Cellular Prion Protein Signaling in Serotonergic Neuronal Cells

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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. Taking advantage of the 1C11 neuroectodermal cell line, endowed with the capacity to convert into $1C11^{5-HT}$ serotonergic or $1C11^{NE}$ noradrenergic neuronal cells, allowed us to ascribe a signaling function to PrP^{C}. Antibody-mediated ligation of PrP^{C} recruits transduction pathways, which involve nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent reactive oxygen species production and target the extracellular-regulated kinases ERK1/2. In fully differentiated cells only, these effectors are under the control of a PrP^{C}-caveolin-Fyn platform, located on neuritic extensions. In addition to its proper signaling activity, PrP^{C} modulates the agonist-induced response of the three serotonergic G protein–coupled receptors present on the $1C11^{5-HT}$ differentiated cells. The impact of PrP^{C} ligation on the receptor couplings depends on the receptor subtype and the pathway considered. The implementation of the PrP^{C}-caveolin complex again is mandatory for PrP^{C} to exert its action on 5-HT receptor signaling. Our current data argue that PrP^{C} interferes with the intensities and/or dynamics of G protein activation by agonist-bound 5-HT receptors. By mobilizing transduction cascades controlling the cellular redox state and the ERK1/2 kinases and by altering 5-HT receptor-mediated intracellular response, PrP^{C} takes part in the homeostasis of serotonergic

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neuronal cells. These findings may have implications for future research aiming at understanding the fate of serotonergic neurons in prion diseases.

KEYWORDS: **GPCR; serotonergic neurons; prion protein; caveolin**

INTRODUCTION

The prion protein PrP^{C} is a normal ubiquitous glycoprotein, 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-Schinker (GSS) syndrome, and fatal familial insomnia (FFI) in humans, as well as scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle.[1] TSE-induced pathology is confined to the central nervous system, with widespread neuronal loss, spongiosis, gliosis, and accumulation of PrP^{Sc}.

In the field of prion research, much efforts aim at unraveling the mechanisms sustaining disease transmission and pathogenesis. It is, for instance, well established that prion propagation strictly depends on the presence of PrP^{C} since prnp knockout mice are resistant to prion infection.[2] More importantly, expression of PrP^{C} at the cell surface of neurons appears to be mandatory for prions to exert their toxicity.[3,4] It is now widely assumed that TSE-associated neurodegeneration involves some corruption of PrP^{C} normal function in neurons. This obviously calls for a better understanding of the function held by PrP^{C} in a neuronal context.

While present in all cell types, PrP^{C} is abundantly expressed in neurons. It is located at the outer leaflet of the plasma membrane, to which it is attached by a glycosylphosphatidylinositol (GPI) moiety. PrP^{C} may actually exist under a variety of isoforms since it is subject to heterogeneous glycosylation on two Asn residues and may also undergo proteolytic cleavage.[5] Against all expectations, mice devoid of PrP^{C} turned out to be viable and did not manifest any major abnormality.[6] In recent years, several studies have reported on the copper binding ability of PrP^{C}, which is thought to reflect some role in the regulation of copper metabolism.[7] A second leading hypothesis is that PrP^{C} would serve a neuroprotective function, for instance, by acting as a sensor of oxidative stress[8] and/or by counterbalancing Bax-mediated apoptotic signals.[9] A third set of studies is centered on the participation of PrP^{C} to cell adhesion processes. Interactions of PrP^{C} with laminin, the laminin receptor precursor (LRP), and the neural cell adhesion molecule (NCAM) have been described.[10,11] In addition, several lines of evidence indicate that PrP^{C} promotes neurite outgrowth.[12–16] At last, we[17,18] and others[19–21] have documented that PrP^{C} may act as a cell surface receptor, endowed with the capacity to instruct downstream intracellular signaling cascades.

The present article focuses on the signaling function of PrP^C. It brings together two sets of data that, first, identify signal transduction pathways coupled with PrP^C, with intracellular targets involved in the control of redox equilibrium and cell homeostasis, and second, define PrP^C as a modulator of neurotransmitter G protein–coupled receptor (GPCR) signaling. We show that the membrane protein caveolin relays both actions of PrP^C, and that some of these downstream events mirror some neurospecific function of PrP^C since they are restricted to fully differentiated cells. We finally discuss the implications of our data with respect to the participation of PrP^C in the homeostasis of neuronal cells and draw some hypotheses as to how prion infection may corrupt PrP^C function in neurons.

