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Nderstanding the neurospecificity of Prion protein signaling

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1. ABSTRACT

The cellular prion protein PrP^C is the normal counterpart of the scrapie prion protein PrP^{Sc}, the main component of the infectious agent of transmissible spongiform encephalopathies (TSEs). It is a ubiquitous cell-surface glycoprotein, abundantly expressed in neurons, which constitute the targets of TSE pathogenesis. The presence of PrP^C at the surface of neurons is an absolute requirement for the development of prion diseases and corruption of PrP^C function(s) within an infectious context emerges as a proximal cause for PrP^{Sc}-induced neurodegeneration. Experimental evidence gained over the past decade indicates that PrP^C has the capacity to mobilize promiscuous signal transduction cascades that, notably, contribute to cell homeostasis. Beyond ubiquitous effectors, much data converge onto a neurospecificity of PrP^C signaling, which may be the clue to neuronal cell demise in prion disorders. In this article, we highlight the requirement of PrP^C for TSEs-associated neurodegeneration and review the current knowledge of PrP^C-dependent signal transduction in neuronal cells and its implications for PrP^{Sc}-mediated neurotoxicity

2. INTRODUCTION

The cellular prion protein PrP^C is membrane-anchored protein, whose pathogenic variant, termed PrP^{Sc} for scrapie isoform of the prion protein, is the main component of the transmissible agent of spongiform encephalopathies (TSEs). These fatal neurodegenerative disorders include Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker (GSS) syndrome, and fatal familial insomnia (FFI) in humans, as well as scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle (reviewed in (1)).

While present in all cell types, PrP^C is most abundantly expressed in neurons (2). It is located at the outer leaflet of the plasma membrane, to which it is attached by a glycosylphosphatidylinositol (GPI) moiety. PrP^C is subject to heterogeneous glycosylation on two Asn residues and may also undergo proteolytic cleavage. Both types of post-translational modifications yield a variety of isoforms whose distribution may differ according to the cell type, brain region or subcellular compartment considered (3-5). While knock-out studies were much expected to shed light on PrP^C function, mice devoid of PrP^C turned out to be viable and did not manifest any major abnormality (6). At closer examination, PrP^C-deficient mice exhibit increased susceptibility to various types of cerebral insults, including ischemia (7, 8) and seizures (9), and were recently shown to suffer from demyelinating disease (10).

In recent years, much effort has been devoted to unravel the physiological function of PrP^C at a cellular level (reviewed in (2)). A converging view is that PrP^C may exert diverse roles according to the cellular context and its local environment, notably in relation with its interaction with binding partners. Besides, much evidence now supports the notion that the recruitment of PrP^C within multimolecular complexes is associated with cell signaling events, some of which are restricted to neurons (2, 11). Here, we highlight the requirement of PrP^C for TSEs-associated neurodegeneration and review the current knowledge of PrP^C-dependent signal transduction in neuronal cells and its implications for PrP^{Sc}-mediated neurotoxicity.

3. PRP^C REQUIREMENT FOR PRP^{SC}-ASSOCIATED NEUROTOXICITY

Despite prion replication in many organs and tissues, TSE-induced pathology is restricted to the central nervous system, with widespread neuronal loss, spongiosis, gliosis, and accumulation of PrP^{Sc}. It is now quite well established that the autocatalytic conversion of PrP^C into PrP^{Sc} lies at the root of prion diseases. The central role played by PrP^C in the development of prion diseases was first illustrated by the observation that disruption of the PrP gene (*Prnp*) in mice confers resistance to prion disease and to the propagation of infectious prions (12). Conversely, PrP-overexpressing (tga20) mice exhibit reduced incubation periods as compared to wild-type mice (13). Neurograft experiments carried out on $PrP^{0/0}$ mice using tga20 mice as brain tissue donors allowed to clearly demonstrate that the presence of endogenous PrP^C is mandatory for PrP^{Sc} to induce pathological alterations (14). Mallucci *et al.* further showed that switching off PrP^C neuronal expression in infected mice, just prior to the clinical phase, blocks TSE pathogenesis, although abundant prion replication still occurs in glial cells (15). These experiments outline that prions require neuronal PrP^C to exert their toxicity. This notion was reasserted and refined in a study based on transgenic mice expressing a GPI anchor-less PrP^C (ΔGPI-PrP) (16). Indeed, ΔGPI-PrP infected mice were found to efficiently replicate scrapie and accumulate high levels of PrP^{Sc} in their brains without any sign of clinical illness. As a whole, these data argue for a primary role of neuronal, GPI-anchored PrP^C in prion neuropathogenesis.

How may corruption of GPI-anchored PrP^C-associated function(s) in neurons, as a result of the conversion of PrP^C into PrP^{Sc}, account for prion-associated toxicity? An ongoing debate in the TSE field is whether PrP^{Sc} triggers a loss of PrP^C physiological function (loss-of-function hypothesis) or promotes a gain of toxic activity (gain-of-function hypothesis), or both (17). This obviously calls for a better understanding of the function held by PrP^C in a neuronal context.

4. ON THE ROADS TO SIGNALING FUNCTION : PRP^C LIGANDS, COPPER BINDING, KNOCK-OUT AND CROSSLINKING

4.1. Insight from PrP^C partners

That PrP^C may act as a receptor or a coreceptor was initially suspected from its location at the cell surface and its orientation towards the extracellular space (18). However, experiments aiming at identifying a soluble ligand of this protein did not succeed. Analyses of the PrP^C fold obtained by RMN also failed to reveal any significant structural relationship with well-known receptors, thus hindering the identification of specific ligands for PrP^C that would be able to stimulate the protein and recruit signaling effectors. To overcome this limitation, several methodologies and strategies were designed to probe for PrP^C partners (reviewed in (2)), including the yeast two-hybrid system (19, 20), chemical cross-linking (21, 22), co-purification with recombinant PrP (23), co-immunoprecipitation (24-26), complementary hydropathy (27), and, more recently, global approaches (28-31).

