioxidant and anti-apoptotic function of PrP^C in cultured neurons, it is tempting to speculate that early upregulation of PrP^C after focal cerebral ischemia may be part of an adaptive cellular response promoting neuronal survival after ischemic injury. (Weise, Crome et al. 2004).

A direct anti-apoptotic function for PrP was also proposed. First, PrP deficient neurons were found to be more susceptible to apoptosis induced by serum deprivation, and this phenotype could be rescued by overexpression of either PrP or Bcl-2, a very well-known anti-apoptotic protein (Kuwahara, Takeuchi et al. 1999).

Second, a soluble cytosolic form of PrP was demonstrated to interact with some Bcl-2 family members (Kurschner and Morgan 1996). In addition, a certain degree of homology was found between PrP and the Bcl-2 homology domain 2 (BH2). Importantly, BH2 is a crucial domain for the anti-apoptotic function of Bcl-2 and for its interaction with the pro-apoptotic Bax protein.

All these clues eventually led to identify a direct anti-Bax effect of PrP, and the anti-apoptotic properties of PrP were demonstrated to be comparable in extent to the classic anti-apoptotic protein Bcl-2 (Bounhar, Zhang et al. 2001).

PrP as a signaling molecule

Through its broad range of functions, PrP appears to be connected with nearly all aspects of neuronal physiology. But how can a single protein serve so many different functions without being indispensable? A possible answer could be that PrP is a signaling molecule involved in the regulation of many redundant pathways.

In support to this hypothesis there is the evidence that PrP interacts with an impressive number of different membrane proteins, potentially influencing their localization and activity. As already mentioned, it also segregates in sphingolipids- and cholesterol-rich membrane microdomains known as lipid rafts. Thanks to their peculiar lipidic composition, lipid rafts bring together receptors, second messengers and effectors, working as *bona fide* "signal transduction centers".

All these aspects are indeed consistent with a possible role of PrP as a signaling molecule, which may work as a receptor, a co-receptor or even a ligand able to induce signal transduction pathways (Hirsch, Hernandez-Rapp et al. 2014).

Several putative ligands for PrP have been identified so far. The most important have been mentioned previously, and include vitronectin, laminin and ST-1 (Hirsch, Hernandez-Rapp et al. 2014). Notably, also amyloid precursor protein (APP) and Aβ oligomers can bind PrP with high affinity, conferring to this protein a relevant role also in the pathogenesis of Alzheimer disease (Lauren 2014). Ligand binding may trigger PrP clustering, thereby activating the signaling activity, as it occurs for other GPI-anchored proteins.

Over the years, many possible downstream effectors were described, including phosphoinositide 3 kinase (PI3K), PKA, the reactive oxygen species (ROS)-generating enzyme NADPH oxidase, the glycogen synthase kinase GSK3b, the mitogen-activated protein (MAP) kinases ERK1/2 and the Src

kinases members Fyn and Lyn (Hirsch, Hernandez-Rapp et al. 2014). Among these, Fyn kinase is probably the main PrP effector, which controls most of the above-mentioned pathways and is involved in both prion and Aβ-induced neurotoxicity (Ochs and Malaga-Trillo 2014).

As a GPI-anchored extracellular protein, PrP is not able to directly induce intracellular signaling cascades, but requires a partner. In 2000, Mouillet-Richard and colleagues identified caveolin as a possible intermediate factor linking PrP with Fyn kinase (Mouillet-Richard, Ermonval et al. 2000).

Fyn and the Src kinases are involved in a wide range of cellular processes, including cell differentiation, proliferation, adhesion, migration and survival (Roskoski 2004), and most of the functions attributed to PrP depend on the activity of proteins that are indeed regulated by Src kinases.

On the other hand, all these general processes are controlled by a very complex network of redundant pathways, each of which can easily compensate for defects in the others. This may likely be the reason why a loss of function of PrP does not produce any remarkable effect.

A role for PrP as a signal transducer of a broad set of redundantly controlled pathways could hence fit quite well to a protein which “is implicated in virtually everything, but responsible for nothing” (Veasey, Fornal et al. 1995).

2.2 PRION GENERATION

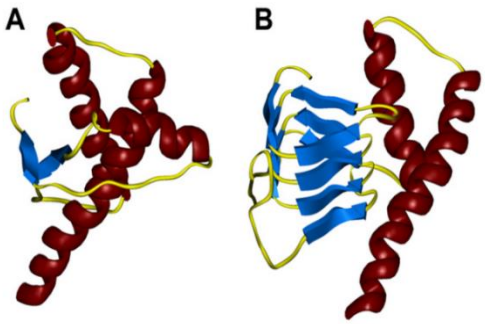
The key event in the development of the disease-causing particle is the conversion of the normal PrP^{C} into the aberrant isoform PrP^{Sc}.

Although identical in sequence and post-translational modifications, the two isoforms show marked differences in their biochemical properties: PrP^{C} has an α-helix-rich structure, is sensitive to proteinase K (PK) and is soluble, whereas PrP^{Sc} is rich in β-sheets, partially resistant to PK digestion and prone to aggregation, being insoluble in non-ionic detergents (Aguzzi, Sigurdson et al. 2008).

Prion propagation is thought to occur by a template-assisted process in which PrP^{Sc} acts as a template onto which PrP^{C} is refolded into the pathological conformation. During this process PrP^{C} may need to acquire a partially unfolded structure to interact with PrP^{Sc}, an intermediate state which is referred to as PrP* (Colby and Prusiner 2011). Conversion may then occur either through an autocatalytic event induced by a heterodimeric association of PrP* and PrP^{Sc} or through a non-catalytic, nucleated polymerization process.

In the former case, the conformational change of PrP^{C} into PrP^{Sc} would be prevented in standard conditions due to a high activation energy. The energy barrier would be lowered by the association of PrP^{Sc} to PrP^{C}, thus permitting the autocatalytic conversion.

The alternative model proposes that conversion of PrP^{C} into PrP^{Sc} is a reversible process, but the equilibrium is strongly in favor of the native conformation. PrP^{Sc} can form and stabilize only when it adds onto a crystal-like seed or aggregate of PrP^{Sc}. Once a seed is present, further monomer addition is accelerated (Aguzzi, Sigurdson et al. 2008).



PrP isoforms biochemical

A	B
Rich in α-helices	Rich in β-sheets
Soluble	Insoluble
PK sensitive	PK resistant
No aggregation	Aggregation

Figure 6- Structural features and biochemical properties of PrP isoforms. The C-terminal globular domains of (A) PrP^{C} and (B) PrP^{Sc} are represented in the upper frame. The normal isoform represented in (A) contains mostly α-helices (indicated in red), whereas in the pathogenic form shown in (B) has a high content of β-sheets. The lower table summarizes the biochemical properties associated to either isoform (from Aguzzi and Polymenidou, 2004).

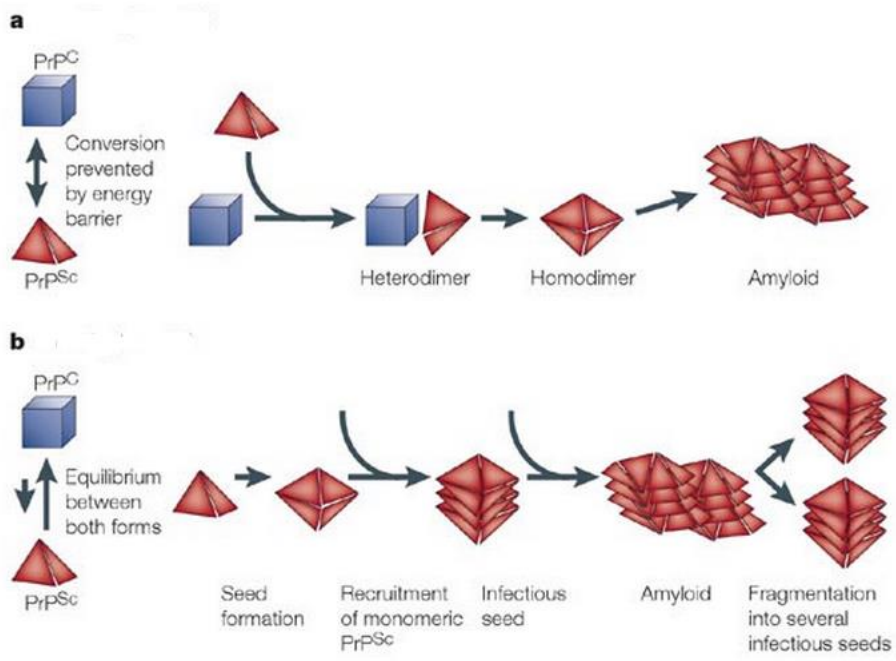


Figure 7- Models of PrP conversion. There are two proposed models for the conversion of the normal prion protein (PrP^{C}) into the infectious form (PrP^{Sc}). (A) An interaction between PrP^{Sc} and PrP^{C}, induces the latter to transform itself into further PrP^{Sc} through an autocatalytic process. Spontaneous conversion to the infectious form does not normally occur due to a high energy barrier between the two isoforms. (B) The conversion of PrP^{C} and PrP^{Sc} is a reversible process, but at equilibrium strongly favors the conformation of PrP^{C}. Converted PrP^{Sc} is maintained only when it adds onto a fibril-like seed or aggregate of PrP^{Sc}. Once a seed is present, further monomer addition is accelerated. PrP^{Sc} can therefore aggregate to form amyloid, and this can fragment to form several infectious seeds (from Aguzzi and Polymenidou, 2004).

These profound structural transitions may also require the activity of other proteins such as chaperones. Several pieces of evidence support the idea that a cofactor may actually exist but, lack of experimental demonstration. This proposed molecule, defined as ”Protein X”, is thought to bind to PrP^{C} facilitating its interaction with PrP^{Sc} (Prusiner 1998; Aguzzi, Sigurdson et al. 2008).

Prion propagation is likely to occur either in late-endosomes or in lysosomes, where PrP is recycled after endocytosis. The environment of these organelles promotes protein unfolding at low pH prior to degradation by acid-activated proteases. According also to *in vitro* studies, it is thus

possible that PrP conversion may occur at this level, facilitated by reducing and moderately acidic milieu (Collinge 2001).

2.3 MECHANISMS OF NEURODEGENERATION

Gain versus loss of function

The nature of prions, their biochemical properties and the mechanisms by which they propagate are now reasonably well understood. In contrast, much less is known about how PrP misfolding induces neuronal death.

Neurodegeneration, that sometimes is restricted to specific neuronal subpopulations, may occur either because of a toxic gain of function or a loss of the physiological function of the protein. Most likely, as suggested below, it can result by a combination of the two (Winklhofer, Tatzelt et al. 2008).

The fact that the inactivation of the PrP gene does not produce any effect on neuronal viability, together with the dominant modality of inheritance of genetic prion diseases tend to exclude that these disorders arise because of a simple loss of the physiological function of PrP (Solomon, Schepker et al. 2010).

Several experimental observations support the hypothesis of a gain-of-function mechanism. For example, the presence of intra-axonal aggregates of misfolded PrP was shown to contribute to neuronal death by disrupting axonal transport (Medrano, Barmada et al. 2008). In addition, *in vivo* crosslinking of PrP^C by antibodies triggers neuronal apoptosis, and, as previously mentioned, mutants lacking the hydrophobic domain of the protein (PrPΔHD) have neurotoxic effects (Winklhofer, Tatzelt et al. 2008). To explain the latter finding it was proposed that PrPΔHD may block some kind of neurotrophic signaling by binding to an unknown receptor (Shmerling, Hegyi et al. 1998). Alternatively, PrPΔHD may compete with PrP^C for binding to a signal-transducing protein. In this scenario, whereas PrP^C induces neuroprotective signaling, binding of PrPΔHD triggers a neurotoxic cascade (Li, Christensen et al. 2007).

However, Li et al demonstrated that the toxic effect of these mutants could be reversed by co-expressing wild type PrP, and observed that a disease-associated mutation impaired the efficiency of the protein in rescuing the phenotype. In addition, PrP was shown to attenuate the activity of N-methyl-D-aspartate receptors (NMDARs), with PrP knock-out neurons being more susceptible to glutamate-induced excitotoxicity (Khosravani, Zhang et al. 2008).

Taken together, these data indicate that a loss-of-function of PrP may also be detrimental in specific contexts. For example, whereas its biological activity is normally dispensable, it may become essential in the disease under cellular stress conditions (Solomon, Schepker et al. 2010).

According to this view, PrP^{Sc} might not be toxic *per se*: it may rather exert a dominant-negative effect on PrP^{C}, either by converting it in an inactive form or by competing for the interaction with its endogenous ligands (Hetz, Maundrell et al. 2003).

Insights from the mutants

Considerable evidence now suggests that aggregation, infectivity and toxicity are distinct properties of PrP that do not necessarily coincide. This concept is exemplified by the existence of several mutants that produce spontaneous neurodegeneration in humans and/or transgenic mice without the formation of infectious PrP^{Sc}. By isolating the neurotoxic effects of PrP from prions, these mutants have provided important - although not completely conclusive- insights into possible pathogenic mechanisms (Solomon, Schepker et al. 2010).

Such mutants can be grouped into two categories.

First, there are the mutations linked to familial prion diseases. When expressed in transfected cells or transgenic mice, these mutants generally display some tendency to aggregation and protease resistance similar to PrP^{Sc}, and sometimes their pathogenic properties actually depend on the toxicity of oligomeric aggregates (Medrano, Barmada et al. 2008). However, they also display other properties that may be responsible for neuronal dysfunction, such as altered trafficking and cellular localization (Ivanova, Barmada et al. 2001) and, possibly, aberrant interaction or loss of interaction with partner proteins. Interestingly, there are also cases in which these mutants are identical to wild type PrP in terms of biochemical properties and cellular localization (Harris 2003), suggesting that prion generation and aggregation are not mandatory for the establishment of the pathology.

The second category includes a variety of artificially-generated mutants. Among these variants, the partial deletions of the protein are particularly interesting. Most of these mutant proteins possess a powerful neurotoxic effect without showing PrP^{Sc}-like properties such as aggregation, protease resistance and infectivity. In some cases, moreover, toxicity is suppressed in the presence of wild type PrP, indicating that the two proteins may compete for the binding to a common target, with opposite effects on neuronal viability (Shmerling, Hegyi et al. 1998; Li, Christensen et al. 2007; Solomon, Schepker et al. 2010).

Combined with the evidence that PrP is a neuroprotective molecule, results obtained on artificial and naturally-occurring mutants suggest that neurotoxicity in prions may ultimately be caused by a subversion of the function of PrP^{C}. Specifically, the interaction with PrP^{Sc} or other aberrant intermediates may alter the physiological activity of the normal protein (Harris and True 2006; Solomon, Schepker et al. 2010). This would then result in cell death because of the suppression of fundamental neuroprotective signals or the direct generation of neurotoxic stimuli. Given the ability of PrP to interact with numerous partners, this functional subversion may also result in further "side effects" caused by alterations of these proteins, which, independent of PrP itself, are important for neuronal function.

Conclusions

Based on what discussed so far, it is evident that prion diseases are not a homogeneous class of diseases. Although pathogenicity is always attributable to a single protein, in fact, many differences exist among the various diseases.

As already pointed out, for example, whereas classic TSEs are defined by the presence of PrP^{Sc} and transmissibility, some of the inherited forms do not possess these features. In this view, probably, a distinction should be made between classic prion diseases and prion proteinopathies (Wadsworth, Asante et al. 2010).

In some cases, prions or prion-like aggregates, are the key neurotoxic intermediates. But in other cases, the toxicity of PrP goes beyond prions, and most likely depends on a subversion of the function of PrP^{C} (Solomon, Schepker et al. 2010).

In general, there are several toxic pathways that can be activated by the abnormal prion protein. These include corruption of PrP interactions at the cell surface, impairment of secretory protein

transport and axonal transport, dysregulation of proteostatic cellular responses and induction of endoplasmic reticulum stress, constitutive recruitment of downstream effectors, alteration of cell membrane properties, and possibly other that are yet to be identified (Medrano, Barmada et al. 2008; Senatore, Restelli et al. 2013; Hirsch, Hernandez-Rapp et al. 2014; Chiesa 2015). These mechanisms are likely to co-exist, but may contribute differently to pathogenesis in different prion diseases (Chiesa 2015).

This complex neurotoxic modality is probably one of the keys of the phenotypic heterogeneity of prion diseases, and has fundamental implications in therapy. Most current approaches to treatment of prion diseases aim at inhibiting accumulation of PrP^{Sc} (Trevitt and Collinge 2006). Identification of cellular pathways activated by neurotoxic forms of PrP or drugs that stabilize the native conformation of the protein would presumably allow development of more specific and effective therapies (Solomon, Schepker et al. 2010; Chiesa 2015).

3. THE PRION PROTEIN AND THE SYNAPSE

3.1 THE SYNAPSE

The synapse is the functional connection between neurons, or between a neuron and another type of cell. Within the CNS, a typical neuron possesses several thousand of these contacts. Most synapses connect axons to dendrites, but contacts may also be created between different parts of the neuron, generating axo-somatic, axo-axonic, and dendro-dendritic synapses. On the basis of the transmission mechanism, these structures are classified into electrical or chemical synapses, the latter being the most abundant in mammalian CNS.

Chemical synapses allow the transfer of information from a presynaptic to a postsynaptic cell and are therefore asymmetric in structure and function. The presynaptic terminal is a specialized area within the axon of the presynaptic cell that contains neurotransmitter enclosed in synaptic vesicles, as well as a number of other supporting structures and organelles, such as mitochondria and endoplasmic reticulum. Synaptic vesicles are organized into different pools on the basis of their availability to be released: the ready releasable pool, readily available on stimulation, the recycling pool, which maintains constant the number of releasable vesicles upon moderate stimulation, and the reserve pool, which contains the highest number of synaptic vesicles in the terminal and works as a storage from which vesicles can be mobilized only under certain conditions (Jahn and Fasshauer 2012).