THE 1C11 NEURONAL DIFFERENTIATION MODEL: A TOOL TO STUDY PRION-PROTEIN-DEPENDENT SIGNALING

Our investigations on prion biology exploit the properties of the murine 1C11 cell line, which behaves as neuroectodermal progenitor and may undergo either serotonergic or noradrenergic differentiation upon induction.[22] The two pathways are mutually exclusive and recruit nearly 100% cells. From a morphological point of view, differentiating cells extend processes, which eventually form a network of neurites (FIG. 1). Neuronal markers, such as $\gamma\gamma$-enolase, neurofilament, and synaptophysin, are induced. Conversion of 1C11 cells into $1C11^{5-HT}$ cells expressing a complete serotonergic phenotype is achieved within 4 days of treatment with dibutyryl cyclic AMP (dbcAMP) and cyclohexane carboxylic acid (CCA). $1C11^{5-HT}$ cells sequentially acquire the capacity to synthesize, store, degrade, and take up serotonin. They selectively implement two serotonergic receptors of the 5-$HT_{1B/D}$ and 5-HT_{2B} subtypes at day 2 of their differentiation program. At day 4, a third subclass of serotonergic receptors—namely 5-HT_{2A}—becomes expressed. These GPCRs act as autoreceptors and regulate the level of serotonin (5-HT) synthesis, storage, and transport in $1C11^{5-HT}$ cells (FIG. 2 and Ref. 22). As for the noradrenergic differentiation program, it reaches completion within 12 days following addition of dbcAMP and CCA in combination with dimethylsulfoxide (DMSO). The resulting $1C11^{NE}$ cells express the overall functions of noradrenergic neurons and selectively implement an α_{1D}-adrenoceptor, which controls the onset of a functional noradrenalin transporter.

Whatever their state of differentiation, 1C11 cells endogenously express the cellular prion protein. In differentiated cells, cell surface PrP^C is located both on cell bodies and on neuritic extensions.[17] These characteristics define the 1C11 cell line as a suitable model to investigate into the biological role of the prion protein in relation with the onset, maintenance, and regulation of a defined neuronal phenotype.

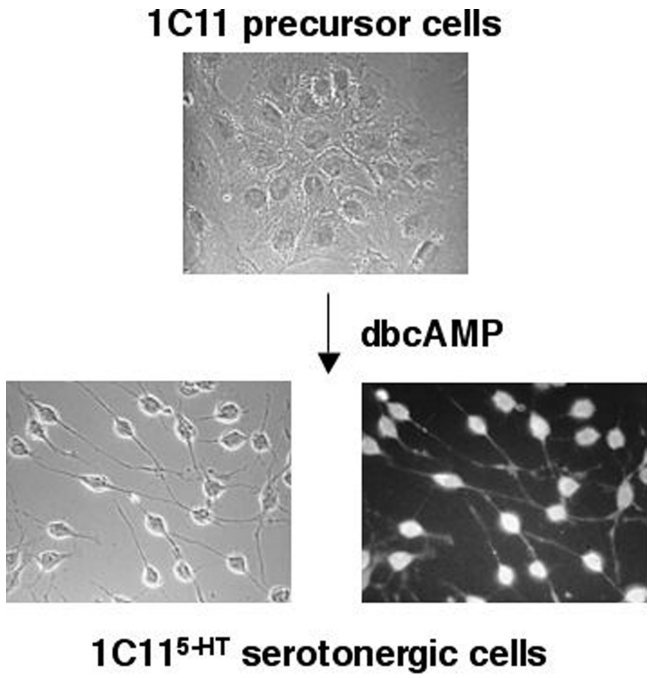

FIGURE 1. Differentiation of neuroepithelial 1C11 cells into 1C11^{5-HT} serotonergic neuronal cells. Conversion is achieved within 4 days of treatment with dbcAMP. The implementation of serotonergic functions is synchronous and homogenous. At day 4, all 1C11^{5-HT} cells are stained with antiserotonin antibodies. The onset of neurotransmitter-associated functions is accompanied by the acquisition of a neuronal morphology with bipolar neuritic extensions.