Among the diverse array of PrP^C partners identified thus far, only a few interactions have been shown to bear functional relevance (Table 1). A first class of ligands are involved in ECM adhesion, neuritic extension and maintenance. Among these is the ECM protein laminin (23), a major constituent of the neuronal basal lamina. The PrP^C-laminin interaction was shown to sustain neurite outgrowth (32), and at functional level, to promote memory consolidation through PKA and ERK-dependent pathways (33). Of note, we recently introduced the tissue-non specific alkaline phosphatase (TNAP) as a novel protagonist in the PrP^C-laminin interplay (26). TNAP specifically interacts with PrP^C in the rafts of differentiated bioaminergic neuronal cells. By controlling the phosphorylation state of laminin, TNAP impacts on its

Table 1. PrP^C signaling partners in neuronal cells

PrP^C signaling partner	Identification methodology	Associated functions	Signaling effectors	References
Laminin	Co-purification with recombinant PrP Interactome	Neurite outgrowth Memory consolidation	PKA; ERK	23, 29, 30, 33
TNAP	Co-immunoprecipitation	Neurite maintenance	laminin	26
Vitronectin	Overlay assay Co-localization	DRG axonal outgrowth		35
NCAM	Chemical cross-liking Interactome	Neurite outgrowth	Fyn	21, 29, 30, 36
LR/LRP	Yeast two-hybrid system Co-immunoprecipitation Interactome	PrP^C endocytosis		19, 30, 38
LRP1	Coimmunoprecipitation	PrP^C endocytosis		39-41
Glypican 1	Coimmunoprecipitation Co-localization	PrP^C endocytosis		42, 43
PrP^C	Yeast two-hybrid system	Stress protective activity		44, 59
STI-1	Complementary hydropathy	Neuronal survival Neuritogenesis Long Term Potentiation	PKA; ERK	48 for review
Caveolin	Co- immunoprecipitation	Neuronal survival Synaptic plasticity	Fyn; NADPH oxidase; ERK; CREB	11, 24, 80
Amyloid Abeta oligomers	Expression cloning Co-localization	Synaptic dysfunction		31

TNAP: Tissue-Non specific Alkaline Phosphatase; NCAM:Neuronal Cell Adhesion Molecule; LR: Laminin Receptor; LRP: Laminin Receptor Precursor; LRP1: LDL Receptor-1

ability to interact with PrP^C (26) and to support neurite maintenance (our unpublished observations). An additional potential player in the PrP^C-laminin complex is the transmembrane protein β-dystroglycan depicted both as a PrP^C (25) and laminin (34) partner, which joins the extracellular matrix to the cell cytoskeleton. A less well-studied PrP^C interaction is that to vitronectin, also involved in axonal growth (35). Finally, the association of PrP^C with NCAM in hippocampal neurons promotes the recruitment of both proteins to rafts and Fyn-dependent neurite outgrowth (36).

Other interactors may contribute to endocytosis of PrP^C, and that of its pathogenic isoform. A salient example is the laminin receptor (LR) and its precursor (LRP) (19, 37). The pathophysiological implications of the LRP/LR interaction with PrP^C have been reviewed elsewhere (38). Two other potently important players in PrP^C endocytosis are the transmembrane protein LRP1 (39-41) and the GPI-anchored heparan sulfate proteoglycan glypican 1 (42, 43). How these proteins may contribute to the induction and/or desensitization of PrP^C-dependent signal clearly deserves further investigation.

It is also of note that PrP^C may interact with itself (44), supporting the possible occurrence of cis or trans interactions, or the binding of soluble PrP to cell-attached counterparts. Soluble PrP^C may alternatively serve as a ligand for (a) yet-to-be-identified receptor(s). This hypothesis is notably supported by the work of Chen *et al* (45) showing that recombinant dimeric PrP^C (PrP-Fc) activates signal transduction pathways sustaining neuroprotection and neurite outgrowth in primary cerebellar neurons. While Kanaani and coworkers confirmed the induction of neuronal polarity and synapse formation following exposure of primary hippocampal neurons to recombinant PrP^C (46), the nature of the cell surface receptors relaying this effect still is elusive.

One candidate is the stress-inducible 1 (STI-1) protein. Following its identification as a putative PrP^C receptor (27), this protein has been further characterized as a soluble trophic factor released from astrocytes (47), whose interaction with PrP^C promotes neuronal survival and neuritogenesis through PKA and ERK-dependent pathways (reviewed in (48)).

The growing list of PrP^C interactors now includes the Alzheimer's disease associated peptide A-beta (31). While debated (49), the notion that PrP^C may serve as a cell-surface receptor for A-beta clearly raises new prospects as to prion and A-beta-mediated signaling and toxicity.

Finally, global approaches have recently allowed to grasp the prion protein interactome in *in vitro* and *in vivo* conditions, to define PrP^C as a component of multiprotein complexes and to enlarge the PrP^C signalosome (28-30, 50). In line with a role for PrP^C in the maintenance of myelin integrity (10), novel PrP^C interactors include families of neuronal glycoproteins and myelin-associated proteins (29). Two metal ion transporters of the ZIP family have also been identified as PrP^C partners (50). ZIP6 and ZIP10 display a PrP-like amino acid sequence within their N-terminal extracellular domain, thus supporting the view of an involvement of PrP^C in the transmembrane transport of divalent cations. Of note, bioinformatics tools reveal that the complex molecular network of PrP^C and interactors has connections with AKT, JNK and MAPK signaling pathways that are essential for cell survival, differentiation, proliferation or apoptosis (28).

4.2. Copper binding and cleavage

Beyond its interaction with diverse partners, several lines of evidence suggest that the PrP^C-dependent recruitment of cell signaling cascades may be promoted by its binding to copper ions and subsequent cleavage. The

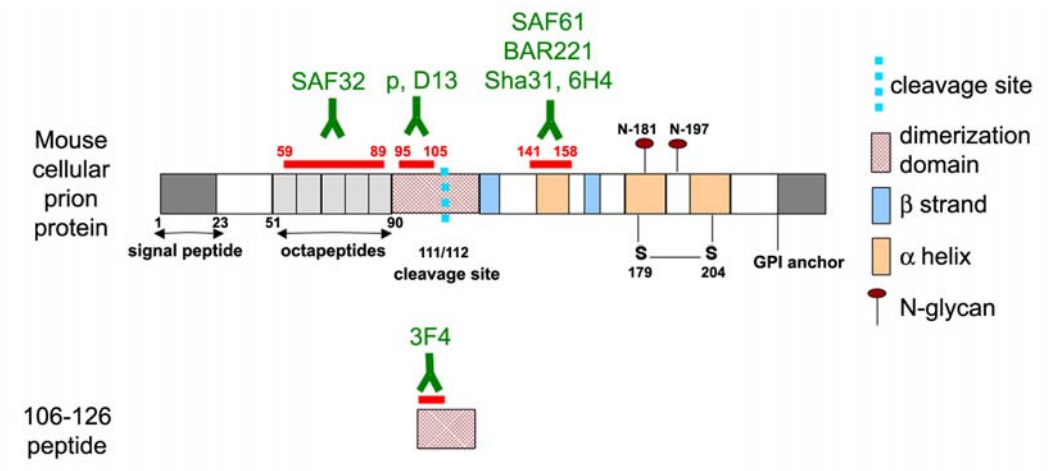

Figure 1. PrP^C primary structure, functional domains and epitope mapping of anti-PrP antibodies. Antibodies used to trigger cellular signaling recognize a C-ter epitope (between residues 59 and 89 for SAF32) or an N-ter epitope (between residues 141 and 158 for SAF61, Bar221, Sha31 and 6H4 antibodies) of the cellular prion protein. The p and D13 antibodies, which induce neuronal cell apoptosis, target the hydrophobic region 95-105. The 3F4 antibody recognizes the N-ter region of the PrP106-126 peptide and does not cross react with mouse PrP^C

interplay between PrP^C copper binding, cleavage and signaling has been extensively reviewed in Haigh *et al* (51) and will not be discussed further here. Of note, these aspects of PrP^C signaling may be connected with its interaction with various partners, as notably suggested from its association with the cell surface metalloprotease ADAM 23 (52) and discussed by (51).