Immediately opposite is a region of the postsynaptic cell containing neurotransmitter receptors. In excitatory synapses, this region consists of an elaborate complex of many different proteins called the postsynaptic density (PSD). Proteins in the PSD are involved in anchoring and trafficking neurotransmitter receptors and modulating their activity, as well as in organizing the signaling proteins which also participate to neurotransmission (Sheng and Kim 2011).

The release of neurotransmitter is triggered by the arrival of an action potential at the presynaptic terminal. The membrane depolarization induced by the action potential opens the voltage-gated calcium channels (VGCCs) resulting in a massive calcium influx into the terminal. Calcium ions then

induce the fusion of docked vesicles with the presynaptic membrane, and the release of their content into the synaptic cleft.

Receptors on the opposite side of the synaptic gap bind neurotransmitter molecules, responding in either of two general ways. First, the receptors may directly open ligand-gated ion channels in the postsynaptic cell membrane, causing ions to enter or exit the cell and changing the local transmembrane potential. These receptors are called ionotropic receptors. The resulting change in voltage is called a postsynaptic potential, and can be excitatory (resulting from membrane depolarization) or inhibitory (resulting from membrane hyperpolarization) depending on which kind of ion channel conducts the current. The second way a receptor can affect membrane potential is by modulating the production of second messengers. These receptors are called metabotropic receptors, and their responses are normally slower and more long-lasting compared to ionotropic receptors.

After being released, the neurotransmitter must be removed from the synaptic cleft to ensure proper duration of stimulation. This removal can occur by simple diffusion, by enzymatic cleavage or through reuptake into neurons and glia.

Synapses in the CNS are mainly integrative. This means that a single synaptic event alone is not able to trigger a response in the postsynaptic neuron. The overall response to synaptic stimulation depends on the integration of excitatory and inhibitory inputs which occur at the same time in the cell: only when the sum of excitatory postsynaptic potentials (EPSPs) overcomes the effect of the sum of inhibitory postsynaptic potentials (IPSPs) the threshold for initiating an action potential can be reached and the nervous signal propagated.

Excitatory synapses

An excitatory synapse is a synapse in which an action potential in the presynaptic neuron increases the probability of an action potential occurring in the postsynaptic cell. Excitatory synapses are usually situated on dendritic spines, specialized membrane protrusions in which receptors and PSDs are concentrated.

Glutamate is the most common excitatory neurotransmitter within the CNS, and it exerts its function by binding to both ionotropic and G protein-coupled metabotropic receptors.

NMDA and AMPA receptors are the two main kinds of ionotropic glutamate receptors. They have different physiological properties and often coexist at the same synapse.

AMPA receptors

AMPA receptors mediate the vast majority of fast excitatory neurotransmission, and are therefore the main responsible for signal propagation in the CNS.

They are composed of four types of subunits, GluA1 to GluA4, which combine to form tetramers. Most AMPA receptors are heterotetrameric, consisting of two dimers of GluA2 and either GluA1, GluA3 or GluA4, with the relative abundance of the different subunits varying across brain regions. Each subunit has an identical membrane topology. The amino terminus is extracellular, and there are three membrane-spanning domains, one re-entrant loop domain and an intracellular C-terminal domain. This C-terminal domain is a highly variable region that provides a platform for both the protein interactions and the post-translational modifications that regulate subunit-dependent trafficking and function (Schwenk, Baehrens et al. 2014; Henley and Wilkinson 2016).

AMPARs are permeable to cations such as sodium, potassium and calcium, and the selectivity of this permeability is governed by the GluA2 subunit. Specifically, the presence of GluA2 renders the channel impermeable to calcium. This is determined by post-transcriptional modification (RNA editing) of the arginine (R)/glutamine (Q) editing site of the GluA2 mRNA. Here, an adenosine is deaminated to form hypoxantine, changing the uncharged amino acid glutamine to the positively-charged arginine in the receptor's ion channel (Higuchi, Single et al. 1993). The presence of a positively-charged amino acid at this site hampers calcium influx through the pore. Most AMPARs in the adult brain contain the GluA2 subunit, and more than 95% of the GluA2 transcripts are edited. This means that the principal ions gated by AMPARs are normally only sodium and potassium (Isaac, Ashby et al. 2007; Mayer 2011).

However, GluA2-lacking, calcium-permeable AMPARs are also present, and may play a critical role in different processes. For example, they are highly expressed during early postnatal development and during the early phases of several forms of synaptic plasticity, and are constitutively present in certain neuronal subpopulations such as inhibitory interneurons (Geiger, Melcher et al. 1995; Kumar, Bacci et al. 2002; Henley and Wilkinson 2016).

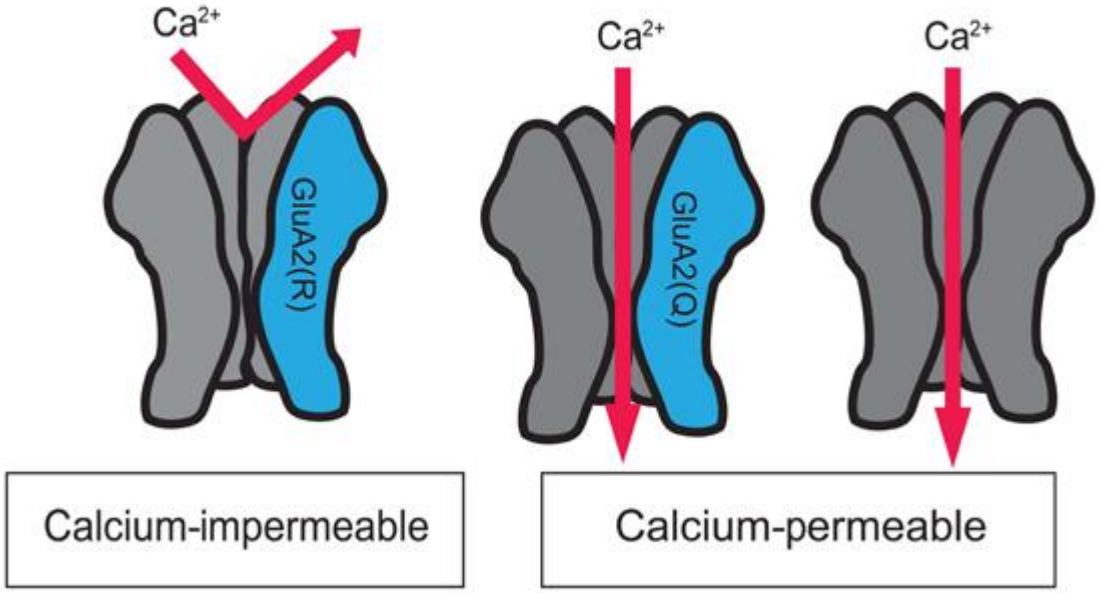


Figure 8- AMPA receptors. AMPA receptors are tetramers that can comprise any combination of GluA1–GluA4 subunits but usually contains GluA2. Receptors containing edited GluA2 are impermeable to calcium (left). AMPA receptors containing unedited GluA2 and AMPA receptors lacking GluA2 are calcium-permeable (right) (modified from Wright and Vissel 2012).

NMDA receptors

NMDA receptors are heteromeric complexes incorporating different subunits grouped in three main types: GluN1, GluN2 and GluN3. The GluN2 family comprises four different proteins (GluN2-A, -B, -C and -D) and the GluN3 family two (GluN3-A and -B). The receptor is formed as a tetramer with two obliged GluN1 subunits associated to two GluN2, or a mixture of GluN2 and 3 subunits (Paoletti, Bellone et al. 2013).

In contrast to AMPARs, NMDARs mediate a slow, indirect form of excitation which results from the modulation in the number and the strength of AMPARs (Horak, Petralia et al. 2014).

Activation of NMDARs requires the presence of both glutamate and a co-agonist (glycine or D-serine), which bind to the GluN2 and GluN1 subunits, respectively. However, NMDARs are silent under resting conditions. The ion channel is in fact normally blocked by a magnesium ion which can be removed only when the membrane is depolarized by concomitant postsynaptic activity. In other words, when a weak activation of the presynaptic neuron causes the release of glutamate from the axon terminal, the neurotransmitter binds to both AMPARs and NMDARs, but it is only

able to activate the former, resulting in a slight depolarization of the postsynaptic neuron. Given a stimulus of sufficient strength, AMPARs can depolarize the membrane sufficiently to expel the magnesium ion from the NMDAR channel, thereby activating the receptor.

NMDARs, like AMPA receptors, are non-specific cation channels, and their activation results in a flux of sodium, potassium and calcium. In particular, calcium entering the postsynaptic terminal through these receptors initiates various metabolic pathways that ultimately affect the strength of the synapse, a process known as synaptic plasticity (see below). Most commonly, these pathways result in changes in the number and properties of AMPARs (Horak, Petralia et al. 2014).

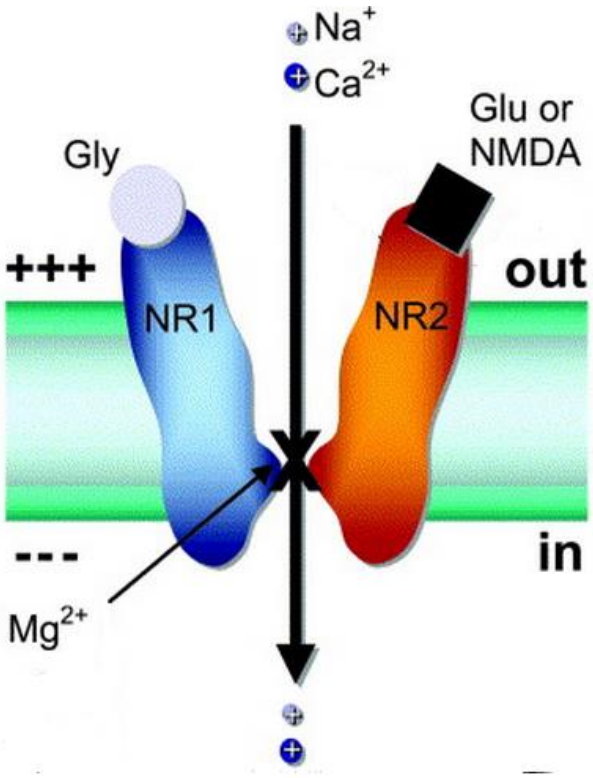


Figure 9- NMDA receptors. NMDARs contain two GluN1 and two GluN2 (or GluN3) subunits, which assemble as a tetramer. The binding sites for agonists (glutamate and NMDA) and co-agonist (glycine and D-serine) are shown. Note that the channel is blocked by a magnesium ion at resting membrane potentials.

Dendritic spines

Dendritic spines are membranous protrusions from the neuronal surface generally consisting of a head connected to the neuron by a thin neck. They are postsynaptic structures specialized for optimizing neurotransmission which receive inputs specifically from glutamatergic neurons. More than 90% of excitatory synapses in the CNS terminate on spines, which are mostly located on the dendrites (Nimchinsky, Sabatini et al. 2002).

The head of a spine contains the PSD, neurotransmitter receptors, cytoskeletal and adaptor proteins, and associated signaling molecules. The role of PSD is to concentrate the receptors close to the active zone of the presynaptic element and to organize the postsynaptic apparatus in order to maximize the efficiency of neurotransmission (Rochefort and Konnerth 2012). Segregation of the PSD in the spine serves to compartmentalize the neurotransmission apparatus to further optimize its function. The presence of a narrow neck, in fact, allows to concentrate signaling molecules and to impair diffusional exchanges between spine and the rest of neuron. This property is very suitable for guaranteeing the specificity of synapse activation, especially during the signal transduction events regulating single-synapse plasticity (Nimchinsky, Sabatini et al. 2002).

Dendritic spines are characterized by morphological diversity. The most common classification criterion divides spines into three major types: mushroom spines, which have a large head and a narrow neck, thin spines with a smaller head and a narrow neck and stubby spines, whit no conspicuous constriction between the head and the attachment to the neuron.

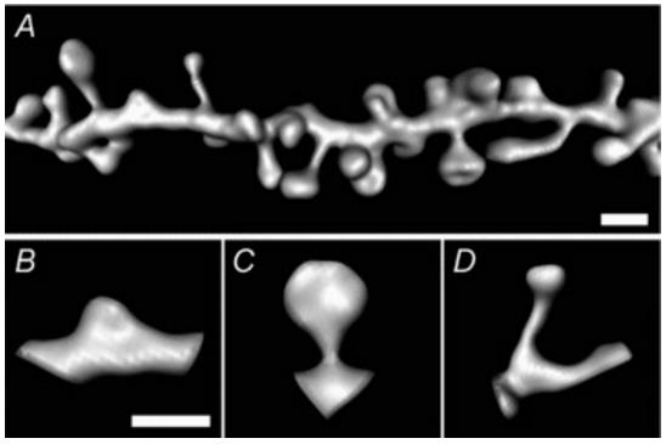


Figure 10- Diversity of spine shape. (A) 3D reconstruction of a dendrite of a hippocampal pyramidal neuron. Representative images of (B) stubby, (C) mushroom and (D) thin spines. Scale bar: 1 µm (from McKinney 2010).

Spines change significantly during development. Neonatal dendrites are relatively bare. Subsequently, the number of spines increases greatly and eventually a process of pruning produces a loss of spines to reach the mature spine density. In the adult brain, mushroom spines are the most abundant type. However, spine morphology does not necessarily correlate with spine maturity, which is rather associated with the presence of a well-developed inner apparatus (Nimchinsky, Sabatini et al. 2002).

Spines and synapses are dynamic structures that undergo continuous turnover throughout the whole life span: they are constantly formed and eliminated, and they undergo profound structural modifications as a function of synaptic activity. (De Roo, Klauser et al. 2008). Such plasticity tends to decrease with age: in adulthood most spines remain persistent, and the half-life of spines increases (Alvarez and Sabatini 2007).

Synaptic plasticity

One of the most important characteristics of synapses is their ability to respond to changes in the incoming patterns of stimuli by modifying their strengths. This fundamental property allows the continuous changes in the efficacy of neurotransmission and neuronal connectivity that are at the basis of the highly adaptable nervous system of vertebrates.

Synaptic plasticity exists in many forms which differ in terms of duration, triggering stimuli and molecular mechanisms. In all cases, changes in synaptic strength are achieved through modifications in two general parameters: the efficacy of neurotransmitter release at the presynaptic side and the sensitivity of the postsynaptic compartment, which in turn depends on modifications in the number, type and properties of neurotransmitter receptors (Anggono and Huganir 2012).

Particularly interesting for the magnitude of their effects and for their relevance in brain pathology are the durable forms of plasticity. They are in fact believed to be the molecular substrates of learning and memory, and alterations in these processes are present in many neurodegenerative conditions, including prion diseases (Johnston, Fraser et al. 1998).

Among the most studied forms of synaptic plasticity are Long-Term Potentiation (LTP) and Long-Term Depression (LTD), which affect synaptic strength in opposite directions. Induction of these forms of plasticity, also known as Hebbian plasticity, is associative in requiring correlated firing of the pre- and postsynaptic neurons and depends on the activation of NMDARs. The resulting change in synaptic strength is rapid, specific to individual synapses and can last from hours to months (Lee, Escobedo-Lozoya et al. 2009). Moreover, Hebbian plasticity produces positive-feedback processes. For example, once LTP is induced, potentiated synapses can undergo further potentiation with greater ease. This mechanism of course has developed to facilitate the reinforcement of significant stimuli, but without a proper counterbalance it may drive neuronal activity to saturation or to unstable states prone to hyperexcitation (Turrigiano 2008).

To prevent these side effects, other homeostatic forms of synaptic plasticity operate as compensatory, negative feedback mechanisms to maintain network stability (Vitureira and Goda 2013). In contrast to Hebbian plasticity, homeostatic plasticity is generally considered to be a slow process elicited by global changes in network's activity and inducing global modifications so that all the synapses of a given neuron are scaled equally. In this way, the overall excitability of the network is maintained within an optimal working range and the relative differences in synaptic strength resulting from Hebbian plasticity are preserved (Turrigiano 2008; Vitureira and Goda 2013).

3.2 SYNPATIC INVOLVMENT IN NEURODEGENERATION

Synapse loss: a key hallmark of neurodegeneration

Neurodegeneration is a complex phenomenon which results from the synergistic effect of several pathogenic mechanisms. Accumulation of neurotoxic substances, inflammation, oxidative stress, autophagy, protein degradation and mitochondrial dysfunction are only some examples, which are often present together in different neurodegenerative conditions. However, it remains unclear whether they are the cause of the disease or a consequence of the primary damage.

Whatever the triggers may be, synapse loss appears to be a major pathophysiological hallmark shared by all neurodegenerative diseases (Lu, Nagappan et al. 2013). More specifically, alterations

of the excitatory synapses are normally early events that strongly correlate with the symptomatology even when neuronal death is not yet evident. For this reason, neurodegenerative disorders are included in the heterogeneous group of the so-called “synaptopathies”, a term which refers to brain disorders which arise from synaptic dysfunction (Lepeta, Lourenco et al. 2016).

This, of course, applies also to prion diseases and is supported by a wealth of evidence. For instance, synaptic changes correlate with early behavioral signs in scrapie infected mice (Jeffrey, Halliday et al. 2000), and local accumulation of PrP^{Sc} is associated with loss of presynaptic compartments and dendritic atrophy before neuronal death occurs (Clinton, Forsyth et al. 1993). Moreover, synaptic alterations have been described in several sporadic or inherited cases of CJD (Ferrer 2002).