IDENTIFICATION OF SIGNALING PATHWAYS COUPLED WITH PrP^{C}

We designed an antibody-based strategy to mimic the interaction with a ligand of the prion protein and thereby trigger an input signal, and then probed for intracellular transduction events. Our current data relating to PrP^{C}-dependent signaling are depicted in FIGURE 3. Antibody-mediated ligation of PrP^{C} at the surface of 1C11^{5-HT} serotonergic or 1C11^{NE} noradrenergic cells promotes the formation of a signaling platform, which associates PrP^{C}, the membrane protein caveolin, and the intracellular kinase of the src family, Fyn.[17] Downstream from this complex, PrP^{C} antibodies trigger the sequential activation of the phosphoinositide 3 kinase (PI3K), protein kinase C δ (PKCδ), and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The reactive oxygen species (ROS) generated by the latter enzyme act as second messengers

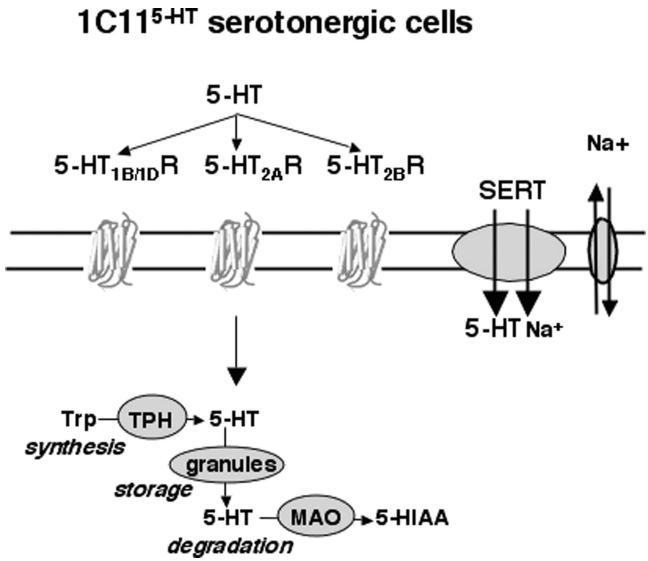

FIGURE 2. Autoregulation of $1C11^{5-HT}$ cells serotonergic functions sustained by 5-HT GRCRs. Along differentiation, $1C11^{5-HT}$ cells selectively implement 5-HT_{2B} (day 2), 5-$HT_{1B/D}$ (day 2), and 5-HT_{2A} (day 4) receptors. This set of receptors transduces signals that allow the regulation of 5-HT metabolism, storage, and uptake.

and contribute to the activation of the extracellular-regulated kinases ERK1/2, a subclass of MAP kinases. PrP^C signaling also partly mobilizes ERK1/2 through an additional pathway, distinct from that involving NADPH oxidase. The Fyn kinase, however, fully controls both cascades converging to ERK1/2. It is noteworthy that the signal transduction events initiated by PrP^C are imparted by prion protein species located on the neurites of $1C11^{5-HT}$ or $1C11^{NE}$ bioaminergic cells.

The implementation of the PrP^C-caveolin-Fyn platform is restricted to fully differentiated $1C11^{5-HT}$ and $1C11^{NE}$ cells, while all protagonists are expressed irrespective of the differentiation state.[17] However, a linear PI3K-PKCδ-NADPH oxidase-ERK1/2 pathway is recruited upon PrP^C ligation in 1C11 precursor cells, through upstream mechanisms that are yet to be identified.

The identification of NADPH oxidase and ERK1/2 as effectors of PrP^C signaling in the 1C11 cell line, as well as in nonneuronal cells, such as GT1 neuroendocrine cells or T lymphocytes[18] argue for a role of PrP^C in redox equilibrium and cell homeostasis. The neuronal-restricted scheme of PrP^C signaling, where the PrP^C-caveolin-Fyn complex controls two pathways converging to ERK1/2, likely mirrors a specific role of PrP^C in mature differentiated cells.