4.3. The knock-out approach

While the knock-out of PrP in mice did not yield any significant contribution to the elucidation of prion-associated signaling, it has nevertheless led to the isolation of neuronal cell lines devoid of PrP^C, which have allowed to shed some light on PrP^C-associated signal transduction (reviewed in (53)). A PrP-null hippocampal cell line was notably exploited to establish a correlation between the expression level of PrP^C and PI3 kinase activity (54). The lack of PrP^C expression appears to be also associated with increased sphingomyelinase activity (55).

In contrast to the mouse model, a clear-cut loss-of-function phenotype was documented in the zebrafish (56). Unlike mammals, the zebrafish possesses two prion protein homologues. Knock-down of PrP-1 causes developmental arrest at the gastrulation stage, with defects in src kinase-dependent calcium signaling and cell adhesion. Besides, an involvement in neuronal development is specifically described for PrP-2. The signaling cascades sustaining the neurogenic role of PrP-2 remain to be elucidated (57).

4.4. The crosslinking paradigm

To bypass the multiplicity of PrP^C partners and assess intracellular signaling events associated to this protein, we designed an antibody-based strategy (Figure 1) to promote the clustering of PrP^C and thereby trigger an input signal (24). It is now quite well established that PrP^C crosslinking at the surface of neuronal or non neuronal cells (11, 58) promotes the recruitment of signaling cascades. Whether antibody-mediated ligation mimics the interaction with a partner (see above) or PrP^C dimerization (59), it allows to transiently freeze a PrP^C-containing signaling platform able to activate sub-membrane effectors. Of note, this property is intimately connected with the location of PrP^C within rafts, as further discussed in Chapter 6 and reviewed in (60).

5. PRP^C SIGNALING IN NEURONS : A COMBINATION OF UBIQUITOUS AND NEUROSPECIFIC EFFECTORS AND PATHWAYS

Understanding the neurospecificity of prion protein signaling is experimentally challenging since it necessitates the availability of appropriate *in vitro* models. Our own strategy has been to exploit an inducible neuroectodermal cell line (1C11), able to convert into fully functional serotonergic (1C11$^{5\text{-HT}}$) or noradrenergic (1C11^{NE}) neuronal cells upon induction, in a homogenous and synchronous manner (61). 1C11 cells endogenously express PrP^C at comparable levels whatever their differentiation state (62), and thus represent a valuable tool to analyze PrP^C function in relation with the onset, maintenance and homeostasis of a neuronal phenotype. Based on our overall findings obtained with the 1C11 cell line and those from the literature, our current view is that PrP^C signaling within a neuronal context shares commonalities with that in other cell types. It also bears neuronal specificity, that may depend on isoforms, partners, cell compartmentation, neuronal polarity (see Chapter 6) and may account for the neurotoxicity of pathogenic prions.

5.1. NADPH oxidase activation, Reactive Oxygen Species production and redox equilibrium

NADPH oxidase was identified as an intracellular effector recruited upon antibody-mediated PrP^C ligation in 1C11 precursor cells and their neuronal

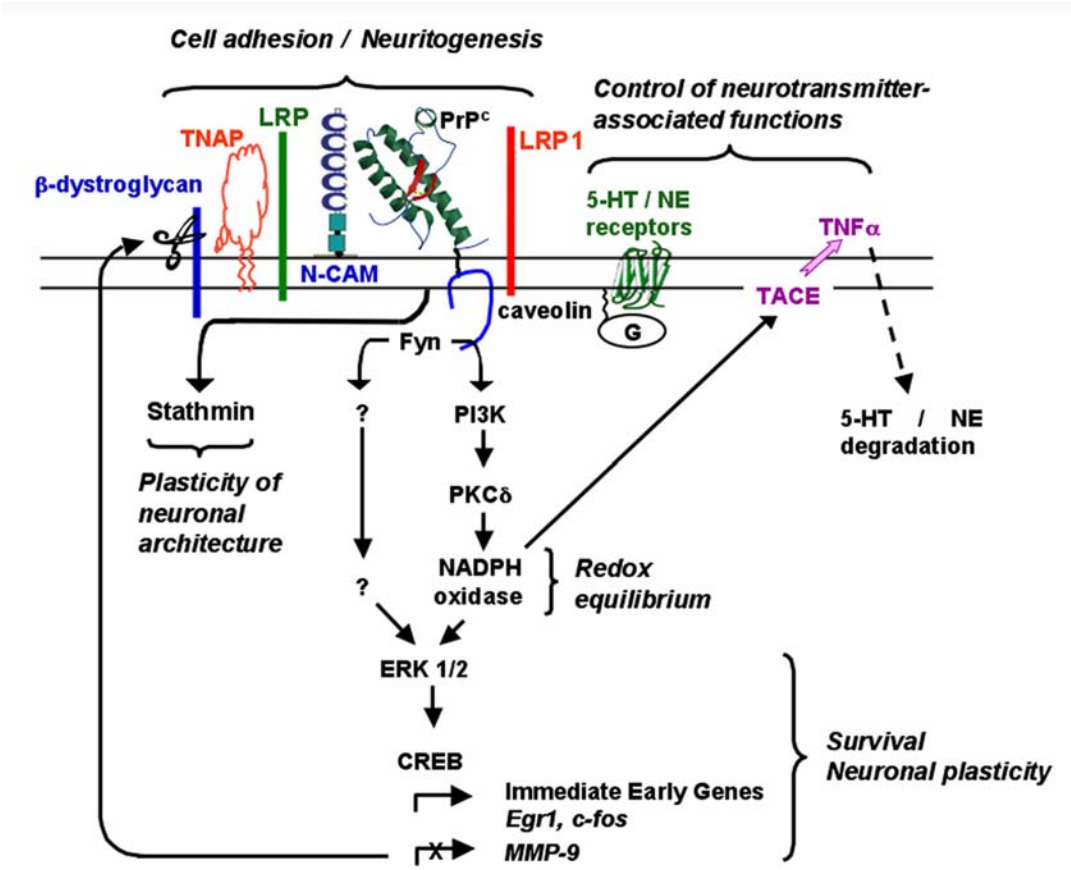

Figure 2. PrP^C signaling network in neurons. PrP^C associates with diverse partners within signaling complexes. PrP^C interactors include extracellular matrix binding proteins, the GPI-anchored phosphatase TNAP, neurotransmitter receptors and the membrane protein caveolin. By activating multiple downstream effectors, PrP^C contributes to redox equilibrium, control of neurotransmitter-associated functions, neurite outgrowth and maintenance, neuronal survival and plasticity.