Other studies reported that impaired synaptic responses associated with early cognitive, behavioral and motor deficits occur before extensive PrP^{Sc} deposition and neuronal loss (Cunningham, Deacon et al. 2003; Senatore, Colleoni et al. 2012), and depend on defective depolarization-induced glutamate exocytosis due to altered calcium influx (Senatore, Colleoni et al. 2012). Interestingly, it was also observed that deletion of the pro-apoptotic gene Bax prevents the death of cerebellar granule neurons in a mouse model of inherited prion disease (Tg(PG14) mice), but does not affect the development of clinical symptoms, nor synaptic shrinkage and loss (Chiesa, Piccardo et al. 2005), reinforcing the hypothesis that pathological signs are somehow independent of neuronal loss at least in the first stages of the disease.

On the other hand, it is now widely agreed upon that synaptic activity is also essential to promote neuronal health and survival (Bell and Hardingham 2011), suggesting that the relationship between synaptic dysfunction and neurodegeneration may not be simply correlative, but causal.

It is known, for instance, that neurotransmission can activate neurotrophic signaling pathways, the blockade of which may result in a dangerous lack of survival stimuli (Mennerick and Zorumski 2000). In some cases, the situation is more complex. For example, NMDARs may activate either neurotrophic or apoptotic cascades depending on their localization: whereas synaptic receptors transduce pro-survival signals, extrasynaptic receptors are coupled to neurotoxic pathways. In this case, thus, is the balance in receptor distribution to be critical for determining neuronal fate (Hardingham and Bading 2010), and the loss of synaptic sites may clearly alter this balance.

Calcium-permeable receptors and excitotoxicity

If, on the one hand, loss of synaptic activity is emerging as a major determinant of neuronal pathology in neurodegeneration, on the other hand an excessive, uncontrolled activation of the excitatory synapses can be detrimental for neuronal survival just as much.

Excitotoxicity is the pathological process by which neurons are killed by the toxic action of excitatory neurotransmitters such as glutamate, and indeed, many lines of evidence demonstrate an increased or deregulated glutamatergic transmission in several neurodegenerative diseases (Dong, Wang et al. 2009).

Overactivation of glutamate receptors can damage neurons in several ways. For example, acute excitotoxicity is thought to be mediated by an osmotic imbalance occurring when high amounts of water follow the exaggerated sodium and chloride influx caused by a sustained membrane depolarization (Dong, Wang et al. 2009).

However, the most relevant mechanism of excitotoxicity is the disruption of cellular calcium homeostasis. This is a direct consequence of the excessive calcium influx through NMDARs and calcium-permeable AMPARs. Uncontrolled rises in intracellular calcium levels result in inappropriate activation of calcium-dependent processes that are normally dormant or operate at low levels, causing metabolic impairment and eventually cell death. Typically, rapid increases in calcium loads activate proteases, lipases and other catabolic enzymes that trigger neurotoxic signaling cascades. These events include mitochondrial membrane depolarization, caspase activation and generation of oxygen and nitrogen free radicals which ultimately lead to apoptosis or necrosis (Arundine and Tymianski 2003; Dong, Wang et al. 2009).

3.3 PrP AS A REGULATOR OF SYNAPTIC FUNCTION

The idea that PrP is a synaptic protein is supported by several observations.

The fact itself that PrP is predominantly located at both pre- and the postsynaptic terminals suggests that it may be involved in regulating synaptic activity (Fournier 2008; Steinert 2015).

Moreover, PrP complex interactome comprises many synaptic proteins, some of which are directly involved in synaptic transmission. They include the pre-synaptic synapsin 1 and synaptophysin, many neurotransmitter receptors such as ionotropic and metabotropic glutamate receptors, serotoninergic receptors and the nicotinic α7 receptor, and ion channels such as VGCCs (Fournier 2008; Senatore, Restelli et al. 2013; Hirsch, Hernandez-Rapp et al. 2014). Although not directly involved in neurotransmission, other interactors have a well-established synaptic role, such as the adhesion molecule NCAM and the signaling protein Fyn (Fournier 2008; Um, Nygaard et al. 2012; Hirsch, Hernandez-Rapp et al. 2014; Ochs and Malaga-Trillo 2014).

PrP and neurotransmission

In addition to the general considerations regarding PrP localization and interactions, a wealth of experimental data indicates a direct role of this protein in regulating neurotransmission.

Most evidence comes from studies in PrP knock-out models. Notably, the only clear abnormalities that were found in these animals were always ascribable to alterations in neurotransmission or to correlated behavioral traits.

For example, one of the first studies assessing synaptic function in PrP knock-out mice found a reduction in the amplitude of hippocampal inhibitory postsynaptic currents and a depolarizing shift in their reversal potential, suggesting a weakened $GABA_A$ receptor-mediated fast inhibition which could explain the epileptic-like activity seen in CJD patients (Collinge, Whittington et al. 1994).

Another general evidence was obtained in a study showing a direct correlation between PrP^C expression levels and the entity of evoked excitatory synaptic responses. This finding also entails that changes in the amount of PrP^C may influence hippocampal synaptic transmission (Carleton, Tremblay et al. 2001). Accordingly, defects in hippocampal LTP in PrP-deficient mice were found in

multiple studies (Collinge, Whittington et al. 1994; Maglio, Martins et al. 2006; Rangel, Madronal et al. 2009).

In addition, a reduction of slow afterhyperpolarization, a specific component of the hyperpolarizing phase of a neuron which can be evoked by trains of action potentials, was observed following acute knock-out of PrP, supporting the idea of a direct role of the protein in the modulation of synaptic transmission (Mallucci, Ratte et al. 2002).

Some behavioral aspects that can be considered important functional correlates of abnormal synaptic transmission have also been reported. In addition to the already mentioned alterations in circadian rhythm and olfactory behavior, PrP knock-out mice exhibit specific cognitive deficits in hippocampal spatial learning which can be rescued by restoring PrP expression in neurons (Criado, Sanchez-Alavez et al. 2005). Even more interesting was the finding that the M129V polymorphism affects long-term memory in humans, with individuals carrying the MM genotype performing better than those with the VV genotype (Papassotiropoulos, Wollmer et al. 2005).

More recently, other works have suggested specific synaptic roles for PrP, two examples of which are described below.

The first example indicates a direct presynaptic involvement in neurotransmission. A study from Senatore et al. demonstrated that PrP interacts with the VGCCs α2-δ1 subunit, an important component which promotes the anterograde trafficking of the channel. The authors also demonstrated that a mutant variant of the protein, the PG14 PrP, is retained in the endoplasmic reticulum, and its interaction with α2-δ1 subunit determines the intracellular retention of the whole channel complex, resulting in reduced membrane delivery of the VGCCs. This is responsible for the lower depolarization-induced calcium influx and the consequent low glutamate release observed in Tg (PG14) mice, and is thought to be at the origin for early motor and behavioral symptoms in these animals (Senatore, Colleoni et al. 2012).

A second interesting example, on the contrary, suggests a postsynaptic function for PrP. In a work from Koshravani et al, PrP deficiency was found to be associated to a strong enhancement of synaptic NMDAR currents, which displayed greater amplitude and prolonged decay time. PrP was also found to co-immunoprecipitate with the GluN2D subunit of the receptor, indicative of a direct role of the protein in downregulating NMDAR activity. Since PrP-deficient neurons were found to be more susceptible to NMDAR-mediated cell death, these data suggest that the putative

neuroprotective function of PrP may be exerted by inhibiting NMDAR excitotoxic potential (Khosravani, Zhang et al. 2008).

Pathological consequences

The concept that PrP is a synaptic protein supports the idea that prion diseases may originate from synaptic dysfunction, and neurodegeneration may follow as a consequence of disrupted neuronal activity.

Indeed, a wealth of data demonstrates that synapse loss is one of the first neuropathological events in prion diseases. Dendritic abnormalities and spine loss have been observed early during the pre-symptomatic phase of the disease, in the absence of evident PrP^{Sc} deposition (Jeffrey, Halliday et al. 2000; Cunningham, Deacon et al. 2003; Fuhrmann, Mitteregger et al. 2007).

This suggests a possible pathological scenario in which abnormal PrP may start to perturb synaptic function early, when morphological alterations are not yet evident. Notably, these very early modification can be reversed (Mallucci, White et al. 2007). Subsequently, increasing accumulation of abnormal PrP would disrupt membrane properties, destabilizing synaptic structure and leading to synapse depletion. In this case, a loss of the trophic action of functional synapses would overlap with the consequences of the subversion of the physiological, neuroprotective function of PrP (see above).

At the same time, alterations of the neurotransmission system would give rise to early symptoms and, later on, to excitotoxic phenomena. For example, the impairment in VGCCs delivery to the pre-synaptic membrane induced by abnormal PrP would lead to weakened glutamatergic transmission, which is likely responsible for motor and behavioral dysfunctions. On the long term, this could also induce compensatory phenomena which may in turn increase VGCC-independent glutamate release. This scenario may be further worsened by the concomitant loss of the physiological function of PrP, which would result in increased NMDAR function and impaired inhibitory GABAergic transmission. All these factors may cooperate synergistically to predispose neurons to excitotoxicity.

The increasing evidence that synapses are privileged targets in prion diseases and that synaptic alterations are the earliest pathological events, open up new avenues for early diagnosis and treatment.

The intrinsic plasticity of these structures is a very appealing property to be targeted, and, indeed, synapse loss has been clearly demonstrated to be reversible, at least in the first stages of the disease (Mallucci, White et al. 2007). Of course, the possibility to intervene at this level requires a full understanding of the specific pathological mechanisms involved.

The study of synaptic dysfunction in prion diseases is still a relatively young field of research, but is well worth pursuing. A deeper understanding of these mechanisms would allow to design specific therapeutic strategies aimed at targeting, if not the primary cause of the disease, the key contributor, maybe taking advantage of already existing molecules which have been developed to target synaptic dysfunction in other diseases. Moreover, identification of early dysfunction would allow to recognize the disease before the terminal situation represented by the full blown clinical symptoms, at a stage in which rescue is still possible.

AIM OF THE WORK

Compromised synaptic function plays a determinant role in the pathogenesis of prion diseases. Understanding the mechanisms of synaptic dysfunction is therefore fundamental for improving our understanding of these pathologies and for devising efficient diagnostic and therapeutic strategies.

In the present work, we investigated this aspect by taking advantage of two different murine models of genetic prion diseases.

Fatal familial insomnia (FFI) and a familial form of the Creutzfeldt-Jakob disease (CJD) are clinically distinct diseases linked to the same point mutation in the PrP gene, the D178N. The specific phenotypes are determined by the M129V polymorphism which is thought to influence D178N PrP misfolding, leading to the formation of distinct prion strains with different neurotoxic properties (Bouybayoune, Mantovani et al. 2015). Specifically, FFI is characterized by severe sleep alterations, autonomic dysfunction and selective neurodegeneration in the thalamus. CJD is a subacute encephalopathy characterized by a rapidly progressive dementia, motor abnormalities and a diffused spongiform degeneration mostly involving the cortex, the cerebellum and the striatum.

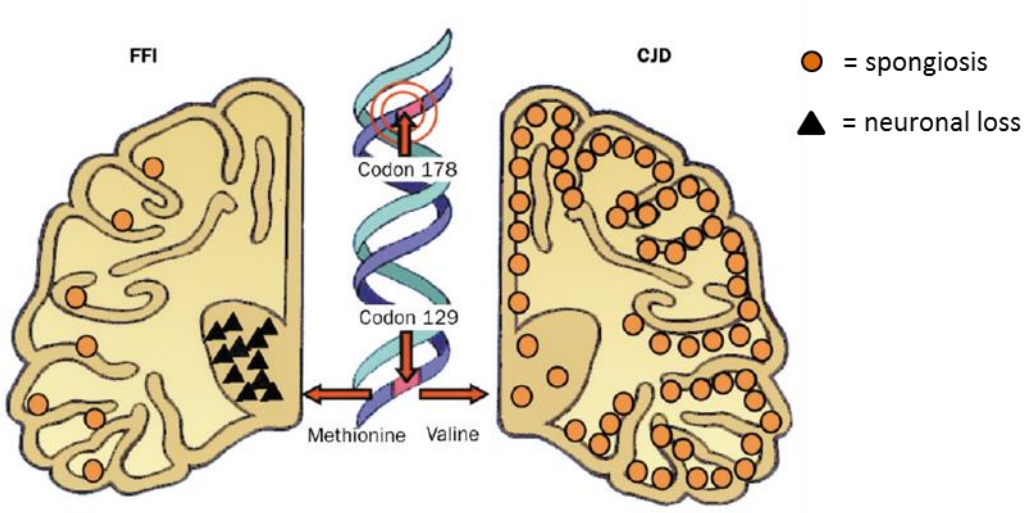


Fig 11- The D178N mutation Schematic drawing representing the main neuropathological differences between the two possible phenotypes of the D1778N mutation in relation to the 129 polymorphism. Note the selective neuronal loss in the thalamus caused by the FFI variant D178N/129M (left) and the diffused corticobasal spongiosis associated to the CJD variant D178N/129V (right). (from Montagna 2003)

Recently generated transgenic mice expressing the murine homologs of these two variants (D177N/128M and D177N/128V) have been shown to recapitulate the key features and the phenotypic differences seen in humans (Dossena, Imeri et al. 2008; Bouybayoune, Mantovani et al. 2015), but very little is known regarding the synaptic effects of the mutations.

Notably, the mutant PrPs expressed in these mice are not infectious, indicating that the proteins possess disease-encoding properties that are independent of their ability to propagate the misfolded conformation (Bouybayoune, Mantovani et al. 2015). This feature is particularly suitable for our purpose, because it allows to isolate the neurotoxic mechanisms of abnormal PrP from the confounding effects of prion replication.

Several pieces of evidence indicate that PrP may be directly involved in synaptic transmission, and this role may be altered in the disease-causing protein. For example, we have recently demonstrated that a different PrP mutation affects the trafficking of the protein causing its retention in intracellular compartments. Due to its ability to interact with VGCCs, the mutant protein impairs the correct delivery of the channels to presynaptic terminals, resulting inefficient glutamatergic neurotransmission (Senatore, Restelli et al. 2013).

Studies from other groups demonstrated that PrP engages functional interactions with other proteins that are important for synaptic function, such as glutamate receptors (Khosravani, Zhang et al. 2008; Watt, Taylor et al. 2012).

Given that also the two variants of the D177N PrP are retained intracellularly (Ivanova, Barmada et al. 2001; Fioriti, Dossena et al. 2005; Dossena, Imeri et al. 2008; Bouybayoune, Mantovani et al. 2015), we asked whether a similar mechanism of interaction and retention could apply also to glutamate receptors. To answer this question, we performed confocal analyses and electrophysiological recordings on primary hippocampal neurons from FFI and CJD mice. We aimed at carrying out a detailed study of the interaction of the mutant proteins with AMPA and NMDA receptors, and of the effects of these proteins on the postsynaptic components of excitatory neurotransmission. Furthermore, we wanted to verify whether the two mutants differed in their effects on synaptic function, because the presence of specific alterations may account at least in part for the phenotypic heterogeneity seen in these diseases.

MATERIALS AND METHODS

REAGENTS

For cell culture, we used Hank's Balanced Salt Solution (HBSS), Neurobasal-A medium, Minimal Essential Medium (MEM), Dulbecco's modified Eagle's medium (DMEM) Basal Medium Eagle (BME) and B-27 supplement, that were supplied by Invitrogen (GIBCO). Ultraglutamine and Penicillin-Streptomicyn (PenStrep) were supplied by Lonza, Glutamax by ThermoFisher, polylysine, papain, trypsin, trypsin inhibitors and aphidicolin by Sigma. Fetal bovine serum (FBS) was acquired from HyperClone (primary hippocampal neurons) or Sigma (HeLa cells and cerebellar granule neurons).

Transfections were carried out by using Lipofectamine®2000 purchased from Invitrogen. P-EGFP-C1 plasmid was provided by Clontech. Plasmids encoding WT, D177N/128V (CJD) and D177N/128M (FFI) PrP constructs containing a monomerized version of EGFP (enhanced green fluorescent protein) inserted after codon 34 of mouse PrP were provided by Roberto Chiesa (Mario Negri Institute for Pharmacological Research) and have been described previously (Massignan, Biasini et al. 2010). The AMPA receptor cDNA for GluA2 (R, flop) long C-term in pCDNA3 vector for expression and the NMDA receptor cDNA for GluN1 in CMV-pRK 7 vector were kindly provided by Professor Peter Seeburg (Max-Planck-Institute for Neurological Research, Heidelberg, Germany).

For immunofluorescence, we used phosphate buffered saline (PBS) and goat serum purchased from Lonza and Invitrogen (GIBCO) respectively.
Primary antibodies were supplied by Synaptic System (anti- Bassoon, anti-Shank2, anti-GluA2, anti-GluN1 and PanAMPA) or Sigma (anti-Tubulin). Secondary antibodies were all purchased from Invitrogen (Alexa Fluor). 4-6-diamidino-2-phenylindole (DAPI) was acquired from Invitrogen.

All drugs used for electrophysiology and calcium imaging were provided by Tocris, except for Fura-2 which was obtained by ThermoFisher. Borosilicate glass capillaries were purchased from World Precision Instruments.

Coimmunoprecipitation was performed by using Igepal, protease inhibitor mixture purchased by Sigma, IgG-conjugated Dynabeads® supplied by Dynal, and immunoglobulin-free BSA acquired from Sigma. Primary anti-PrP antibody 94B4 was donated by Jan Langeveld (Virologie Immunologie Moléculaires, UR892, INRA, Jouy-en-Josas, France). Protein quantification was carried out by using BCA assay provided by Pierce.