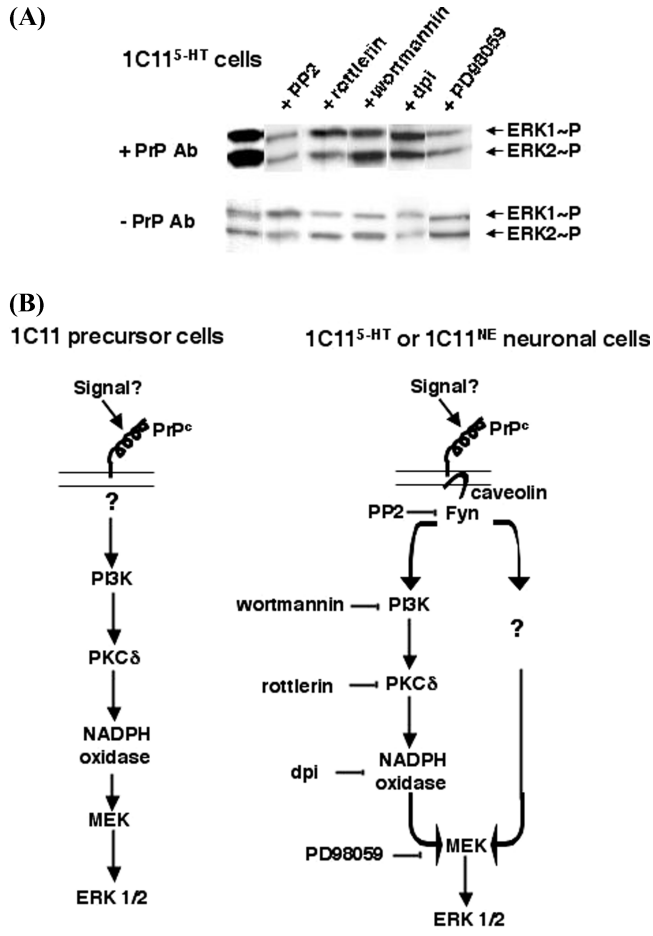

FIGURE 3. Signaling pathways coupled with PrP^C in the 1C11 cell line. (**A**) Western blot experiments showing the PrP^C ligation-induced phosphorylation of ERK1/2 in $1C11^{5-HT}$ serotonergic cells. The use of various inhibitors of defined signaling intermediates allows to draw a scheme of PrP-associated signaling cascades in 1C11 precursor cells or $1C11^{5-HT}$ serotonergic cells (**B**).

It may relate to the selective expression of some prion protein isoforms, such as the particular PrP^C glycoform that is specifically transported to the axonal compartment.[23] A differentiation-dependent onset of other partners could also account for this neuronal specificity. Finally, the colocalization of the signaling components in proper spatial compartments allowing for functional association may depend upon the maturation of the membrane properties of the cells along neuronal differentiation.

PrP MODULATES THE COUPLINGS OF THREE SEROTONERGIC RECEPTOR SUBTYPES

Because in $1C11^{5-HT}$ cells, the cellular prion protein might reside in close proximity to serotonergic GPCRs within lipid rafts, we sought to investigate whether antibody-mediated engagement of PrP^C would affect the signaling activity of the three serotonergic receptors present at the cell surface. FIGURE 4 summarizes the second message signals coupled with these GPCR as well as the impact of PrP^C ligation on these responses in $1C11^{5-HT}$ serotonergic cells.[24] 5-$HT_{1B/D}$ receptors are negatively coupled with the adenylate cyclase (AC) pathway through a Gi subtype of G proteins. Agonists of 5-HT_{2B} receptors promote nitric oxide (NO) production, through direct interaction of NO synthase with a C-terminal PDZ motif. 5-HT_{2B} receptors also recruit the phospholipase A2 (PLA2)/ arachidonic acid (AA) pathway through Gz.