progenies but also in GT1-7 neuroendocrine cells and BW5147 T lymphocytes (63). NADPH oxidase is an oligomeric enzyme composed of a catalytic core (NOX) and adapter subunits ($p47^{PHOX}$, $p67^{PHOX}$, $p22^{PHOX}$, rac) that assemble at the plasma membrane or at the surface of endosomes and catalyzes the conversion of O_2 into superoxide anion $O_2^{\cdot-}$ (64). Both $p47^{PHOX}$ and $p67^{PHOX}$ subunits are phosphorylated following PrP^C ligation (63). Although the detailed mechanisms through which PrP^C activates NADPH oxidase remain unclear, we depicted an involvement of PI3 kinase (PI3K) and Protein kinase C delta (PKC delta) (11) in the PrP^C-dependent control of NADPH oxidase (Figure 2).

In contrast to oxidative stress conditions where Reactive Oxygen Species (ROS) saturate antioxidant defenses, the PrP^C-induced production of ROS by NADPH oxidase is transient and devoid of toxicity. The ubiquitous PrP^C-NADPH oxidase-ROS cascade thus introduces PrP^C as an important regulator of cellular redox equilibrium.

The free radical properties of the superoxide anion $O_2^{\cdot-}$ and other ROS derived from $O_2^{\cdot-}$ allow ROS to covalently modify macromolecular molecules, like proteins, lipids and nucleic acids (65). As second message signals, ROS have emerged as regulators of many redox-sensitive protein activities. Accordingly, our search for downstream targets of the PrP^C-ROS coupling has lead to the identification of the MAP kinases ERK1/2 (63) and the metalloprotease TACE (66) as effectors of PrP^C signaling.

5.2. ERK1/2 MAP Kinases, cell survival and homeostasis The ERK1/2 MAP kinases have been documented as targets of PrP^C signaling in many different cell lines and experimental paradigms. A PrP^C-ERK1/2 coupling was notably evidenced in the 1C11 neuronal cell line (63), primary neurons (45, 67), neuroendocrine cells (63, 68) and various types of immune cells (58, 63). Diverse strategies have converged onto a biochemical link between PrP^C and ERK1/2 activation, including antibody-mediated ligation of PrP^C (63, 68), cell exposure to recombinant PrP-Fc (45), soluble STI-1 (69) or blockade of the PrP^C-laminin interaction *in vivo* (33). It is of note that, in physiological settings, PrP^C does not couple to JNK and p38, two stress-associated protein kinases that appear to contribute to the neurotoxicity of pathogenic prions or their PrP106-126 peptide mimetics (63, 70, 71).

At a mechanistic level, relatively few studies have addressed the issue of the molecular cascades relaying the PrP^C-dependent activation of ERK1/2. We were able to show an involvement of ROS, downstream from NADPH oxidase, in the control of ERK1/2 phosphorylation. This fits in with the notion that ROS, as signaling transducers, promote kinase activation and trigger phosphatase inhibition (72, 73). While ROS thoroughly control ERK1/2 activation in non-neuronal cells, they only partly contribute to the phosphorylation of these MAP kinases in a neuronal context (63). This prompted us to postulate the recruitment of a neuronal-specific cascade downstream from PrP^C that would additionally converge onto ERK1/2 (Figure 2). This second cascade may involve the Shc-Grb2/Sos-Ras-Raf pathway, whose role in ERK1/2 activation has been largely documented (74, 75). In this respect, it is of note that PrP^C binds Grb2 in neuronal cells (20).

The functional link between PrP^C and the pleiotropic ERK1/2 MAP Kinases (76) strengthens the view that PrP^C participates to cell survival and homeostasis and that this pathway may account in part for the ubiquitous protective role exerted by PrP^C against diverse insults (77).

5.3. The CREB Transcription factor

The contribution of ERK1/2 to cell homeostasis is assumed to be relayed by transcription factors including AP1, Elk-1, NF-κB or CREB. CREB, for cAMP-responsive element binding protein, is activated in response to several stimuli, including growth factors, hormones, inflammatory cytokines or neurotransmitters and triggers the transcription of genes involved in cell survival, differentiation, proliferation or neuronal plasticity (78, 79). With the 1C11 cell line, we identified CREB as a downstream effector of the PrP^C-ERK pathway, in both undifferentiated and neuronal cells (80).

The PrP^C-dependent activation of CREB results in the transcription of two immediate early genes (IEG) : egr-1 and c-fos (80) (Figure 2). Egr-1 and c-fos are transcription factors that relay the action of CREB in cell survival and proliferation (81). The identification of a functional link between PrP^C-CREB-Egr-1/c-fos in 1C11 precursor cells as well as in 1C11 neuronal derivatives indicates that PrP^C may exert a ubiquitous role in the control of cell homeostasis. CREB inhibition or c-fos deletion in mice alter nerve excitability and trigger neurodegeneration in stress conditions (82, 83). Besides, the signaling pathway CREB-Egr-1/c-fos is indeed involved in long-term potentiation and memory consolidation (84). Within a neuronal context, the coupling of PrP^C to CREB and PrP^C-induced egr-1/c-fos transcription may thus reflect some contribution of PrP^C to synaptic plasticity.

6. NEURONAL SPECIFICITY OF PRP^C SIGNALING

6.1. Neuronal specificity of PrP^C signaling : a matter of isoforms ?

Despite the identification of PrP^C-controlled signaling effectors (PI3 kinase, NADPH oxidase, ERK1/2, CREB) shared by neuronal and non-neuronal cells, much evidence points to a neurospecific action of PrP^C, which may be the clue to PrP^{Sc}-mediated neurotoxicity.

The neuronal signature of PrP^C signaling may be accounted for by the selective expression of some prion protein isoforms in particular neuronal compartments (85). In agreement, we observed differences in the set of PrP^C glycoforms expressed in the 1C11 neuronal cell line according to the differentiation state (86). However, there is no strategy to specifically monitor the cell signals imparted by a given isoform of PrP^C. The use of antibodies targeting various epitopes may help discriminate between some subsets of PrP^C (Figure 1). The comparable NADPH oxidase-dependent ROS production or ERK1/2 activation obtained with antibodies against either the carboxy- or the amino-terminus of PrP^C (63) leads us to propose that both truncated and full-length PrP^C are competent to transduce protective signals. Notwithstanding, the copper-binding N-terminal region of PrP^C may be critical for mobilizing some cell survival signals, as suggested from *in vitro* (54) and *in vivo* (87, 88) observations. The 95-125 central region further deserves special consideration. Indeed, not only does it overlap the cleavage site (Figure 1) but it also encompasses the dimerization domain (59). This may explain the massive apoptosis of hippocampal neurons observed upon *in vivo* cross-linking of PrP^C with antibodies targeting the 95-105 epitope of the protein (89). A critical role imparted by this PrP region may also be inferred from the very severe neurodegenerating phenotype observed in mice with a deletion in the central domain (Δ105-125 or Δ94-134) (90, 91).