For Western blot, we used Tween®20 and Tris obtained from VWR International. Primary anti-GluR1 and anti-GluR2 antibodies were purchased from Synaptic System. Secondary antibodies were acquired from GE Healthcare. Nitrocellulose membranes were purchased by BioRad, and Chemiluminescent ECL kit was purchased by Amersham Biosciences.

For toxicity assays we used Hoechst and propidium iodide (PI) acquired from Sigma or the CytoTox96® assay from Promega.

Other chemicals were of the purest grade available from regular commercial sources.

EXPERIMENTAL PROCEDURES

Animals

Procedures involving animals and their care were conducted in conformity with the institutional guidelines at the IRCCS – Mario Negri Institute for Pharmacological Research in compliance with national (D.lgs 26/2014; Authorization n. 19/2008-A issued March 6, 2008 by Ministry of Health) and international laws and policies (EEC Council Directive 2010/63/UE; the NIH Guide for the Care and Use of Laboratory Animals, 2011 edition). They were reviewed and approved by the Mario Negri Institute Animal Care and Use Committee that includes ad hoc members for ethical issues, and by the Italian Ministry of Health (Decreto nr 212/2016-PR). Animal facilities meet international

standards and are regularly checked by a certified veterinarian who is responsible for health monitoring, animal welfare supervision, experimental protocols and review of procedures.

All efforts were made to minimize the number of subject used and their suffering and housed in cages with free access to food and water at 22°C and with a 12-hours alternating light/dark cycle.

We used C57 black 6 (C57BL/6) mouse strain as control animals, whereas mutant neurons and tissues were obtained from transgenic mice expressing the D177N/128M and the D177N/128V mutations, referred to as Tg(FFI) and Tg(CJD) mice respectively. The production of these mice is reported in (Ref. Dossena 2008 and Boubayoune 2015). These animals are maintained on a C57BL/6J/Prnp0/0 (PrP knock-out) background, therefore they express transgenic but not endogenous wild-type PrP.

Cell cultures

Primary hippocampal neurons were obtained from 2-4-days old pups.
Animals were sacrificed and hippocampi were isolated under surgical stereomicroscope. Separated tissues were digested with Papain (200 U/ml) in CNDM medium (5.8 mM $MgCl_2$, 0.5 mM $CaCl_2$, 3.2 mM HEPES, 0.2 mM NaOH, 30 mM K_2SO_4 and 90 mM Na_2SO_4. pH 7.4, 292 mOsm) supplemented with 0.4% glucose for 30 min at 34°C. Enzymatic activity was subsequently blocked by incubating the tissue with tripsin inhibitors (10 μg/ml) in CNDM plus 0.4% glucose for 45 min at room temperature (RT). Dissociation was performed in MEM supplemented with 10% FBS and 0.4% glucose. Cells were then spinned down at 1500 rpm for 2 min, resuspended in MEM with 10% FBS, 0.4% glucose and PenStrep (100 U/ml) and plated on polylisine-coated 18 or 24 mm round coverslips. The total number of plated cells was adjusted to 130.000 and 260.000 per each 18 mm and 24 mm coverslip, respectively. Culture medium was prepared by adding B-27 supplement (2%), PenStrep (100U/ml) and glutamine (200 mM) to Neurobasal-A. Medium was changed after 7 days in culture.

Cerebellar granule neurons were obtained from 6 days old pups. Cerebella were dissected, sliced into ~1-mm pieces and incubated in HBSS containing 0.3 mg/ml trypsin at 37 °C for 15 min. Trypsin inhibitor was added to a final concentration of 0.5 mg/ml, and the tissue was mechanically dissociated. Cells were plated at 350–400,000 cells/cm^2 on polylysine-coated coverslips. Cells were maintained in BME supplemented with 10% FBS, PenStrep (100U/ml), and KCl 25 mM. To reduce

the number of non-neuronal cells, aphidicolin (3.3 µg/ml) was added to the medium 36 h after plating. Medium was replaced every 2 days.

HeLa cells were grown in a 1:1 mixture of DMEM plus Glutamax and αMEM plus Glutamax, supplemented with 10% FBS, nonessential aminoacids and PenStrep, and maintained at 37°C in an atmosphere of 5% CO_2, 95% air. When cells reached 90% confluence in 75-cm^2 flask, they were split by trypsinization. Medium was removed and after washing in PBS, cells were incubated with 1.5 ml of trypsin/EDTA solution for 3 minutes at 37°C. Trypsin was inhibited by adding 10 ml of complete medium, and the cell suspension transferred in a 15 ml sterile tube and centrifuged at 900 rpm for 5 minutes. The cell pellet was re suspended in fresh medium and the cell suspension was diluted and seeded in 75-cm^2 flasks.

All cells were maintained at 37 °C in an atmosphere of 5% CO_2, 95% air.

Transfection

For dendritic spine analysis, primary neurons were transfected at days in vitro (DIV) 11-DIV 13 with GFP-expressing plasmids.

For each coverslip, 0.5 µg of DNA were combined with 2.5 µl of Lipofectamine to a final volume of 90 µl in Neurobasal-A medium, and the mix was incubated for 20 minutes at RT. Culture medium was removed from neurons and 210 µl of Neurobasal-A were added to each coverslip. DNA-lipofectamine complexes were then applied to neurons in a total transfection volume of 300 µl, and cells were incubated for 30 minutes at 37°C. Transfection medium was eventually replaced with the old culture medium.

For AMPA and NMDA receptors localization analysis HeLa cells were co-transfected with either WT or mutant PrP and GluA2 or GluN1.

HeLa cells were transfected with the cDNA combinations stated above using the ratio PrP-EGFP (WT or FFI or CJD): GluA2 or GluN1 = 0.06: 0.47. Transfection was performed with Lipofectamine according to the manufacturer's instructions. Briefly, 0.25 µg DNA and 0.5 µl Lipofectamine were used to prepare the DNA-lipid complexes in 8-well micro-slides (Ibidi). Cells suspended in culture medium were subsequently added at a density of 20.000 cells per well. Following transfection, the AMPAR antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or the NMDAR antagonist 2-

amino-5-phosphonopentanoate (AP5) were added to the medium to prevent glutamate-induced excitotoxicity.

Immunofluorescence staining

Neurons were used for immunofluorescent staining on DIV 13-DIV15.

First, cells were fixed in paraformaldehyde 4% for 8 minutes and washed in PBS twice for 10 minutes.
Then, they were washed 3 times for 5 min in low salt and high salt buffer, and incubated for 30 minutes in goat serum dilution buffer (GSDB) for blocking non-specific reactivity and for permeabilization. Primary antibodies were diluted in GSDB, incubated with cells for 2 hours at RT and used at the following concentrations: anti-Bassoon (guinea pig) 1:300; anti-Shank2 (rabbit) 1:500; anti-Tubulin (rabbit) 1:200.

Afterwards, coverslips were washed in high salt buffer 3 times for 10 minutes and incubated with the corresponding secondary antibodies diluted 1:200 in GSDB for 45 minutes at RT.
Coverslips were washed again 3 times for 5 minutes first in high salt buffer, then in low salt buffer and one last time in phosphate buffer and eventually mounted onto slide glasses with Mowiol® mounting solution.

For staining of surface AMPARs, living neurons were incubated with a monoclonal antibody directed against the extracellular portion of the receptors (PanAMPA, mouse, 1:100) dissolved in Krebs-Ringers-Henseleit solution (KRH, see below) for 5 min at 37°C and washed 3 times with fresh KRH. Cells were subsequently fixed and stained as described above.

Immunofluorescence staining of HeLa cells was performed 48 h after transfection.

Cells were washed in PBS and fixed with 200 mM Hepes/NaOH pH 7.4 for 10 minutes at RT. After washing with PBS and incubation in blocking solution (50mN NH_4Cl, 1% bovine serum albumine, 10% FBS and 0.1% saponin in PBS pH 7.4 for AMPA receptors or 5% FBS, 0.2% bovine serum albumine and 0.2% TritonX-100 in PBS pH 7.4 for NMDA receptors) cells were incubated for 1 hour at RT or overnight at 4°C with the primary antibody. Anti-GluA2 (rabbit, 1:500) and anti-GluN1 (mouse monoclonal, 1:1000) were used.

After three washing steps in PBS and incubation secondary antibodies (1:500) for 1 hour at RT, nuclei were stained by exposing cells to 300 nM (DAPI) in PBS for 10 min at RT.

Confocal microscopy

Morphological analyses of primary neurons and HeLa cells were performed on an Olympus FV1000 confocal microscope equipped with 458, 488 and 515 nm Argon lasers and 405, 559, 594 and 635 diode lasers, and the associated software FV10-ASW.
For dendritic spines analysis images of proximal dendrites were acquired with a 60X oil immersion objective using a scan format of 1024x1024 pixels.
Each image consisted of a stack of images taken through the *z*-plane of the cell. Microscope settings were kept constant in each experiment.
Spines were manually classified with the NeuronStudio software, and density was calculated as number of spines/µm.

The analysis of synaptic markers was carried out on single plan images using ImageJ software (National Institute of Health, USA). Pixel size was 115 nm x 115 nm. The minimum puncta size was set to 3 pixels (0.034 μm^2). Colocalization of two selected markers was measured using the Boolean function "and" for the selected channels. The resulting image was binarized and used as a colocalization mask to be subtracted to single channels. The number of puncta resulting from colocalization mask subtraction were calculated for each marker. A colocalization fraction was set as colocalizing puncta/total puncta number.

The analysis of synaptic density was performed on clusters lying along dendritic branches. Density was calculated as the number of either single channel or colocalizing puncta/µm.

Coimmunoprecipitation

For coimmunoprecipitation (co-IP) from hippocampus, the tissue was homogenized in ice-cold co-IP buffer (75 mM NaCl, 1% Igepal, protease inhibitor mixture, 50 mM Tris-Cl, pH 7.4). After centrifugation at 1000 x g for 5 minutes, the supernatant was recovered and protein content quantified by BCA assay. 500 µg of protein was diluted in 1 ml of co-IP buffer and after five passages through a 23-gauge needle and incubation at 4°C for 30 minutes on a rotating wheel, the sample was centrifuged at 1000 x g for 3 minutes to remove the non-soluble material. The supernatant was transferred into a new tube and incubated for 1 hour at RT with 20 µl of anti-

mouse IgG-conjugated Dynabeads. The lysates were then incubated overnight at 4°C with 25 µl/ml of anti-mouse IgG-conjugated Dynabeads coated with the primary antibody anti-PrP 94B4. Primary antibody (1.5 µg) was incubated with 25 µl of anti-mouse IgG-conjugated Dynabeads for 2 hours at RT in PBS plus 0.1% immunoglobulin-free BSA. After three washes with 150 mM NaCl, 0.5% Igepal, 50 mM Tris-Cl, pH 7.4, immunoprecipitated proteins were eluted by 20 µl DTT-containing Laemmli sample buffer and analyzed by Western blot.

Western blot

30 or 50 µg of proteins were separated on standard 10-15% SDS-PAGE. For Western blotting, gels transferred to nitrocellulose filters were first blocked for 1 h with 5% nonfat dry milk in PBS, and then incubated over night with the primary antibodies diluted in PBS with 5% nonfat dry milk (1:1000 for anti-GluR1 and anti-GluR2, 1:2500 for anti-PrP 94B4) washed three folds for 10 minutes in Phosphate Buffered Saline with Tween (PBST), incubated for 1 hour with the peroxidase-conjugated anti-rabbit secondary antibody (1:5000), washed again in PBST as described above and once in PBS. Photographic development was by chemiluminescence. Western blot bands were quantified by the ImageJ program.

Toxicity assay

For toxicity assessment cultures were exposed to the excitotoxic agents for 24 hours. Following the stimulation period cell death was determined either with the DAPI/PI method or by measuring lactate dehydrogenase release (LDH).

For DAPI/PI staining cells were incubated with with Hoechst (10 µg/ml) and PI (2 µg/ml) for 30 min and mortality was calculated as PI/Hoechst positive nuclei.

To measure LDH release, the CytoTox96® kit was used according to the manufacturer's instructions.

Electrophysiology

Voltage-clamp whole cell recordings were obtained from cultured neurons on DIV 13-DIV16 under visual guidance using fluorescence and transmitted light illumination.
Extracellular solution (KRH) contained 125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM HEPES sodium salt, 2 mM $CaCl_2$ and 6 mM glucose.

For standard miniature excitatory postsynaptic currents (mEPSCs) recordings, patch pipettes (2.5-4.5 MΩ resistance) made from borosilicate glass were filled with a potassium gluconate-based solution containing 10 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES sodium salt, 130 mM potassium gluconate, 1 mM ethylene glycol tetraacetic acid (EGTA), 4 mM Mg-ATP and 0.3 mM Tris-GTP. For rectification index experiments we used cesium gluconate-based solution containing 130 mM CsOH, 8 mM CsCl, 2 mM NaCl, 10 mM HEPES sodium salt, 4 mM EGTA, 4 mM Mg-ATP and 0.3 mM Tris-GTP and 120 µM spermine.
Synaptic AMPA receptor-mediated currents were measured by holding neurons at a membrane potential of -70 mV in the presence of 1 µM tetrodotoxin (TTX) to block action potentials, 20 µM bicuculline and 20 µM AP5 to block GABA and NMDA receptors respectively.
Rectification experiments were carried out in extracellular solution containing TTX 10 nM, bicuculline 20 µM and AP5 100 µM. AMPAR-mediated responses were first recorded at -70 mV and then at +50 mV.

To measure NMDAR-mediated currents, cells were kept in Mg^{2+} -free KRH in the presence of 1 µM TTX, 20 µM bicuculline, 10 µM 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), 1 µM strychnine and 20 mM glycine. 200 mM NMDA was applied locally using a Perfusion Fast-Step System SF-77B (Warner Instruments, Hamden CT, USA) during 5 sec and then washed.

Recordings were performed at RT in voltage clamp mode using an Axopatch 200b or a Multiclamp 700B amplifier (Molecular devices) and pClamp-10 software (Axon Instruments). Series resistance ranged from 5 to 20 MΩ and was monitored for consistency during recordings. Cells with leak currents >200 pA, V_m> -40 mV and mEPSCs frequency < 0.2 Hz were excluded from the analysis. Signals were amplified, sampled at 10 kHz and filtered to 4 KHz, and acquired using pClamp 10 data acquisition program.
Analyses were carried out using Clampfit-10.6 software. Traces were lowpass filtered at 1 KHz. mEPSCs amplitude and frequency were automatically calculated, and rectification index was determined as the ratio of current amplitude at +40 mV and -70 mV.

Whole-cell NMDA currents were calculated as the maximum peak of the current divided by cell capacitance (current density).

Calcium Imaging

Hippocampal neurons of 13-16 DIV were loaded with 5 µM Fura-2 pentacetoxymethylester in culture medium for 45 min at 37 °C, washed in the KRH and transferred to the recording chamber of an inverted microscope (Axiovert 100; Zeiss, Oberkochen, Germany) equipped with a Polychrome IV (TILL Photonics, Germany). After excitation at 340 and 380 nm, the emitted light was acquired in selected regions of interest (ROI) corresponding to neuronal somata at 505 nm at 1-2 Hz. The exposure time was set at 10 msec for 340 nm and 20 msec for 380 nm. A constant value corresponding to the emitted light at 340 nm excitation in the empty area of the coverslip was subtracted as a background. After a period for baseline acquisition, neurons were stimulated with 30 µM AMPA for 30 sec in the presence of 1 µM TTX, 1 µM Cd^{2+},100 µM AP5 and 20 µM nifedipine, or 50 mM KCl in the presence of 1 µM TTX and 100 µM AP5.

Statistical analysis

Statistical analyses were carried out using Excel (Microsoft, Redmond, WA, USA) or PRISM 5 software (GraphPad, Software Inc.). After testing whether data were normally distributed or not, the appropriate statistical tests were used (see figure legends). Data are presented as mean ± standard error of the mean (s.e.m.) from the indicated number of elements analysed. The differences were considered to be significant if $P<0.05$ and are indicated by an asterisk; those at $P<0.01$ are indicated by double asterisks; those at $P<0.001$ are indicated by triple asterisks.

RESULTS

Mutant PrP impairs membrane delivery of the GluA2 subunit of AMPA receptors

Deregulation of excitatory neurotransmission is a common and early hallmark of many neurodegenerative disorders, including prion diseases (Mallucci 2009; Miladinovic, Nashed et al. 2015). AMPA receptors are central elements of the excitatory synapse, as they mediate the majority of fast excitation in the CNS. It follows that alterations in their function or trafficking can be crucial in the pathogenesis of such conditions.

PrP was described to interact with AMPARs, and some evidence suggests that this interaction may influence the surface expression of the receptors (Watt, Taylor et al. 2012).

On these bases, we aimed at studying the relationship between AMPARs and two mutant forms of prion protein, the D177N/128V and the D177N/128M, linked to CJD and FFI, respectively.

First, we wanted to verify whether PrP mutations affected the ability of the protein to interact with the receptor. To this purpose, we performed coimmunoprecipitation on hippocampal homogenates of wild type, CJD and FFI mice of PrP and the two main subunits expressed at hippocampal synapses, GluA1 and GluA2 (Schwenk, Baehrens et al. 2014). As shown in fig. 12A, we observed that both the wild type and the mutant isoforms of the protein selectively interacted with the GluA2 but not with the GluA1 subunit.

Given that several mutations in the *PRNP* gene, including the two under investigation in this study, were shown to cause intracellular retention of the protein (Ivanova, Barmada et al. 2001; Fioriti, Dossena et al. 2005), and since previous work of our group demonstrated that the interaction of the mutant protein with the $\alpha_2\delta$-1 subunit of the voltage-gated calcium channels (VGCCs) impairs the membrane delivery of the receptor complex (Senatore, Restelli et al. 2013), we aimed to define whether a similar mechanism of retention could also apply to AMPARs.