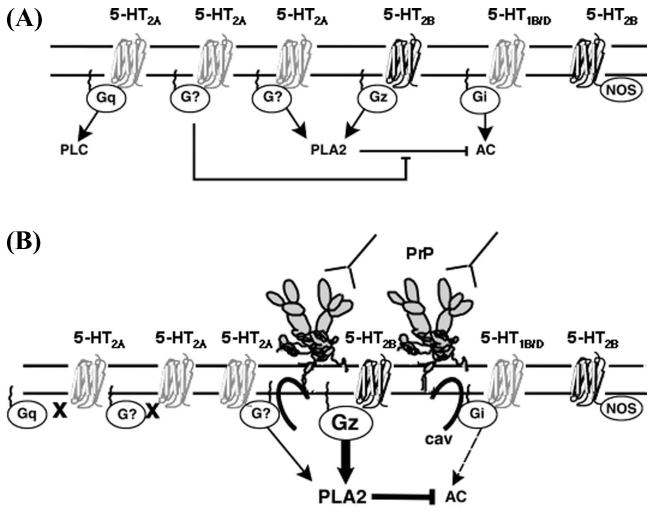

FIGURE 4. Modulation of 5-HT receptor signaling by PrP^C. (**A**) 5-HT_{2B} receptors couple to NOS through direct interaction at a C-terminal PDZ motif and to PLA2 through Gz. 5-HT_{2A} receptors constitute distinct reserves connected to PLC via Gq or to PLA2 through a yet-to-be-identified G heterotrimer. The 5-$HT_{1B/D}$ receptor is negatively coupled to AC through Gi. The 5-HT_{2B} receptor cancels the functionality of 5-$HT_{1B/D}$ receptors through a PLA2-dependent metabolite. The 5-HT_{2A} receptor counterbalances the 5-HT_{2B} receptor-related inhibition of 5-$HT_{1B/D}$ receptor function through a yet-unknown pathway. (**B**) Ligation of PrP^C concomitant to agonist exposure selectively cancels the 5-HT_{2A} receptor-PLC coupling, decreases the AC response imparted by 5-$HT_{1B/D}$ receptors, and potentiates the 5-HT_{2B} receptor-PLA2 coupling. Altogether, PrP ligation alters the cross-talks among the three serotonergic receptors, by notably reinforcing the regulatory loop involving 5-HT_{2B} and 5-$HT_{1B/D}$ receptors and abrogating the antagonistic action of 5-HT_{2A} receptors.

Importantly, this PLA2 pathway cross-talks with the 5 $HT_{1B/D}$–AC coupling, and inhibits the 5-$HT_{1B/D}$ receptor response. 5-HT_{2A} receptors display promiscuous couplings, including PLA2 and phospholipase C (PLC)-dependent inositol trisphosphate (IP3) release, the latter being mediated by Gq proteins. Another yet-to-be-identified pathway associated with these 5-HT_{2A} receptors counterbalances the blocking action exerted by 5-HT_{2B} receptors on the 5-$HT_{1B/D}$ receptor function.

PrP^C ligation by itself does not induce any NO, PLA2, IP3, or AC response. In contrast, concomitant exposure of $1C11^{5-HT}$ serotonergic cells to PrP antibodies and receptor agonists modify the output response normally monitored under receptor activation. PrP^C ligation decreases the AC response imparted by 5-$HT_{1B/D}$ receptors. It abolishes the coupling of the 5-HT_{2A} receptor to the PLC pathway, while having no effect on its PLA2 response. At last, it does not impact on the 5-HT_{2B}-mediated NO production, but augments the PLA2 activity induced by these receptors subclass. By modulating the signaling activity of the three serotonergic receptors, PrP^C also affects the cross-talks that occur between the receptors pathways. The outcome is a reinforced antagonistic action of 5-HT_{2B} receptors on the functionality of 5-$HT_{1B/D}$ receptors.

This set of data introduces PrP^C as a modulator of GPCR signaling and thereby shows that PrP^C is a multifacetted protagonist of signal transduction processes.