The notion of neurospecific isoforms cannot be disconnected from that of partners. Indeed, in line with the notion that PrP^C may drive the assembly of multicomponent complexes at the cell surface allowing for the instruction of cell signaling events (2), a specificity of PrP^C-containing platforms according to the cell type and subcellular microdomain is very likely.

6.2. The PrP^C-Caveolin-Fyn signaling platform

A salient example of a cell-type specific formation of a PrP^C-containing complex is the implementation of a platform associating PrP^C, the raft component caveolin and Fyn, a member of the Src tyrosine kinase family, that is restricted to the neuronal progenies of the 1C11 cell line (24). In 1C11 cells, PrP^C, caveolin and Fyn are expressed at similar levels whatever the differentiation state, but their functional association is specific to cells that have acquired a complete mature serotonergic or noradrenergic neuronal phenotype. This restriction may in part relate to the onset of neuronal polarity since the coupling of PrP^C to Fyn only occurs in neurites of fully differentiated neuronal cells, while PrP^C expressed at the surface of the cell body weakly activates Fyn. One explanation for this neuronal and sub-cellular restriction may be the contribution of specific PrP^C isoforms or partners to the activation of Fyn. Another possible important parameter is the local membrane composition. The spatial organization of receptors, transporters, scaffold proteins… within microdomains

(caveolae, rafts) governs many aspects of signal transduction (92). The specific lipid composition of rafts indeed allows for stable and effective interactions between cell signaling partners. Beyond their importance for prion protein trafficking and conversion into PrP^{Sc}, lipid rafts indeed drive PrP^{C} signaling (reviewed in (60)).

A frequently raised issue is how GPI-anchored proteins may transduce intracellular signals. It is now acknowledged that the crosslinking of cell surface proteins by antibodies may induce the clustering of rafts and thereby initiate cell signaling events (60, 93). The raft constituent caveolin may play an important role in these processes (94). Accordingly, although it lacks any extracellular domain, converging evidence defines caveolin as a major relay in the PrP^{C}-dependent activation of Fyn (24, 67). For instance, immunosequestration of caveolin hinders the activation of Fyn in response to PrP^{C} (24). Also supporting the importance of rafts in PrP^{C} signaling is the observation that PrP^{C} recruits NCAM to lipid rafts to trigger Fyn activation and neurite outgrowth (36).

As summarized in Figure 1, the neurospecific PrP^{C}-caveolin-Fyn platform controls the recruitment of downstream ubiquitous effectors : PI3 kinase, NADPH oxidase, ERK1/2 and CREB (11, 63, 80). Fyn therefore appears as a proximal effector of PrP^{C} that orchestrates the repertoire of signaling cascades coupled to PrP^{C} in neurons.

6.3 Linking PrP^{C} signaling to synaptic plasticity : MMP-9 and beta-dystroglycan

A further aspect of PrP^{C} neurospecific action is associated with extracellular matrix remodeling. Recently, we documented a decrease in the level of MMP-9 encoding mRNAs following antibody-mediated ligation of PrP^{C} that is restricted to the neuronal progenies of the 1C11 cell line (80). The downregulation of MMP9 transcripts is accompanied by a raise in the mRNA level of TIMP-1, the main MMP-9 physiological inhibitor. While both events depend on the coupling of PrP^{C} to CREB, the mechanisms through which PrP^{C} interferes with MMP-9 transcription downstream from CREB remain elusive.

There are multiple potential targets of MMP9 in neurons, among which laminin, growth factors (95) and beta-dystroglycan (96). Beta-dystroglycan, is a transmembrane protein that bridges the extracellular matrix to the cell cytoskeleton and whose cleavage triggers a loss of cell adhesion to its substratum (97). Building upon the observation that beta -dystroglycan interacts with PrP^{C} in the brain (25), we established that PrP^{C} signaling protects beta -dystroglycan from cleavage by MMP-9, as a result of inhibition of MMP-9 transcription and reduced MMP-9-associated proteolytic activity (80). This may enhance the interaction between dystroglycan and laminin (34). By down-regulating MMP-9 expression and activity, it is likely that PrP^{C} contributes to the stability of cell/matrix interactions and improves synaptic activity.

Beyond the regulation of beta -dystroglycan cleavage, the PrP^{C}-dependent control on MMP-9/TIMP1 expression may also account for the neuroprotective role exerted by PrP^{C}. In cerebral ischemia, tissue damage is largely associated to an increase in MMP-9 activity (98). The size of infarct of the ischemic brain is reduced in MMP-9-null mice and, conversely, enlarged in PrP knockout mice (8, 99). On another hand, CREB activation, TIMP-1 expression or PrP^{C} overexpression facilitates neuronal recovery (7, 98, 100). The functional link between PrP^{C}, CREB and MMP-9 may thus constitute one of the facets of PrP neuroprotective action.

6.4. PrP^{C} and neuromodulation: TACE-dependent TNF-alpha shedding and bioamine metabolism

Starting from the coupling of PrP^{C} to ROS production, we recently identified the TNF-alpha Converting Enzyme (TACE) metalloprotease as a novel target of PrP^{C} signaling in neuronal cells (66). Indeed, TACE activation notably necessitates oxidoreduction reactions that unmask a catalytic cystein residue (101). TACE primarily catalyzes the cleavage and shedding of membrane-anchored pro-TNF-alpha into soluble TNF-alpha. Accordingly, antibody-mediated PrP^{C} ligation triggers a time-dependent accumulation of TNF-alpha in the surrounding medium of bioaminergic neuronal cells. TNF-alpha is essentially viewed as a proinflammatory cytokine but it may also act as a modulator of neuronal functions (102). It exerts pleiotropic roles through its binding to two distinct receptors : TNFR1, assumed to transduce pro-apoptotic signals, and TNFR2, associated with cell survival (103). The picture may in fact be more complex since TNF-alpha –mediated activation of TNFR1 can mobilize various signaling pathways resulting in either a survival or a death response, according to compartmentalization (104).

In our experimental paradigm, TNF-alpha shed upon PrP^{C} stimulation does not affect cell viability (66). In support of an action as a neuromodulator, we reported that TNF-alpha triggers the degradation of bioamine neurotransmitters in 1C11-derived neuronal cells.