To this end, we investigated the effect of our mutants on the cellular localization of GluA2. We co-transfected HeLa cells with plasmids encoding for either the wild type or the mutant PrP and

GluA2, and we analyzed the localization of the two proteins by confocal microscopy after immunofluorescent staining. Consistent with previous studies protein (Ivanova, Barmada et al. 2001; Fioriti, Dossena et al. 2005), most wild type PrP localized at the cell surface, whereas both the mutant isoforms were predominantly found in intracellular compartments. As for GluA2, we observed that it was efficiently expressed at the plasma membrane only in cells expressing WT PrP, whereas in CJD and FFI PrP-expressing cells most GluA2 signal localized intracellularly (fig. 12B).

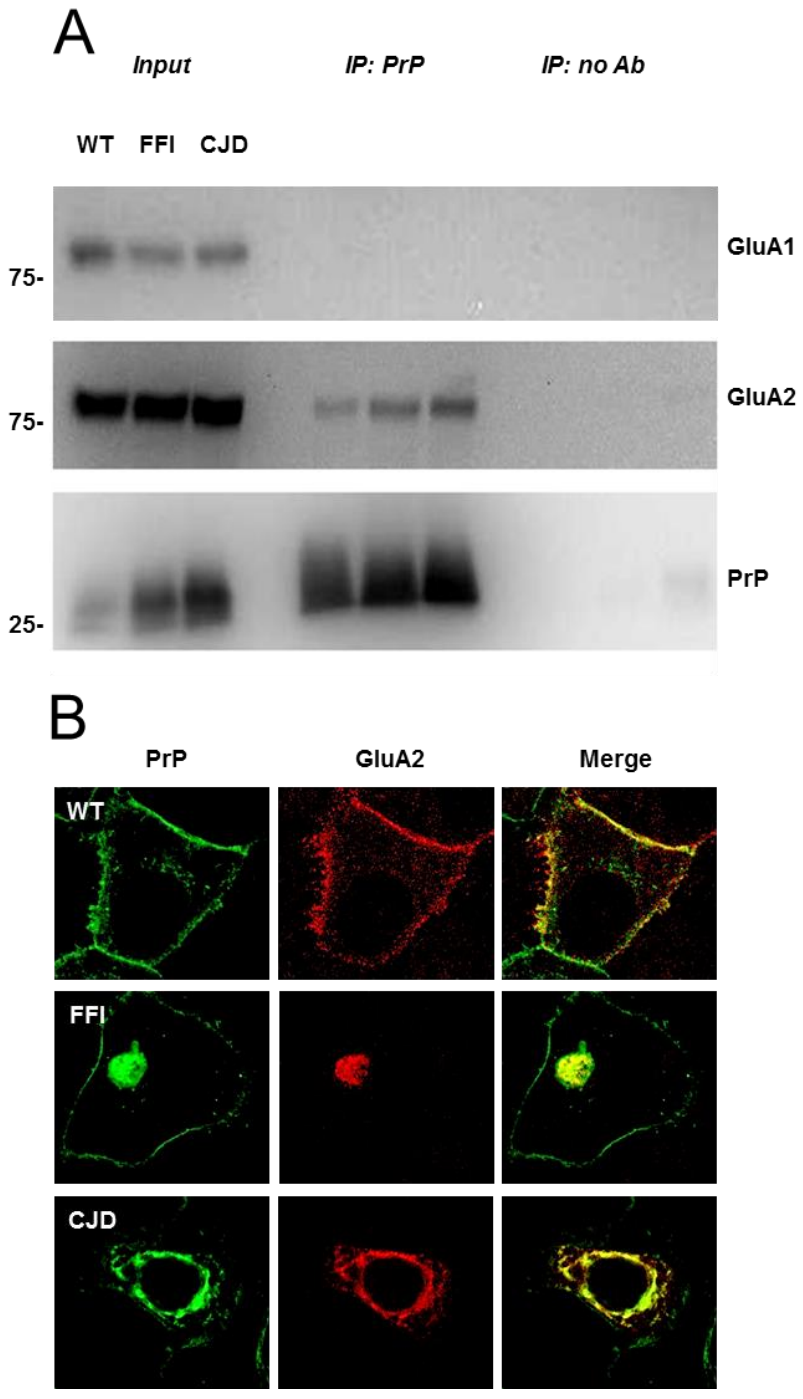


Figure 12- Mutant PrP interacts with the GluA2 subunit and impairs its membrane delivery. (A) Co-immunoprecipitation of PrP and GluA1 or GluA2 from hippocampal protein extracts of WT, FFI and CJD mice. The image is representative of 3 independent experiments. (B) Co-expression of WT (upper row) or mutant (central and lower rows) PrP and GluA2 in HeLa cells.

These results indicate that PrP is involved in the trafficking of GluA2, and that this trafficking is impaired in the presence of both variants of the D177N mutation.

Mutant PrPs induce alterations in dendritic spines

Transgenic CJD and FFI mice have been described to efficiently recapitulate the corresponding human diseases (Dossena, Imeri et al. 2008; Bouybayoune, Mantovani et al. 2015). Nevertheless, a systematic study of the structure and function of the excitatory synapses was never performed so far.

It has been suggested that synaptic dysfunction and dendritic spine loss may have a central role in the pathogenesis of prion diseases (Jeffrey, Halliday et al. 2000; Cunningham, Deacon et al. 2003; Fuhrmann, Mitteregger et al. 2007). Moreover, impairment in a mechanism of primary importance for synaptic function such as AMPARs trafficking is expected to result in synaptic alterations (Hanley 2008; Bassani, Folci et al. 2013). For these reasons, we carried out a detailed synaptic analysis of CJD and FFI neurons.

Dendritic spines are the main sites at which excitatory neurotransmission takes place, and modifications in their number and shape has been described in several neurodegenerative disorders (Fiala, Spacek et al. 2002; Maiti, Manna et al. 2015). To verify whether mutant PrP affected spine morphology, we transfected WT and transgenic primary hippocampal neurons with GFP, which served as a cell-filler (fig. 13A). Spine morphological analysis was carried out in mature cultures at 14 DIV.

We calculated the overall spine density (number of spines/dendrite µm) associated to the two genotypes, and compared it to that of control neurons. As shown in fig. 13B, a significant reduction in spine density was present in both FFI and CJD neurons.

Next, we performed a detailed analysis of spine morphology. We classified spines into four general categories: mushroom, stubby, thin and “other” spines. Spine quantitation shown in fig. 13C revealed that in both the genotypes there was a significant reduction in mushroom spines, accompanied by a parallel increase in thin spines.

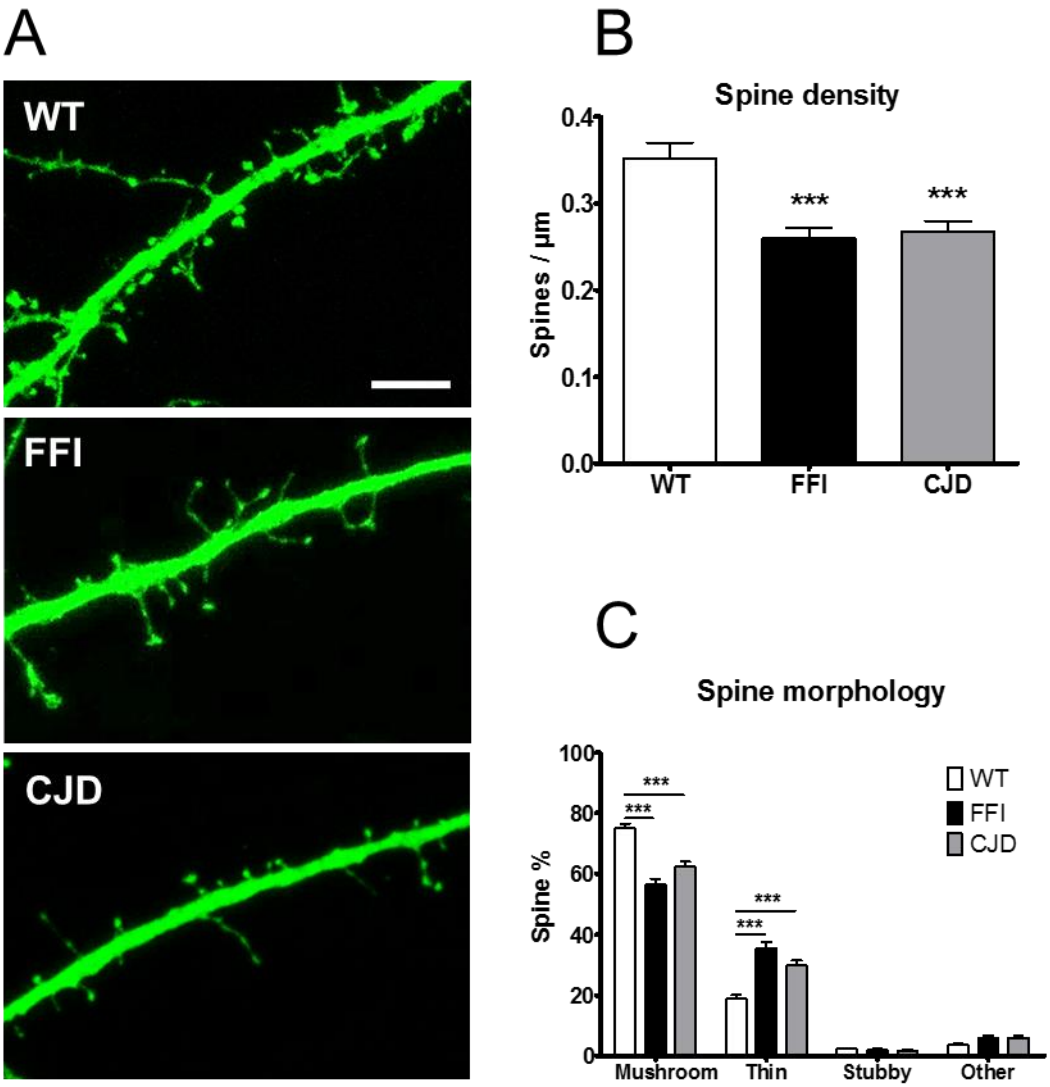


Figure 13- Neurons expressing mutant PrP show reduced dendritic spine density and altered spine morphology. (A) Confocal images of GFP-transfected primary hippocampal neurons from WT or transgenic mice. Scale bar: 5 µm. (B) Quantitative analysis of total spine density (spines/µm). (C) Quantitative analysis of mushroom, thin, stubby and atypical spines. Spine percentage was calculated as the number of spines of a given class divided by the total number of spines per field. All values are mean ± s.e.m. Statistical test: Kruskal-Wallis test followed by Dunn's correction for multiple comparisons. ***P< 0.001. n= 4 independent experiments.

These data indicate that the expression mutant PrP is associated to a defective dendritic spine development, characterized by a reduction in the overall number of protrusions and a morphological conversion from a more mature, mushroom-rich phenotype to a more immature, thin-rich phenotype.

CJD and FFI mutations differently impact functional synapses

Although most excitatory synapses are formed on dendritic spines, these two elements cannot be strictly assimilated to one another, because functional excitatory synapses can also form outside spines, for example directly on the dendritic shaft (Aoto, Ting et al. 2007). On the other hand, although characteristic mature spines are normally considered to be mushroom-shaped, synaptic contacts may sometimes develop also at the level of filopodial protrusions (Menna, Zambetti et al. 2013). Therefore, a caveat must be kept in mind when drawing conclusions about the functional state of synapses from dendritic spines.

Accordingly, we aimed at deepening the synaptic characterization of CJD and FFI neurons through more specific structural and functional analyses.

First, to uncover possible differences in the overall number of receptors at the synapse we stained neurons for surface AMPARs and we calculated the density of the clusters (number of AMPAR puncta/µm, fig. 14B). A significant reduction in the number of postsynaptic sites was observed in CJD neurons. A similar trend, although not statistically significant, was present also in FFI cells.

Next, we wanted to determine how many postsynaptic sites were coupled to presynaptic terminals to form *bona fide* synaptic contacts. To this purpose, we stained neurons for Bassoon and Shank2 as pre- and postsynaptic markers, respectively. We performed a colocalization analysis and we calculated the fraction of postsynaptic puncta colocalizing with presynaptic terminals (number of Shank2-Bassoon colocalizing puncta/total number of Shank2 puncta or number of colocalizing puncta/total number of Bassoon puncta). As shown in fig. 14C and D, the fractional colocalization was lower in both CJD and FFI neurons compared to the controls, suggesting that both PrP mutations negatively impact the formation of structurally mature synapses. The effect did not vary when the number of colocalizing puncta was compared to either the pre- or the postsynaptic marker (fig 14D), arguing against the presence of mutation-specific effects on the overall amount of Shank and Bassoon.

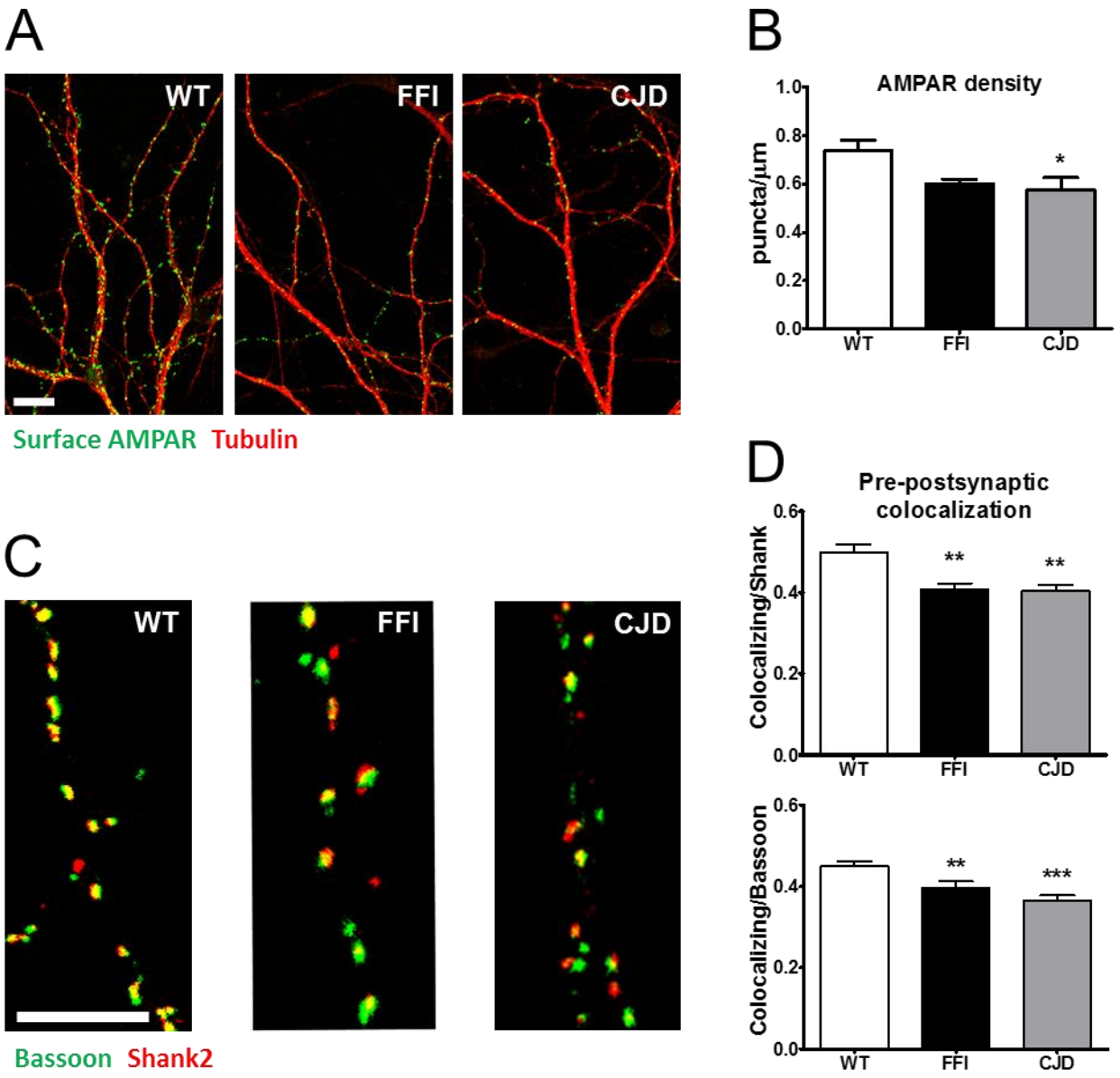


Figure 14- Neurons expressing mutant PrP show abnormal synaptic structure. (A) Immunocytochemical staining for surface AMPARs (green) and tubulin (red) in WT and transgenic neurons. Scale bar: 10 µm. (B) Quantitative analysis of surface AMPAR density (AMPAR puncta/µm). Statistical test: one-way ANOVA followed by Bonferroni correction for multiple comparisons. (C) Immunocytochemical staining for bassoon (green) and shank2 (red). Scale bar: 5 µm. (D) Quantitative analysis of pre- to post-synaptic colocalization. Fractional colocalization is calculated as the number of colocalizing pre- and post-synaptic puncta divided by the total number of shank2 puncta (upper panel) or bassoon puncta (lower panel). Statistical test: Kruskal-Wallis test followed by Dunn's correction for multiple comparisons. All values are mean ± s.e.m. $^{**}P < 0.01$, $^{*}P < 0.05$. $n = 4$ independent experiments.

Conversely, electrophysiological recordings of AMPAR-mediated mEPSCs shown in fig. 15 revealed a clear difference between the two mutations.