TWOFOLD CONTRIBUTION OF PrP^C TO THE HOMEOSTASIS OF SEROTONERGIC NEURONAL CELLS THROUGH CAVEOLIN

A salient finding is that the PrP^C-associated effects on the 5-HT receptors output signals are restricted to fully differentiated cells (day 4), despite the functional expression of both 5-$HT_{1B/D}$ and 5-HT_{2B} receptors from day 2 of the $1C11^{5-HT}$ cell differentiation program.[24] This supports the notion that the subcellular localization of the various signaling protagonists varies in the time course of differentiation and that a proper compartmentalization is achieved at the terminal stage, when cells have acquired a mature neuronal phenotype. The differentiation-dependent modulation of 5-HT receptor signaling by PrP^C is in keeping with the neurospecificity of implementation of the PrP^C-caveolin-Fyn complex at the neurites of 1C11-derived bioaminergic progenies, which controls multiple pathways converging to ERK1/2.

The integration of the PrP^C-caveolin platform within defined subcellular compartments was actually visualized through quick-freeze deep etch electron microscopy (FIG. 5 A). As shown on this micrograph of neuritic replica of $1C11^{5-HT}$ serotonergic cells, at day 4 of differentiation, PrP^C (large-diameter gold particles) is concentrated on caveolae, themselves stained with anti-caveolin 1β antibodies (small-diameter gold particles). Such a colocalization

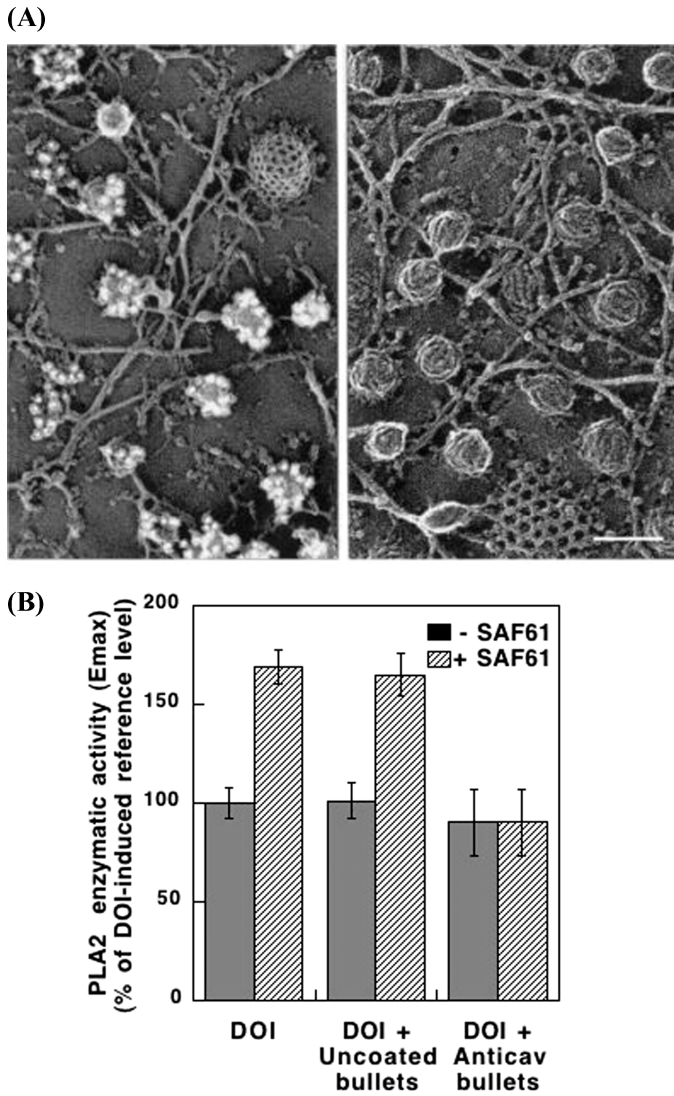

FIGURE 5. Involvement of caveolin in PrP^C-mediated signal transduction events in $1C11^{5-HT}$ serotonergic cells. (**A**) Quick-freeze deep etch electron micrograph of $1C11^{5-HT}$ cells neuritic extensions highlighting the colocalization of PrP^C (large-diameter gold particles, left panel) with caveolin-1 (small-diameter gold particles, both panels). Note that clathrin-coated pits are not stained with PrP^C-antibodies. Scale bar = 100 nm. (**B**) Immunosequestration of caveolin using antibody-coated tungsten bullets cancels the PrP^C-induced potentiation of the $5\text{-}HT_{2B}$ receptor-mediated PLA2 response, as monitored following DOI addition. PrP^C ligation was achieved using SAF61 monoclonal anti-PrP antibodies.