All these cell signaling events are under the control of the PrP^{C}-caveolin-Fyn platform (Figure 2), indicating that TACE activation and TNF-alpha release occurs at the neurites of differentiated cells. The mechanisms by which TNF-alpha stimulates neurotransmitter catabolism remain unknown. Nevertheless, linking PrP^{C} to neurotransmitter metabolism provides additional support for a key role of PrP^{C} in the regulation of neuronal functions and synaptic activity. PrP^{C}-induced degradation of bioamines likely impacts on the content of neurotransmitter at the synapse and therefore modulates synaptic transmission. Increases in bioamine turn-over (*i.e.* variations in the concentration ratios of serotonin or norepinephrine *vs* their respective catabolites) in the hippocampus are assumed to be associated with a control of long term memory consolidation (105-107).

Thus, the identification of the PrP^{C} coupling to TACE-mediated TNF-alpha shedding and bioamine degradation defines a molecular basis that may account for the contribution of PrP^{C} to long-term memory consolidation processes (33, 108). Further, in view of the

relationship between synaptic plasticity and sleep (109), this cascade may also sustain the involvement of PrP^C in sleep regulation that is inferred from observation in $PrP^{-/-}$ mice (6, 109, 110).

6.5. The interaction with TNAP : physiological implications

In an alternative approach to get insight into the function exerted by PrP^C in neurons and in relation with its raft location, we sought to identify proteins that would specifically interact with PrP^C in rafts of the neuronal progenies of the 1C11 cell line. Raft isolation and co-immunoprecipitation allowed us to establish an interaction of PrP^C with the Tissue-Non specific-Alkaline-Phosphatase (TNAP) (26). This phosphatase has recently been recognized as an important enzyme in the central nervous system, which may contribute to neurotransmission (111). TNAP notably emerges as an important player in bioaminergic neurons since it indirectly contributes to the regulation of serotonin and norepinephrine synthesis by regulating the level of pyridoxalphosphate, a cofactor necessary to aromatic acid decarboxylase (AADC) activity (26).

We further assessed the functional outcome of the association of PrP^C with TNAP, focusing on the ecto-phosphatase activity of the enzyme. TNAP acts on phospho-laminin and, by maintaining laminin in a non-phosphorylated state, it favors the interaction of this ECM protein with PrP^C. In line with the notion that the binding of laminin to PrP^C supports neurite outgrowth, our unpublished data indicate that neurite retraction occurs when the PrP^C-laminin interaction is destabilized following an inhibition of TNAP activity.

6.6. From effectors to functions : neuroprotection, neurite outgrowth and plasticity, myelin maintenance

The neuroprotective action of PrP^C is well-described (2). PrP^C upregulation is notably proposed to sustain post-traumatic recovery of the ischemic brain (7, 8). The neuroprotection afforded by PrP^C appears to be imparted by its N-terminal region (88, 112), and may involve various effectors including PI3K and Akt (113). An interference with the p53 pathway has also been described (112). A neuroprotective action of PrP^C is also triggered in response to its binding to STI-1, (48), or through trans-interactions (45), both via PKA.

As already discussed, the interaction of PrP^C with various of its partners including laminin, NCAM, STI-1 and TNAP supports neurite outgrowth and maintenance (see Chapter 4). On another hand, several lines of evidence point to Fyn and the ERK pathway as important mediators relaying the role of PrP^C in neuritic elongation (36, 45, 46, 48). The PrP^C-induced phosphorylation of stathmin, which inhibits the microtubule destabilizing activity of the latter protein, may also mirror a role for PrP^C in the plasticity of the neuronal architecture (68).

Finally, studies in knock-out mice recently uncovered a role for PrP^C in the maintenance of myelin in peripheral nerves (10). The molecular pathways sustaining this action remain to be identified. In line with this, PrP^C appears to form complexes with myelin-associated proteins (29).

6.7. PrP^C and copper

Work over the past fifteen years has unveiled a very complex interplay between copper, PrP^C and signaling (reviewed in (51)). Copper may notably influence PrP^C expression, cleavage, interaction with partners and, thereby, signaling. In neuronal cells, it has been proposed that PrP^C may regulate the concentration of copper at the synapse (114). In addition, PrP^C appears to protects neurons against copper toxicity through its capacity to redox cycle divalent copper into monovalent copper ions (reviewed in (51)).

6.8. Calcium homeostasis

A link between PrP^C and calcium influxes has been established in various cell types including neurons and lymphocytes (reviewed in (2)). In the zebrafish embryo, the PrP-mediated increase in intracellular calcium is relayed by src kinases and sustains cell adhesion (57). Similarly, the src kinase Fyn controls calcium influxes downstream from PrP^C in hippocampal neurons (115). Besides, hippocampal neurons from $PrP^{-/-}$ mice exhibit a reduced calcium influx via L-type voltage-gated calcium channels (VGCCs), as compared to PrP-expressing neurons (116). The involvement of PrP^C in calcium homeostasis is again consistent with a role in synaptic transmission and plasticity. At present, the neuronal response to the PrP^C-mediated calcium signals is however still unclear. Diverse outcomes may be investigated, including regulation of the activity of calcium sensing proteins, calcium-dependent gene transcription, or functional interaction with other cell signaling pathways (117).

6.9. The GPCR connection

As mentioned, the cellular prion protein concentrates within specialized membrane microdomains (60), where its interaction with caveolin is critical for instructing downstream cell signaling events in neuronal cells (see Chapter 6.2). Rafts also play a major role in neurotransmitter receptor signaling (118). Assuming that PrP^C might reside in close proximity to neurotransmitter receptors within lipid rafts, we exploited the 1C11 cell system to probe an impact of antibody-mediated engagement of PrP^C on the signaling activity of the serotonergic receptors present at the cell surface of $1C11^{5\text{-}HT}$ neuronal cells. These three serotonergic receptors (5-$HT_{1B/D}$, 5-HT_{2B} and 5-HT_{2A}) are metabotropic receptors coupled to G-proteins (GPCR). Most, but not all, of their effectors are recruited downstream from G-proteins. We observed a neutral effect of PrP^C ligation on the couplings of the 5-HT_{2B} receptor subtype to neuronal nitric oxide synthase (nNOS), which is G-protein independent (119), and to NADPH oxidase, whose intermediates are still unclear (66). In contrast, concomitant PrP^C ligation and agonist stimulation of each receptor impacts on the G-protein couplings of the receptor (119). The positive or negative action of PrP^C depends on the G-protein and the pathway considered. For instance, PrP^C ligation downregulates the Gi-mediated function of the 5-$HT_{1B/D}$ receptor. It also impairs the 5-HT_{2A} receptor-dependent

phospholipase C (PLC)-inositol trisphosphate (IP3) response, which is relayed by Gq proteins. This inhibitory effect is somewhat reminiscent of the defects in acetylcholine receptors coupling to Gq induced by accumulation of the A-beta amyloid peptide (120). On another hand, PrP^C enhances the Gz-dependent coupling of the $5\text{-}HT_{2B}$ receptor to phospholipase A2 (PLA2) (119). By modulating the signaling activity of the three serotonergic receptors, PrP^C affects the cross-talks occurring between the receptors. *In fine*, the function of $5\text{-}HT_{2B}$ receptors is enhanced while that of $5\text{-}HT_{1B/D}$ receptors is blocked. Because $5\text{-}HT_{1B/D}$ receptors act at a presynaptic level to limit neurotransmitter release (121), our hypothesis is that PrP^C reinforces neuronal activity.