In particular, CJD neurons displayed a significant decrease in mEPSCs frequency, in line with a genuine reduction in the number of functional synapses. In parallel, we observed an increase in the amplitude of the events which might be attributable to either an increased strength of the residual postsynaptic sites or a modification in the conductance of the receptors.

In contrast, FFI neurons fully compensated for the structural defects, as both the frequency and the amplitude of mEPSCs were undistinguishable from those recorded in WT neurons.

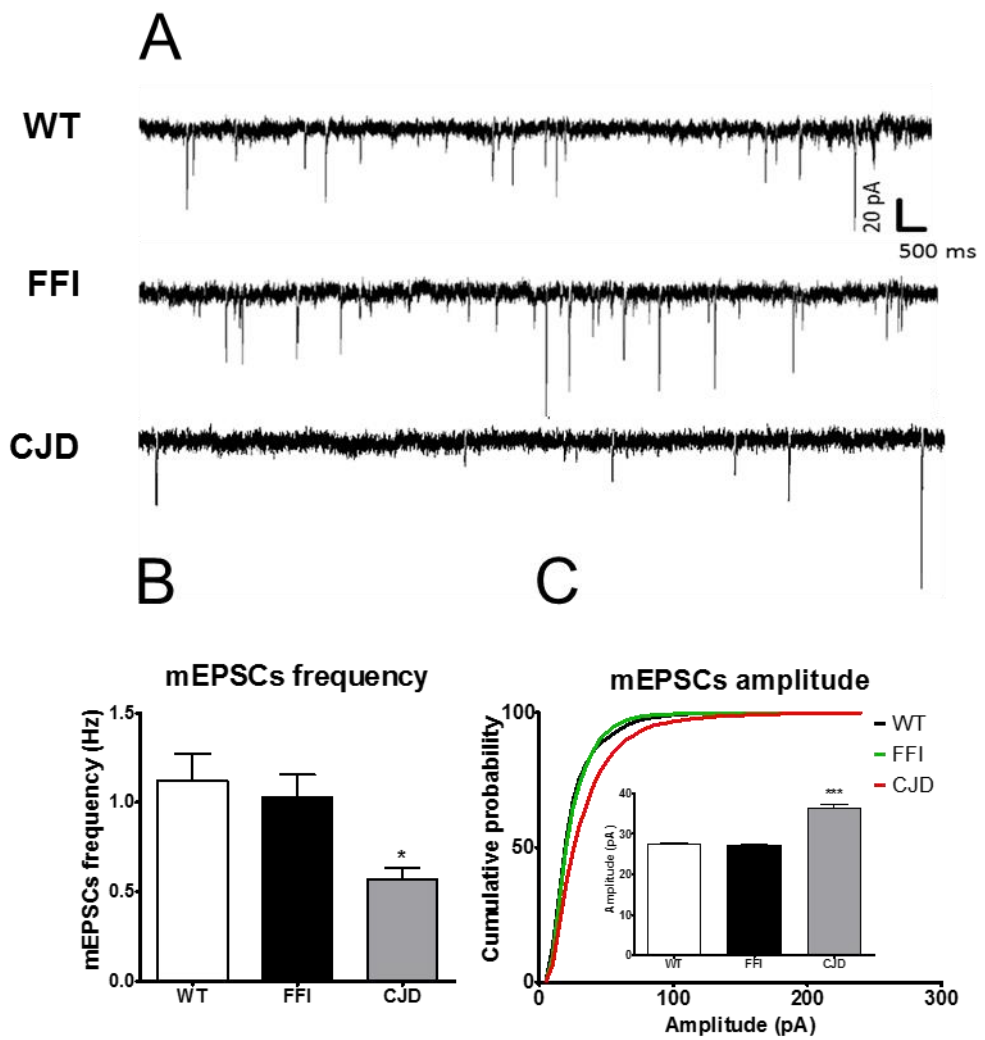


Figure 15- Altered synaptic function in CJD but not in FFI neurons. (A) Representative traces of mEPSCs. (B) Mean mEPSCs frequency in the different genotypes. (C) Cumulative mEPSCs amplitude histogram for WT and transgenic neurons. Inset shows the mean mEPSC amplitude. All values are mean ± s.e.m. Statistical test: one-way ANOVA followed by Bonferroni correction for multiple comparisons. ***P< 0.001, **P< 0.01, *P< 0.05. n= 9 independent experiments.

Taken together, these results suggest that although both the mutants show similar structural alterations, the CJD mutation seems to have a more prominent functional impact, whereas FFI neurons have a more preserved synaptic function.

AMPA receptors trafficking is altered in CJD but not in FFI neurons

Glutamate receptor levels at the synapse are very dynamic, and their ability to move in and out the postsynaptic plasma membrane is crucial for the modulation of synaptic transmission. Trafficking of these proteins is, in fact, a key process which not only depends on, but also determines the structural and functional integrity of the synapses (Hanley 2008).

Data obtained so far demonstrated that mutant PrP causes mislocalization of GluA2 and loss of dendritic spines and functional synapses. This could be consistent with an impaired glutamate receptors homeostasis.

We aimed, therefore, to explore the possibility that mutant PrP affected AMPARs trafficking, and we used the induction of synaptic plasticity as a method for assessing the neuronal ability to deliver AMPA receptors to the plasma membrane. In particular, we focused our attention on synaptic scaling.

Synaptic scaling is a homeostatic form of plasticity that is triggered by global decreases or increases in the network activity. Such changes elicit a compensatory response of opposite sign, by which neurons scale up or down the strength of all the synapses to normalize their output without changing the relative strength of synaptic inputs. This allows to maintain neuronal responsivity within an optimal working range, without disrupting information storage or processing mechanisms that rely on differences in synaptic weights (Turrigiano 2008). Like most of the other forms of plasticity, synaptic scaling is achieved through both pre- and postsynaptic adjustments (Pozo and Goda 2010). At a postsynaptic level, it depends on the accumulation or removal of AMPA receptors.

A widely-established protocol for inducing synaptic scaling consists of blocking neuronal firing over several hours to induce a scaling-up of synaptic strength. Accordingly, we treated mature cultures at DIV 13-14 with 1 μM TTX and we analyzed them 48 hours later.

Electrophysiological recordings showed that WT neurons underwent a significant increase in synaptic strength upon TTX treatment, as demonstrated by a concomitant increase in both

mEPSCs amplitude and frequency. FFI neurons also displayed a strong postsynaptic potentiation, which appeared in the form of increased mEPSCs amplitude which was not paralleled by an increase in frequency. Conversely, CJD neurons showed no response to TTX treatment, arguing for the presence of a significant defect in AMPARs trafficking (fig 16A and B).

We next examined the TTX-induced AMPARs clustering to determine whether the functional discrepancies that we observed in FFI and CJD neurons could in fact be ascribable to differential effects of the mutations on receptors trafficking. To this purpose, we stained control and TTX-treated neurons for surface AMPARs and for the presynaptic marker Bassoon, and we performed a colocalization analysis (see above). This allowed us to consider only the synaptic fraction of AMPA receptors. In line with the functional data, the experiments shown in fig. 16C and D revealed that the TTX- induced increase in synaptic clustering of the receptors occurred in WT and FFI neurons, but was impaired in the presence of the CJD mutation.

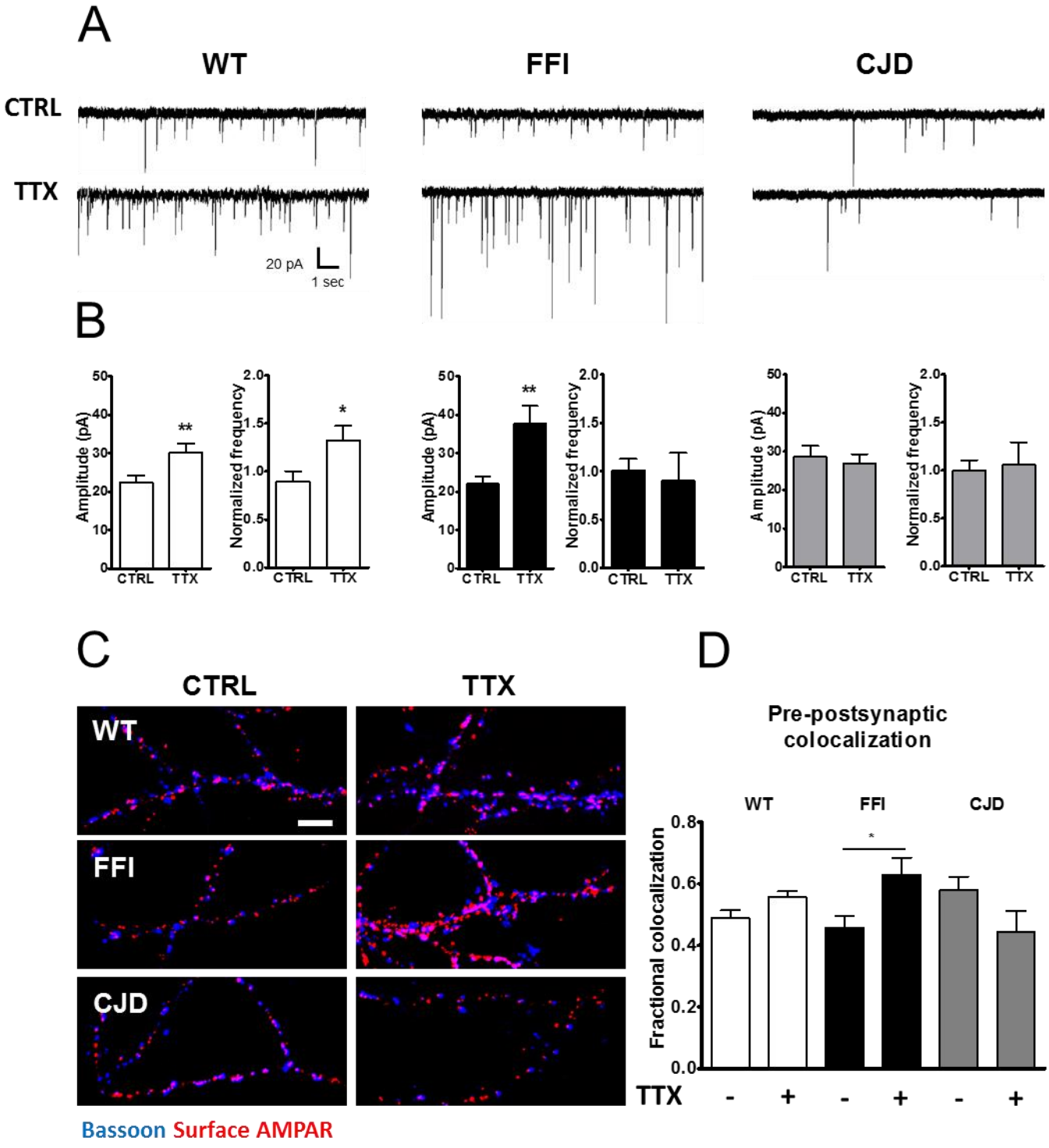


Figure 16- Synaptic scaling is impaired in CJD but not in FFI neurons. (A) Representative mEPSCs traces before (upper traces) and after (lower traces) the induction of synaptic scaling in the different genotypes. (B) Mean mEPSCs amplitude and frequency in control conditions and after 48 hours-treatment with 1 µM TTX in WT, FFI and CJD neurons (left, central and right pair of graphs, respectively). Statistical test: unpaired Student's *t*-test. *n*= 4 independent experiments. (C) Representative images of WT or mutant dendrites showing the colocalization between bassoon (blue) and surface AMPARs (red) before (left panels) and after (right panels) the induction of synaptic scaling. Scale bar: 5 µm. (D) Quantitative analysis of pre- to postsynaptic colocalization in control conditions or after TTX treatment. Fractional colocalization is calculated as the number of colocalizing bassoon and AMPAR puncta divided by the total number of bassoon puncta. Statistical test: one-way ANOVA followed by Bonferroni correction for multiple comparisons. All values are mean ± s.e.m.**P< 0.01, *P< 0.05.

Once again, these results confirmed that CJD and FFI mutations have a different impact on the function of excitatory synapses. In particular, CJD PrP seems to have a greater effect on AMPARs trafficking compared to the FFI variant. Notably, however, none of the mutant genotypes showed an increase in mEPSCs frequency following TTX treatment. This suggests the occurrence of additional defects, possibly involving the presynaptic compartment.

Impaired trafficking of AMPA receptors specifically involves the GluA2 subunit and results in exposure of GluA2-lacking receptors in different types of genetic prion diseases

After having demonstrated that mutant PrP, and especially the CJD variant, was associated to a defective trafficking of AMPA receptors we aimed to verify whether this impairment selectively affected the GluA2-containing receptors.

A reasonable hypothesis was, in fact, that the mutant PrP-dependent intracellular retention of GluA2 resulted in a rearrangement in the subunit composition of the remaining receptors.

To examine the GluA2 content of synaptic AMPARs in the different genotypes we measured the rectification index of AMPAR-mediated miniature currents.

The current-voltage relationship (I/V) of AMPA receptors is largely determined by the GluA2 subunit. GluA2-containing receptors show a linear I/V relationship, whereas GluA2-lacking receptors are inwardly rectifying; in other words, they conduct little outward current at positive potentials (fig. 17). It follows that the ratio between mEPSCs amplitude calculated at positive and negative potentials (we chose +50 and -70 mV, respectively), which is referred to as rectification index, will be close to 1 for GluA2-containing receptors, and lower for GluA2-lacking receptors.

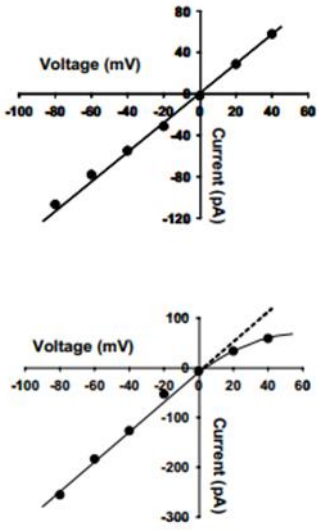


Figure 17- Representative I-V curves for AMPA receptors. Current- voltage plots for GluA2-containing (upper panel) and GluA2-lacking (lower panel) AMPA receptors. The solid line is obtained by plotting EPSC amplitude as a function of holding potential. Note that current-voltage relationship deviates from linearity at positive potentials in case of GluA2-lacking receptors. This behavior is due to the inwardly rectifying properties of these receptors, which have a reduced outward conductance with respect to the GluA1-containing receptors. Modified from Billa et al., 2010.

The analysis of the rectification index shown in fig. 18B revealed that CJD neurons displayed a significantly lower ratio compared to the controls, arguing for the presence of an increased synaptic population of GluA2-lacking AMPARs. A similar trend, although not statistically significant, was observed for FFI neurons.

GluA2 is a critical subunit in determining many of the major biophysical properties of AMPA receptors, including calcium permeability (Isaac, Ashby et al. 2007). In particular, GluA2-containing receptors are only permeable to sodium and potassium, whereas GluA2-lacking receptors are also permeable to calcium.

To verify whether the higher expression of GluA2-lacking AMPARs in CJD neurons resulted in increased neuronal calcium permeability, we measured the intracellular calcium levels following AMPA receptors activation. AMPA was administered to Fura-2-labeled WT and CJD neurons, and calcium imaging was performed in the presence of the NMDAR blocker AP5, the general VGCCs blocker cadmium and the L-type calcium channel inhibitor nifedipine, to isolate the AMPAR-mediated component. As shown in fig. 18D, the peak calcium increase in CJD neurons was greater

than in WT neurons, confirming our hypothesis. Fig. 18C shows the mean responses in two independent experiments.

Excessive activity-dependent calcium influx is a key mediator of excitotoxicity (Arundine and Tymianski 2003). Interestingly, an increasing body of literature demonstrates that upregulation of calcium permeable AMPARs (CP-AMPARs) occurs in different pathological conditions, and calcium entry through these receptors can directly trigger neuronal death (Brorson, Manzolillo et al. 1994; Liu and Zukin 2007). Therefore, activation of CP-AMPARs can play a major role in excitotoxic processes.

To verify whether the mutant PrP-dependent increase in CP-AMPARs resulted in enhanced susceptibility to excitotoxicity, we exposed neurons to AMPA for 24 hours and we measured cell death through dual DAPI/PI staining. Preliminary data shown in fig. 18E indicated that, indeed, CJD neurons displayed an increased sensitivity to AMPA toxicity compared to the controls. Surprisingly, a similar effect was observed also in FFI neurons, suggesting that increased expression of GluA2-lacking AMPARs may be a common hallmark of the two mutations.

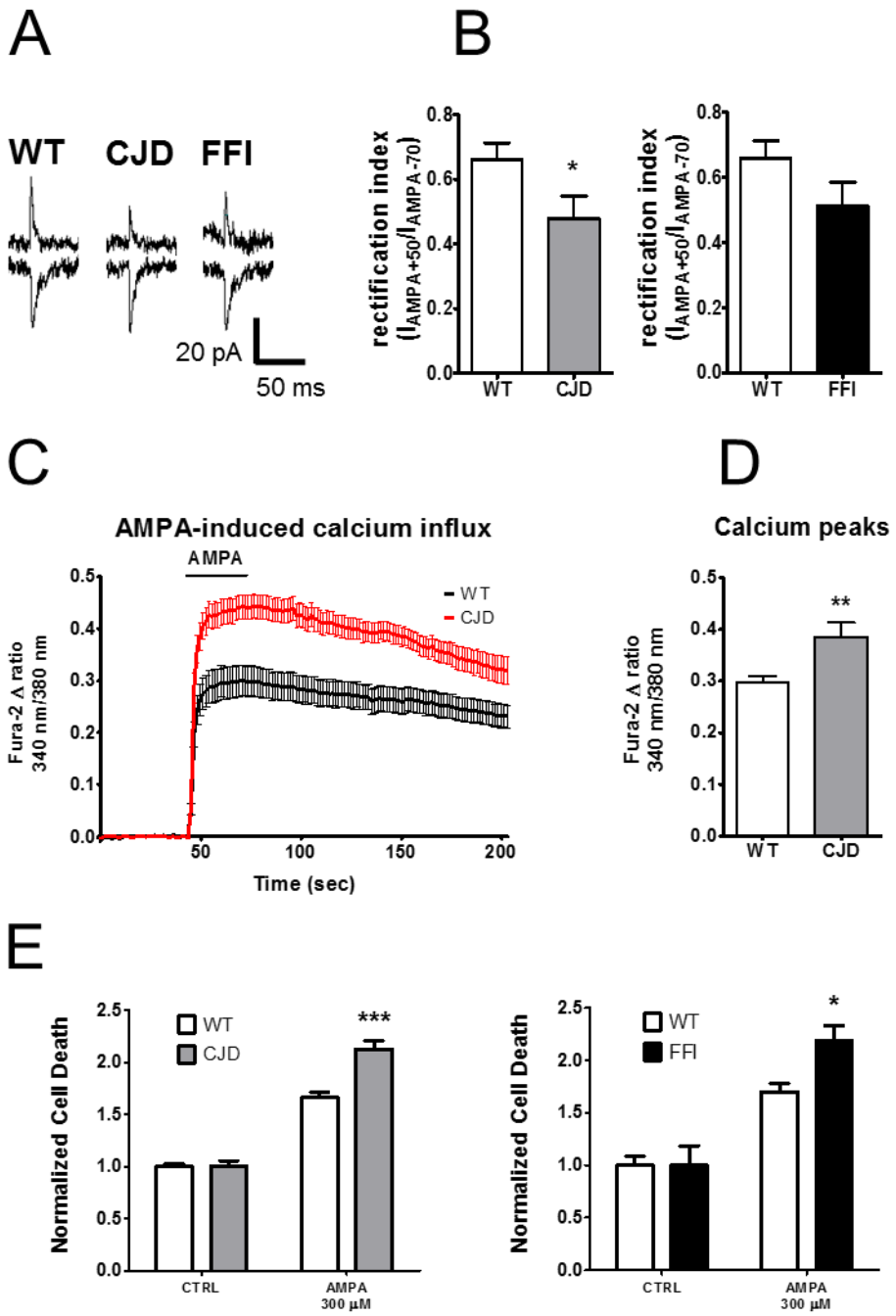


Figure 18- Mutant neurons display increased exposure of GluA2-lacking AMPARs, increased AMPA-induced calcium permeability and enhanced susceptibility to excitotoxic cell death. (A) Representative mEPSCs at +50 (top) and -70 (bottom) mV in the different genotypes. (B) Rectification index values for WT, CJD and FFI neurons. Statistical test: Mann-Whitney *t*-test for WT vs CJD; unpaired Student's *t*-test for WT vs FFI. *n*= 3 independent experiments. (C) Representative traces of intracellular calcium levels (normalized fluorescence 340 nm/fluorescence 380 nm in Fura-2-treated cells) in WT (black) and CJD (red) neurons stimulated with AMPA (30µM for 30 sec). (D) Quantification of AMPA-induced calcium peaks in WT and CJD neurons. Statistical test: unpaired Student's *t*-test. *n*= 2 independent experiments. (E) Normalized cell death (PI puncta/Hoechst puncta) for CJD (left) and FFI (right) neurons compared to WT. Statistical test: unpaired Student's *t*-test. All values are mean ± s.e.m. ***$P< 0.001$, **$P< 0.01$, *$P< 0.05$.

Given the relevance of increased expression of CP-AMPARs in a neurodegenerative context, we investigated whether this mechanism was shared by other PrP mutations.

Unpublished data of our group indicate that interaction and retention of GluA2 also occurs in the presence of PG14 mutation. PG14 PrP contains a nine-octapeptide insertion (fig. 19) and in humans is associated to a neurological disease characterized by progressive dementia and ataxia. In 2000, Chiesa et al. reported that mice expressing the murine homolog of the mutation develop a fatal neurological disorder characterized by cerebellar atrophy with massive degeneration of cerebellar granule neurons (CGNs) (Chiesa, Drisaldi et al. 2000). We therefore performed the same set of experiments in CGNs from WT and PG14 mice.

Figure 19- PG14 PrP. Schematic representation of the PG14 allele

Once again, we observed that mutant neurons displayed reduced rectification index (fig. 20A and B). Calcium imaging experiments revealed a significantly higher AMPA-induced calcium influx in PG14 neurons, and the mutant cells maintained elevated intracellular calcium concentrations 10 minutes after stimulation. Finally, we found that PG14 CGNs were more susceptible to AMPA- but not to NMDA-induced cell death than the controls (fig. 20C-F).

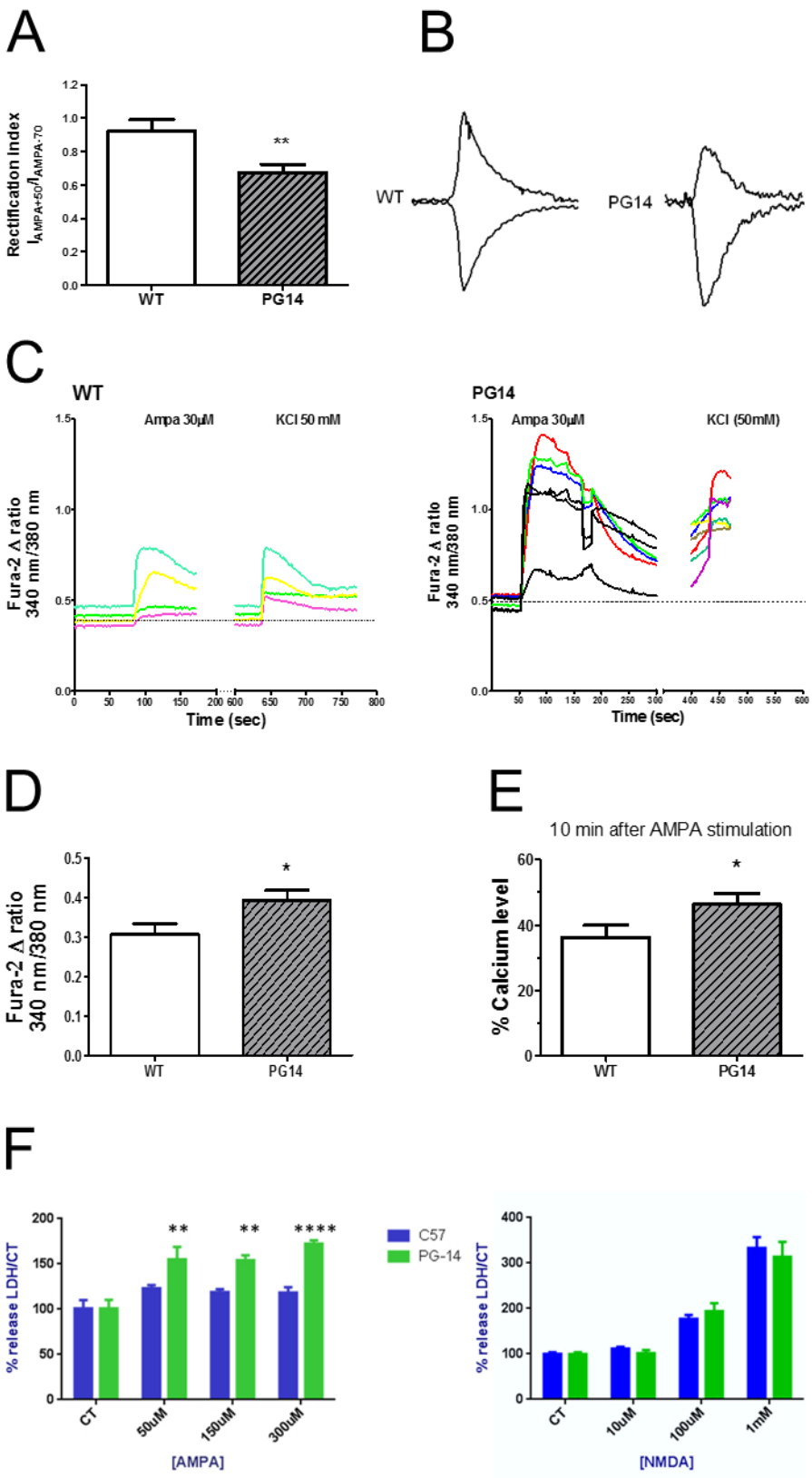

A
B
Rectification index
I AMPA+50/I AMPA-70
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
WT
PG14
WT
PG14
C
WT
Ampa 30µM
KCl 50 mM
Fura-2 Δ ratio
340 nm/380 nm
1.5
1.0
0.5
0.0
0
50
100
150
200
600
650
700
750
800
Time (sec)
PG14
Ampa 30µM
KCl (50mM)
Fura-2 Δ ratio
340 nm/380 nm
1.5
1.0
0.5
0.0
50
100
150
200
250
300
400
450
500
550
600
Time (sec)
D
Fura-2 Δ ratio
340 nm/380 nm
0.5
0.4
0.3
0.2
0.1
0.0
*
WT
PG14
E
10 min after AMPA stimulation
% Calcium level
60
40
20
0
*
WT
PG14
F
% release LDH/CT
200
150
100
50
0
**
**
****
CT
50uM
150uM
300uM
[AMPA]
C57
PG-14
% release LDH/CT
400
300
200
100
0
CT
10uM
100uM
1mM
[NMDA]

Figure 20- PG14 PrP alters AMPAR subunit composition, affecting calcium permeability and susceptibility to excitotoxicity. (A) Rectification index values for WT and PG14 PrP-expressing neurons. Statistical test: unpaired Student's *t*-test. *n*= 3 independent experiments. (B) Representative mEPSCs at +50 (top) and -70 (bottom) mV in WT and PG14 neurons. (C) Representative traces of calcium imaging experiments showing calcium responses to AMPA and KCl in CGNs from WT (left) and PG14 (right) mice. Quantification of AMPA-induced calcium peaks (D) and intracellular calcium levels 10 minutes after AMPA stimulation (E) in WT and PG14 CGNs. Statistical test: Mann-Whitney *t*-test. *n*= 6 independent experiments. (E) Normalized cell death (LDH release) for WT (C57) and PG14 CGNs following AMPA (left) and NMDA (right) treatment. Statistical test: unpaired Student's *t*-test. *n*= 3 independent experiments. All values are mean ± s.e.m. ****P< 0.0001, ***P< 0.001, **P< 0.01, *P< 0.05.

Taken together, these results suggest that a common pathological mechanism operates in different prion diseases, and may therefore represent a useful therapeutic target.

NMDAR function is differentially affected by CJD and FFI mutations

Data obtained so far indicated that a substantial difference existed between the two mutant genotypes in terms of AMPARs trafficking capacity. This phenomenon, however, hardly depended on the direct action of PrP on AMPA receptors, as we had demonstrated that interactions with the receptor and retention of GluA2 were similar in CJD and FFI neurons.

We therefore reasoned that the cause may lay elsewhere, at the level of different pathways not directly associated to AMPAR trafficking.

NMDAR activity is necessary for regulating AMPAR mobility during synaptic development and plasticity, and plays a critical role in dendritic spine formation and maintenance (Hanley 2008; Sala, Cambianica et al. 2008). Since PrP has been described to interact and regulate also NMDARs (Khosravani, Zhang et al. 2008), we hypothesized that differences between the two mutations could occur at this level.

To test this hypothesis, we coimmunoprecipitated PrP and the NMDAR subunits GluN1 and GluN2B from hippocampal homogenates of WT and transgenic mice. These experiments, shown in fig. 21A, revealed interesting differences. Whereas WT and CJD PrPs interacted with both the subunits, FFI PrP showed only a weak association to GluN2B and lost completely its ability to interact with GluN1.

To verify whether this defect could differentially impact the cellular localization of NMDAR, we co-transfected HeLa cells with either WT or mutant PrP and GluN1. Once again, we observed that the

localization of the subunit depended on its interaction with PrP. GluN1 was expressed at the plasma membrane in the presence of the WT protein, whereas it was intracellularly retained when co-expressed with the CJD variant. On the other hand, the subunit was correctly delivered to the cell surface in FFI neurons, where the interaction with PrP was lost (fig. 21B).

To assess the functional significance of our findings, we investigated the expression of functional NMDA receptors. Since NMDAR-mediated mEPSCs can be difficult to detect, due to their small amplitude which is often close to noise level (Bardoni, Magherini et al. 1998; Townsend, Yoshii et al. 2003), we measured whole-cell currents elicited by local application of NMDA. Whole-cell currents are large enough to measure, because they result from the activation of all the synaptic and nonsynaptic receptors (including somatic receptors) reached by the agonist. Since the amplitude of the current depends also on the size of the cell, which affects the absolute number of available receptors, we normalized the peak amplitude to the cell capacitance, which provides an estimate of cell size, thus allowing to correct for cell dimension. The resulting measure was referred to as current density.

In line with GluN1 localization, we observed that the current density of FFI was comparable to that of WT cells, whereas it was significantly decreased in CJD neurons (fig. 21C and D).

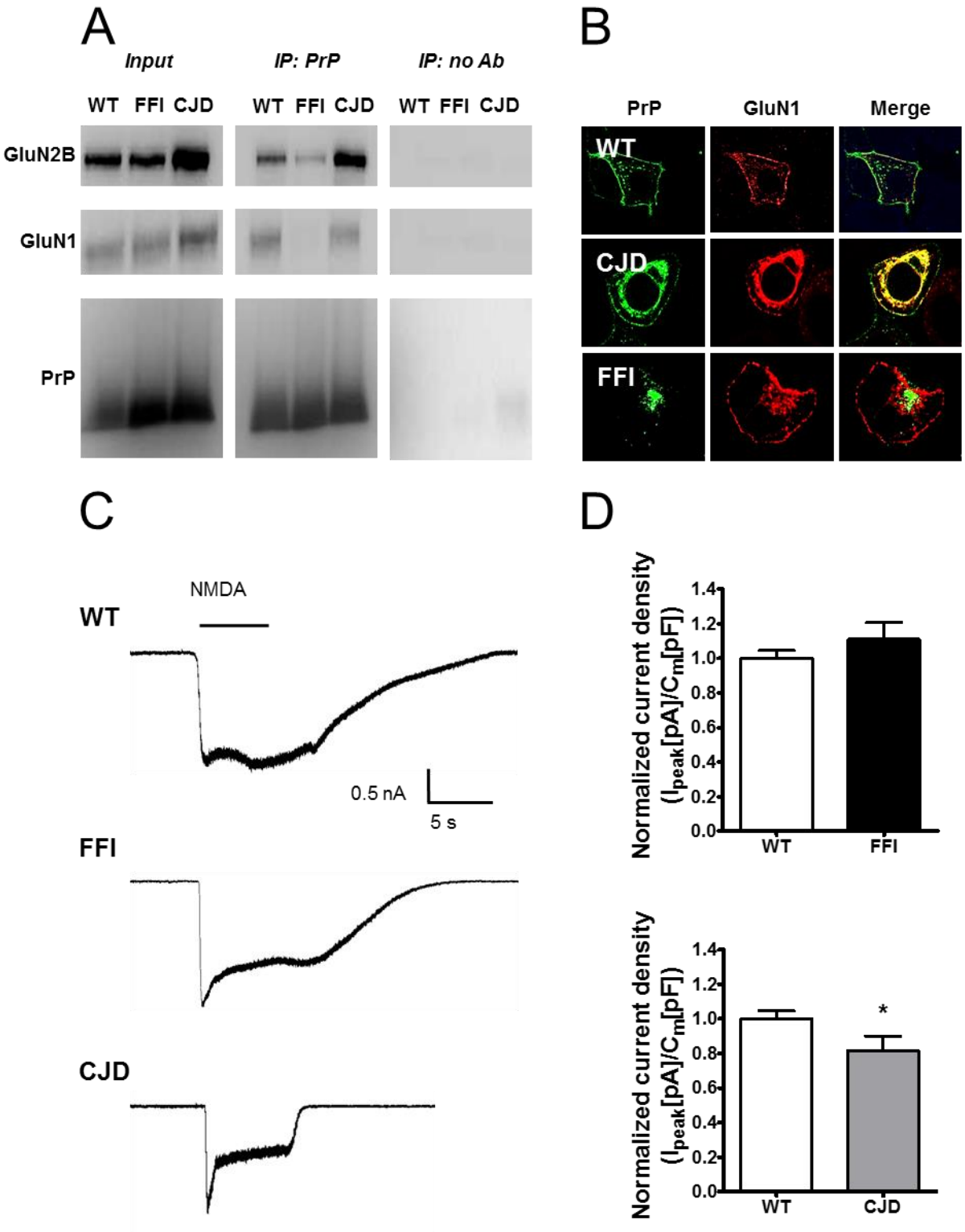


Figure 21- Differential interactions and functional effects of FFI and CJD PrPs on NMDAR. (A) Co-immunoprecipitation of PrP and GluN1 or GluN2B from hippocampal protein extracts of WT, FFI and CJD mice. (B) Co-expression of WT (upper row) or mutant (central and lower rows) PrP and GluN1 in HeLa cells. (C) Representative traces of whole-cell currents evoked by 5 sec exposure to 200 µM NMDA in the different genotypes. (D) Normalized current density in FFI and CJD neurons compared to the controls. Statistical test: unpaired Student's *t*-test. *n*= 4 independent experiments. All values are mean ± s.e.m. **P*< 0.05.