As observed for its proper neurospecific couplings (see Chapter 6.2), the modulatory effect of PrP^C on serotonergic GPCR function is restricted to mature neuronal cells and caveolin-dependent (119). The involvement of caveolin allows to propose some mechanistic explanation for the action of PrP^C. Caveolin interacts with diverse protagonists of GPCR signaling, including GPCRs themselves (122), G proteins (123), or GPCR-related kinases (GRK) (124). The spatio-temporal availability of proximal effectors (e.g. G proteins) and other partners of signaling, such as arrestins, GRKs, or regulators of GPCR signaling (RGS) greatly influences the duration and strength of GPCR signaling (125). By mobilizing caveolin, PrP^C ligation most likely interferes with the recruitment of such GPCR partners and *in fine* modulates the intensities and/or dynamics of G protein activation by the agonist-bound serotonergic receptors.

Whether PrP^C exerts similar regulatory action on other types of GPCR in different neuronal populations clearly deserves further attention. Notwithstanding, our data obtained in serotonergic neurons fully fit in with *in vivo* evidence for an involvement of PrP^C in the homeostasis of the brain serotonergic system (110).

7. PRP^{SC} NEUROTOXICITY : EVIDENCE FOR A SUBVERSION OF PRP SIGNALING

7.1. Prion-induced neurotoxicity : a loss of neuroprotective signals ?

Our understanding of the mechanisms underlying PrP^{Sc}-induced neurodegeneration still is far from complete. In view of the requirement of PrPC for the neurotoxic action of PrPSc, as summarized in chapter 3, it is now admitted that neuronal demise in TSEs primarily originates from alterations of PrPC function(s). The « loss-of-function hypothesis » posits that PrPSc behaves as a dominant negative and disrupts PrPC signaling. This scheme accommodates well with the increased sensitivity of PrP-depleted neurons to various types of insults, as compared to PrP-expressing counterparts (reviewed in (126)). The loss of PrP^C neuroprotective function in an infected context may also be accounted for by the inability of PrP^{Sc} to undergo proteolytic cleavage at position 111/112 (127) and thereby to generate the PrP N-terminal fragment endowed with neuroprotective activity (112).

It is generally claimed that the loss-of-function hypothesis cannot fully account for PrP^{Sc}-mediated neurodegeneration since PrP knockout mice are viable and do not exhibit any sign of brain neurodegeneration (128). A more severe phenotype of PrP null mice may nonetheless be masked by regulatory mechanisms that compensate PrP^C silencing during embryogenesis and development. Irrespective of compensation, using behavioral tests in mice, we observed that some of the deficits progressively induced by prion infection recapitulate phenotypic changes monitored in PrP knock-out animals (110). It is finally noteworthy that the neuroprotective role exerted by PrP^C *in vivo* mostly manifests under stress conditions (6). Whether prion infection increases the vulnerability to brain insults such as ischemia or stroke has not been tested so far.

7.2. Evidence towards the gain of function hypothesis

Comparatively to the loss of function hypothesis, there is much more experimental evidence to support the gain of function model. In this scheme, it is proposed that PrP^{Sc} exacerbates PrP^C signals to toxic levels (17). Mechanistically, this could arise from an increased stability of PrP^{Sc}-containing signaling complexes. This is directly supported by *in vivo* observations obtained in transgenic mice overexpressing wild type PrP^C by 5 to 10-fold (129). In these mice, the aggregation of normal PrP^C is associated with synaptic loss and a neurological disease that is not transmissible. Besides, in line with the role of rafts in normal PrP^C signaling (see Chapter 6.2 and (60)), drugs that disrupt the integrity of rafts impair the toxicity of prions in neuronal cells (130). It is further proposed that the infection of neurons triggers a durable aggregation of signaling platforms within rafts where PrP^C is replaced by its pathogenic isoform (131).

7.2.1. Can the gain-of-function hypothesis be tested *in vivo*?

The validity of the gain of function hypothesis is difficult to address *in vivo*, notably because TSEs are chronic disorders with very long latencies. Global analyses indicate that the changes induced by prions in the brain vary according to the stage of disease incubation and to the prion strain (132). In regard to the effectors and pathways of PrP^C signaling in neuronal cells (Chapter 6), it is noteworthy that increases in the activation states of Fyn (133) and ERK1/2 (134) in the brains of infected mice have been reported. Finally, the occurrence of oxidative stress in prion diseases is well-established, both in animal models and patients (135-138). The primary origin of uncontrolled ROS production cannot however be traced *in vivo*. Other pathways (NFkappaB (139), PLD, (140)) are altered in prion infected mice and may provide candidate targets of PrP^C normal signaling in neurons.

7.2.2. Lessons from the Prion peptide PrP 106-126

Cellular models offer an alternative approach to overcome the complexity of *in vivo* studies. In some prototypic assays, the toxicity of PrP^{Sc} is mimicked by the PrP106-126 prion peptide. PrP106-126 shares several physicochemical properties with PrP^{Sc}, including a beta-rich structure and the capacity to aggregate. Our work with the 1C11 cell line has allowed to shed some light on the

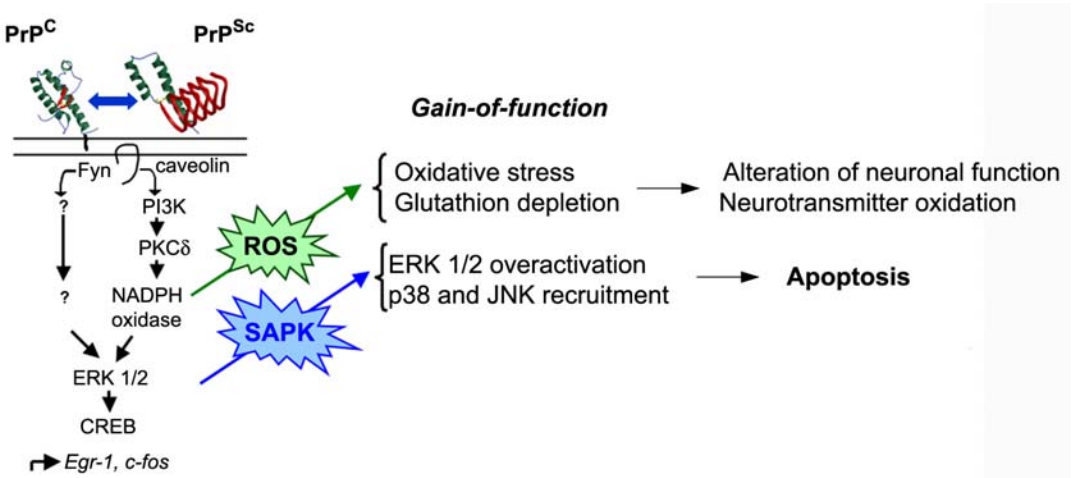

Figure 3. Deregulation of PrP^C signaling within an infected context, according to the gain-of-function hypothesis. PrP^{Sc}-mediated recruitment of PrP^C signaling pathways triggers an overactivation of proximal cell signaling targets, leading to oxidative and cellular stresses. related Protein 1; STI-1: STress-Inducible protein 1 (Numbering corresponds to amino acid position in the mouse PrP^C)