Given that FFI neurons display normal synaptic scaling, we examined whether preserved NMDAR function could contribute to this process. To this purpose we treated WT and FFI neurons with TTX (1µM) alone, with TTX together with the NMDAR antagonist AP5 (100 µM), or with AP5 alone, and we recorded AMPAR-mediated mEPSCs after 48 h. Preliminary data obtained in WT neurons showed that all the treatments produced an increase in the amplitude of mEPSCs in WT neurons (fig. 21, left panel), consistent with previous reports (Sutton, Ito et al., 2006).

Conversely, in FFI neurons (fig. 21, right panel) we observed the increase in mEPSCs amplitude only after either TTX or AP5 treatment. Surprisingly, synaptic potentiation did not occur when blockade of action potential firing was associated to the suppression of NMDAR activity (TTX+AP5).

This preliminary finding suggests that FFI neurons possess a compensatory mechanism that allows the establishment of synaptic scaling in response to chronic blockade of presynaptic firing, which requires NMDA receptors activity.

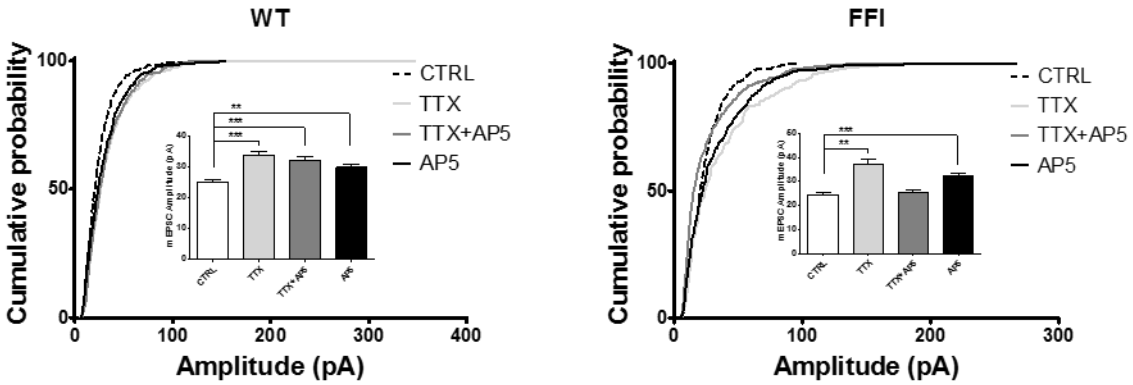


Figure 22- Activity blockade requires NMDAR function to induce synaptic scaling in FFI neurons. Cumulative mEPSCs amplitude histogram in control conditions and after 48 hours-treatment with TTX (1 µM), TTX + AP5 (100 µM) and AP5 for WT (left) and FFI (right) neurons. Insets show the mean mEPSC amplitude. Statistical test: Kruskal-Wallis test followed by Dunn's correction for multiple comparisons. All values are mean ± s.e.m. ***P< 0.001, **P< 0.01.

Taken together, these results indicate that the two variants of the D177N mutation impact differently on NMDA receptors. Our data also suggest that preserved NMDAR function may be particularly important for the regulation of AMPAR trafficking during synaptic plasticity. We therefore speculate that the differential interaction of mutant PrP with NMDAR may explain some aspects of the phenotypic heterogeneity observed in these neurons.

DISCUSSION

Although the neurodegenerative pathways activated by the abnormal PrP are not known, synaptic dysfunctions appear to be a major pathological determinant of prion diseases.

Synaptic alterations have been described to occur before and independently of neuronal loss (Clinton, Forsyth et al. 1993; Jeffrey, Halliday et al. 2000; Ferrer 2002), suggesting that synaptic deregulation may be at the origin of the symptomatology, and eventually lead to neuronal death due to a combined action of a lack of activity-dependent trophic signals and excitotoxicity (Cunningham, Deacon et al. 2003; Chiesa, Piccardo et al. 2005; Bell and Hardingham 2011; Senatore, Colleoni et al. 2012).

Several pieces of evidence indicate that PrP is directly involved in synaptic regulation, and indeed pathological mutations of the protein negatively affect this function. For example, our group has recently found that mutant PrP is retained in the endoplasmic reticulum where it interacts with the VGCCs. This impairs the correct delivery of the channels to the cell surface, impacting glutamatergic transmission and leading to the onset of neurological symptoms (Senatore2012). However, this phenomenon alone does not account for neurodegeneration. Other studies have demonstrated that PrP engages functional interactions with postsynaptic proteins such as AMPA and NMDA receptors (Khosravani, Zhang et al. 2008; Watt, Taylor et al. 2012), but the relevance of these interactions in prion diseases was never explored so far.

The aim of the present work was to mark a step forward in linking synaptic dysfunction and neurodegeneration in these disorders. We hypothesized that interactions between the pathological prion protein and glutamate receptors could result in postsynaptic defects, producing adverse effects on neuronal function and survival.

To verify this, we took advantage of two different models of inherited prion diseases: FFI mice which express the D177N/128M mutation, and CJD mice which express the D177N/128V. Both variants of the protein accumulate intracellularly (Ivanova, Barmada et al. 2001; Fioriti, Dossena et al. 2005; Dossena, Imeri et al. 2008) and have specific neurotoxic properties which are

independent of prion replication (Bouybayoune, Mantovani et al. 2015; Chiesa, Restelli et al. 2016).

We used primary hippocampal neurons from these mice to characterize the interactions of the mutant proteins with AMPA and NMDA receptors, and to analyse the effects of the mutations on postsynaptic structure and function.

We first observed that both the WT and the mutant isoforms of PrP interact with the GluA2 but not with the GluA1 subunit of AMPA receptors. This finding contrasts with a previous report which showed that the protein interacts with both subunits (Watt, Taylor et al. 2012). The authors also showed that the interaction with AMPAR is lost in the presence of pathogenic PrP mutations, including the two under investigation in our study, and suggest that functional deficits may arise from a loss of interaction of the mutant protein with the receptor. Once again, this contrasts with our observation that the interaction with GluA2 is conserved in the presence of the mutations, causing the intracellular retention of the subunit.

The intracellular retention of GluA2 in mutant neurons did not result in a decreased basal amplitude of AMPAR-mediated mEPSCs, as would be expected in the presence of a general reduction in the number of receptors. This is in line with several studies demonstrating that a lack of GluA2 does not affect basal synaptic transmission, because of a compensatory increase in the insertion of GluA1 (possibly through formation of homomeric GluA1 receptors) (Meng, Zhang et al. 2003; Gainey, Hurvitz-Wolff et al. 2009). Consistently, we observed in mutant neurons a lower rectification index relative to WT cells, indicative of a high synaptic content of GluA2-lacking AMPARs. Our functional data, thus, support the idea that PrP does not interact with GluA1, which is free to compensate for the lack of GluA2, at least under basal conditions. Moreover, as discussed below, many of the defects that we found in the mutant neurons can be largely explained by the presence into the membrane of AMPARs lacking GluA2. We therefore suggest that mutant PrP impacts AMPAR trafficking and function due to the selective, intracellular retention of GluA2.

Given the central role of dendritic spines in synaptic transmission, it is not surprising that several brain disorders, including neurodegenerative diseases, are associated to alterations in these structures. In prion diseases, dendritic spine pathology has been reported as an early manifestation of prion infection (Jeffrey, Halliday et al. 2000; Cunningham, Deacon et al. 2003;

Fuhrmann, Mitteregger et al. 2007). We found that dendritic spine defects and synaptic alterations also occur *in vitro*, in models of inherited diseases in which neuronal pathology is independent of prion replication. These findings endorse the centrality of synaptic pathology in these disorders, which is most likely attributable to cell-autonomous mechanisms depending on the abnormal PrP.

When speculating about the nature of these mechanism, it must be considered that spine and synapse development are complex processes, regulated at many different levels.

Relevant to our interests is the notion that a close correlation exists between the size of the spine and the density of AMPARs at the synapse. In other words, regulation of spine size is linked to regulation of AMPAR number, and AMPAR trafficking has been proposed to be one of the primary driving forces in spine morphogenesis (Hanley 2008). In particular, the N-terminal domain of GluA2 was identified as a direct modulator of spine morphogenesis, and a decrease in spines and functional synapses was observed upon GluA2 knock-down (Passafaro, Nakagawa et al. 2003). Although the precise molecular details are still unclear, the mechanism for this effect appears to involve an interaction between GluA2 and N-Cadherin, which is important for pre- and postsynaptic adhesion (Saglietti, Dequidt et al. 2007).

The intracellular retention of GluA2 may therefore, at least in part, explain the altered spine morphology and the reduced juxtaposition between pre- and postsynaptic terminals observed in mutant neurons. However, PrP is also involved in dendritic spine regulation via Fyn kinase (Toni, Spisni et al. 2006; Babus, Little et al. 2011; Um, Nygaard et al. 2012; Koleske 2013), and it is likely that a subversion of the physiological function of the protein at this level may contribute to the phenotype.

Interestingly, despite the common structural defects of spines and synapses, the two mutations show different functional effects.

CJD neurons display a severe but straightforward phenotype. Basal synaptic transmission is clearly impaired in CJD neurons, in line with the reduced number of functional synapses formed. Moreover, the lower rectification index is indicative of a reduced content of GluA2-containing receptors. This may also explain the increased amplitude of the miniature events, because GluA2-lacking AMPARs are known to display higher conductance (Isaac, Ashby et al. 2007). However, these neurons seem to be unable to compensate for the loss of GluA2 with other subunits upon

the induction of synaptic plasticity: the absence of synaptic potentiation in response to TTX treatment indicates that the impaired trafficking of GluA2 is paralleled by an overall alteration in AMPAR homeostasis. The increased expression of GluA2-lacking receptors, which is linked to higher calcium permeability (Isaac, Ashby et al. 2007), is in fact confirmed by the increased AMPAR-mediated calcium influx in CJD neurons. Notably, we show that this results in higher susceptibility to AMPA toxicity with respect to WT neurons.

This finding may have relevant clinical implications. Epileptic seizures are, in fact, frequently observed in CJD patients (Ng, Westover et al. 2014), and activation of CP-AMPARs in these conditions may significantly contribute to excitotoxicity. Our data therefore suggest that targeting of CP-AMPARs is a potentially interesting therapeutic option to be explored in CJD.

Evidence obtained in FFI neurons are more complex to interpret. The surprising finding here is that the presence of significant structural alterations does not seem to affect synaptic transmission and plasticity. This indicates that other mechanisms of functional compensation are active in these neurons.

For example, rectification index is not significantly reduced in FFI neurons, arguing for the presence of a higher proportion of synaptic GluA2-containing AMPARs, with respect to CJD cells. Interestingly, it has been reported that FFI PrP tends to be more expressed at the cell surface than the CJD variant in CGNs (Fioriti, Dossena et al. 2005). If this were the same in hippocampal neurons, lower intracellular retention of the protein might imply lower retention of the interacting partners too. In this case, a higher amount of GluA2 would be free to reach the plasma membrane.

Although controversial, some authors suggested that GluA2 plays a specific role in driving AMPAR insertion during synaptic scaling (Gainey, Hurvitz-Wolff et al. 2009; Pozo and Goda 2010). A different availability of GluA2 in FFI and CJD neurons may therefore contribute to the differential ability to support homeostatic plasticity.

A lower proportion of GluA2-lacking, CP-AMPARs should also result in lower AMPA-induced calcium influx compared to CJD neurons. In perspective, it will be important to verify this hypothesis.

In a way, experiments on PG14 neurons support the idea of a correlation between the amount of membrane protein and the severity of the phenotype: Fioriti and colleagues found that surface

PrP expression in PG14 neurons was much lower than in FFI (Fioriti, Dossena et al. 2005). In line with this observation, we found that PG14 CGNs, like CJD neurons, display a markedly reduced rectification index and increased AMPA-induced calcium influx. Importantly, these data also suggest that increased expression of CP-AMPARs may be a common pathological mechanism in genetic prion diseases.

However, increased susceptibility to AMPA-induced cell death was observed in all the mutants analyzed, including FFI. This is not necessarily in contrast with what was discussed so far, because a defect in membrane delivery of GluA2 does exist in FFI neurons, although possibly milder than in the other mutants. Increased expression of CP-AMPARs is therefore likely to occur also in this case, and may be sufficient to trigger excitotoxicity upon the prolonged and massive activation of the receptors elicited in our experimental protocol.

Notably, PG14 CGNs are more sensitive to AMPA and not to NMDA toxicity. This is the proof of concept that increased expression of CP-AMPARs is a specific mechanism of neurodegeneration in these mutants.

Our analysis of the interaction between PrP and NMDARs provides another interesting basis for speculating about the mechanisms of functional compensation that operate in FFI neurons. We report that, unlike the CJD variant, FFI PrP loses the ability to interact properly with NMDARs. In the absence of a physical association, the receptors are not retained intracellularly by the mutant protein, and their localization and function are, therefore, unaffected. Preserved NMDAR function may, in turn, contribute to support AMPAR trafficking and thus normal synaptic activity.

Different mechanisms may be involved in a possible NMDA-mediated support of AMPA receptor trafficking. For example, SynGAP is a downstream component of NMDAR signaling complex that is physically associated to GluN2B-containing receptors and which negatively regulates AMPAR trafficking. Activation of NMDARs leads to SynGAP phosphorylation by CaMKII, which reduces its negative control on Ras and Rap, two other components of NMDAR signaling pathways involved in the regulation of AMPAR trafficking. This results in increased exocytosis and decreased recycling of the receptors, thereby promoting AMPAR insertion at the synapse (Jeyabalan and Clement 2016). Notably, SynGAP signaling operates also in basal conditions (Wang, Held et al. 2013). It is therefore tempting to speculate that NMDAR activity in FFI neurons may compensate via SynGAP for the defects in AMPAR trafficking, potentiating the insertion of the receptors which escape PrP

retention. Conversely, this mechanism would not be possible in CJD neurons due to a lack of functional NMDARs. Experiments are currently being performed to verify this possibility.

Our last experiments, showing that synaptic potentiation is impaired in FFI neurons when blockade of neuronal firing is associated to inhibition of NMDARs, argue for an NMDAR dependence of synaptic scaling in these cells, further upholding the hypothesis that the functionality of these receptors provides cells with an important compensatory capacity. It is essential to state, though, that these are only preliminary data, and therefore they must be considered with caution.

A role for NMDARs in homeostatic plasticity has been proposed by several authors, although findings are controversial and the precise mechanisms involved remain to be elucidated. In particular, NMDAR-mediated miniature activity was shown to exert a tonic suppression of local protein translation in the dendrites, thereby limiting synthesis and incorporation of AMPARs. Blockade of this activity with AP5 results in a rapid scaling-up of synaptic strength (Sutton, Ito et al. 2006; Wang, Held et al. 2011; Wang, Held et al. 2013). This is consistent with our observations in WT neurons, but contrasts with findings in FFI cells, in which synaptic scaling was suppressed in the presence of concomitant blockade of neuronal firing and NMDARs. This may suggest that mutant neurons develop a compensatory mechanism which shifts NMDAR spontaneous activity from repressing to promoting AMPAR insertion. For example, since GluN2B-containing NMDARs and SynGAP were demonstrated to be required for the proper establishment of homeostatic plasticity (Wang, Held et al. 2011; Wang, Held et al. 2013) we can hypothesize that NMDAR-dependent regulation of SynGAP may become essential in FFI neurons for supporting synaptic scaling.

On the other hand, PrP exerts an inhibitory function on NMDARs (Khosravani, Zhang et al. 2008), and a loss of physical interaction of the mutant protein may result in a reduced control of NMDAR activity. In view of this, it will be interesting to verify whether FFI neurons are more susceptible to NMDA toxicity.

In summary, findings from this work confirm that PrP exerts a regulatory function on glutamate receptors homeostasis, and indicate that the aberrant interactions of mutant PrP with these proteins is responsible for structural and functional alterations of the postsynaptic compartment which affect excitatory neurotransmission and sensitizes neurons to excitotoxicity. In addition, our

findings may shed a light on the unsolved issue of phenotypic heterogeneity of prion diseases. Phenotypic heterogeneity is explained by postulating that different mutations assume specific conformations that are selectively toxic to different neuronal populations. However, the underlying mechanisms are unclear. Here, we provide evidence that distinct PrP mutations interact differently with glutamate receptors, altering their localization and function in different ways. Based on our results, we propose that the differential interaction with specific glutamate receptors subunits, and most likely with other proteins which are differentially expressed across neuronal subpopulations, may be a determinant of phenotypic variability of prion diseases. Of course, however, the site-specific outcome of the interactions with synaptic proteins cannot be addressed by using a single *in vitro* model such as hippocampal neuronal cultures. For this reason, it will be essential to confirm our results in vivo, by focusing on the brain regions specifically affected in the different diseases.

Given the broad, pleiotropic function of PrP, glutamate receptors homeostasis is likely to be only one of the many aspects of synaptic physiology that are affected in prion diseases. For example, we only focused on the postsynaptic compartment, but a role of PrP in regulating presynaptic neurotransmission has also been described (Senatore, Restelli et al. 2013; Robinson, Nugent et al. 2014), and indications arguing for the presence of presynaptic defects were obtained in our experiments as well.

These considerations underscore the need for elucidating all the possible mechanisms of synaptic pathology in prion diseases. As already mentioned, synapses are a very appealing target for therapy, not only because synaptic dysfunctions are the earliest pathological events, but also because, due to their intrinsic plasticity, synaptic alterations can be reversed. Ideally, the identification of a panel of specific synaptic dysfunctions for each disease may help to optimize therapeutic intervention for these multifaceted, untreatable disorders.

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