detrimental events triggered by PrP106-126 (70). In line with the neurospecificity of PrP^C signaling, the toxicity of PrP106-126 to 1C11 cells is restricted to neuronal serotonergic or noradrenergic progenies and is relayed by PrP^C and its downstream effectors (70). Notably, PrP106-126 triggers a NADPH oxidase-dependent chronic production of ROS that saturates the cellular antioxidant capacity. These oxidative stress conditions are associated with the prolonged and sustained phosphorylation of ERK1/2, the activation of the stress-activated kinases JNK and p38 and cell apoptosis (70). Similar cascades appear to operate in primary cortical neurons (71) and SH-SY5Y neuroblastoma cells (141, 142).

If PrP106-126 allows to unveil some mechanisms of prion-induced neurotoxicity, it obviously cannot fully mimic PrP^{Sc}-induced neurodegeneration. PrP106-106 is neurotoxic but not infectious and the kinetics of cell events induced by the prion peptide are not compatible with the high latency of prion diseases that may range between a few months up to several decades. Rather, the cascades monitored in cells exposed to PrP106-126 may reflect the acute response to pathogenic prions.

7.2.3. The contribution of chronically-infected cells

Despite the availability of diverse cellular models of prion infection (143), there is still little information on the cellular and molecular impact of pathogenic prions on neuronal physiology. In GT1-7 neuroendocrine cells, infection is associated with reduced viability (144) and increased susceptibility to oxidative stress (145). Primary cerebellar granule neurons infected by several prion strains also exhibit increased rates of apoptosis (146). The ability of neurospheres to propagate prions (147-149) may offer a promising tool to assess prion-induced cellular dysfunctions. The 1C11 neuronal cell line also can replicate several prion strains (150). In these cells, endogenous PrP^C expression is sufficient to sustain chronic infection. PrP^{Sc} accumulation does not induce any major phenotypic change in 1C11 undifferentiated cells. Prion-infected 1C11 cells retain the ability to engage into serotonergic or noradrenergic differentiation programs. However, we monitored specific alterations of the overall neurotransmitter-associated functions in the bioaminergic neuronal progenies of 1C11 infected cells. The decreases in neurotransmitter synthesis, storage and transport and the increases in neurotransmitter catabolism are reminiscent of the serotonergic dysfunctions reported in TSE-affected animals or patients (110, 151). Of note, we detected the production of oxidized derivatives of either serotonin or catecholamines that are classified as neurotoxins and alter neuronal function (150). The formation of serotonin and catecholamine oxidation products clearly mirrors elevated levels of ROS in prion-infected neuronal cells. We favor the view that the enhanced ROS level originates from a deviation of the PrP^C coupling to the ROS-generating enzyme NADPH oxidase (Figure 3) (63).

A subversion of the signaling pathways downstream from the PrP^C-caveolin-Fyn complex on neuronal processes would be in line with the neuritic transport of PrP^{Sc} in infected cells (152). It could also account for the occurrence of synaptic dysfunction and dendritic atrophy prior to neurodegeneration in TSEs (153).

8. CONCLUSIONS AND FUTURE DIRECTIONS

Considerable advance has been made over the past years on the relationship between the toxicity of PrP^{Sc} and the normal function of its cellular counterpart PrP^C in neurons. The binary model according to which PrP^{Sc} may promote either a loss or a gain of PrP^C function is clearly oversimplistic. Indeed, it does not take into account the multiplicity of PrP^C isoforms, which may not all be equivalent substrates for conversion into PrP^{Sc}. The relative contribution of a given isoform to the overall signaling events associated to PrP^C is another important parameter,

which is very difficult to challenge experimentally. Adding to this complexity is the notion that the subset of PrP^C species eligible for PrP^{Sc} formation varies according to the prion strain. Finally, in view of the diversity of PrP^C partners, it is likely that the conversion into PrP^{Sc} exerts promiscuous impact on the PrP^C interactome. For all these reasons, a combination of loss and gain of PrP^C activity upon prion infection appears more realistic. The outcome of PrP^{Sc} accumulation on PrP^C function may not only depend on the isoform, signaling complex, effectors and cell type considered. It may also evolve in time and space, as notably suggested by longitudinal studies in infected animals at a global scale.

Despite the progress achieved, there are still many questions to be answered. What is the significance of the plethora of PrP^C isoforms? Why can PrP^{Sc} be found in normal brain without any sign of pathology (153)? How can we reconcile the critical role of the caveolin-Fyn platform in PrP^C signaling and the normal transmission of TSEs to mice deficient for caveolin or Fyn (154)?

As highlighted in this review, the continuous synergy of multidisciplinary *in vivo* and *in vitro* approaches will be mandatory for deciphering the complexity of the mechanisms sustaining prion-induced neurodegeneration. The reciprocal and evolving dialogue between infected neurons and the surrounding glia cells is an important issue to address. Another challenge is to diagnose some of the prion-induced brain dysfunctions at a stage when therapy can be effective.

This quest in fact extends beyond the TSE field. Intricate networks of cellular dysfunctions –altered neurotransmitter signaling, loss of neuronal homeostasis, oxidative stress, microglial activation, synaptic deficits- are at play in other neurodegenerative diseases (155). Dissecting the molecular pathways sustaining prion-induced neurotoxicity may thus have implications for amyloid- and aging-associated brain disorders.

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Abbreviations: CREB : cAMP-responsive element binding protein, ERK1/2 : extracellular-regulated kinases 1 and 2, GPCR : G-protein coupled receptor, MAPK : mitogen activated protein kinase, NADPH oxidase : nicotinamide adenine dinucleotide phosphate-dependent oxidase, NE :norepinephrine, PI3K : phosphatidylinositol 3 kinase, PKC : protein kinase C, PrP : prion protein, PrP^{C} : cellular prion protein, PrP^{Sc} : scrapie prion protein, ROS : reactive

oxygen species, TACE : TNF-alpha Converting Enzyme, TNF-alpha : Tumor Necrosis Factor-alpha, 5-HT : 5-hydroxytryptamine.

Key Words: Prion, Signaling, Infection, Neuron, Review

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