mirrors our previous demonstration of an interaction between PrP^C and caveolin through coimmunoprecipitation experiments.[17]

From a mechanistic point of view, it is worthy to note that caveolin immunosequestration cancels the action of PrP^C on 5-HT receptor signaling (FIG. 5 B and Ref. 24). This observation reinforces the importance of the scaffold protein caveolin in mediating the overall action of PrP^C in mature neuronal cells. It also gives some clue as to how PrP^C may exert its modulatory effects on the serotonergic receptors signaling. It is unlikely that PrP^C directly alters the conformational state of the receptors since PrP^C ligation does not affect their affinities for drugs, i.e., their pharmacological profiles.[24] The involvement of caveolin adds weight to the idea that PrP^C impacts at the level of postreceptor components of 5-HT GPCR signaling. Indeed, caveolin has been reported to interact with diverse protagonists of GPCR signaling, including GPCRs themselves,[25] G proteins,[26] GPCR-related kinases (GRK).[27] The spatio-temporal availability of proximal effectors (e.g., G proteins) and of other partners of signaling, such as arrestins, GRKs, or regulators of GPCR signaling (RGS) greatly influences the duration and strength of GPCR signaling.[28] We thus propose that, by mobilizing caveolin, PrP^C ligation interferes with the recruitment of such GPCR partners and in fine modulates the intensities and/or dynamics of G protein activation by the agonists-bound serotonergic receptors.

In $1C11^{5-HT}$ cells, the overall serotonergic functions are regulated by external 5-HT, through the set of 5-HT receptors. For instance, excess of 5-HT in the cell-surrounding milieu is associated with reduced 5-HT synthesis, storage, and uptake.[24] This autoregulation allows a tight control of neurotransmitter metabolism and thereby contributes to the maintenance of cell homeostasis. The demonstration that PrP^C modulates the 5-HT receptors couplings and cross-talks indicates that PrP^C participates to the fine-tuning of the serotonergic functions of fully differentiated $1C11^{5-HT}$ cells. This specific effect is accompanied by a wider involvement of PrP^C in the homeostasis of neuronal cells that is sustained by its proper signaling activity.

SEROTONERGIC NEURONS AND PRION INFECTION

An important feature of prion diseases is the restriction of pathogenic lesions to the central nervous system, while the pathogenic prion protein PrP^{Sc} accumulates in various tissues and organs.[1] *De novo* PrP^{Sc} formation arises from the posttranslational conversion of host normal PrP^C, with existing PrP^{Sc} molecules acting as a conformational template. The pattern of PrP^{Sc} deposition and the vacuolation profile in the brain may vary according to the TSE strain. It is, however, unclear whether the vulnerability of a brain area to a given prion strain is related to a neurotransmitter-associated phenotype. Prion strain specificity is believed to be enciphered by the conformation of PrP^{Sc}.[29]

In view of the regional heterogeneity of PrP^C isoforms in the brain,[30] it is likely that according to the prion strain, only a defined subset of compatible PrP^C species is converted into pathogenic counterparts. Such substrate selectivity could account for the differential neuronal targeting associated with TSE strains.[31] Whatever the strain, however, infectious prions rely on GPI-anchored PrP^C at the neuronal cell surface to exert their toxicity.[3,4]

In this context, an obvious issue raised by our findings is how the presence of pathogenic prions impact on PrP^C-associated transduction events in neuronal cells. Several hypotheses can be made. PrP^{Sc} may behave as a dominant negative and disrupt PrP^C signaling and cross-talks. In an alternative gain of function scheme, it may exacerbate PrP^C-associated signals. In any case, PrP^{Sc} accumulation would create an imbalance in PrP^C function leading to a loss of cell homeostasis, at the level of the redox equilibrium and/or as a result of aberrant 5-HT receptor signaling. The latter putative feature could account for the reported alterations of serotonin metabolism in prion-infected animals[32–35] and patients.[36] It may also confer to serotonergic neurons an increased vulnerability to TSE agents.[37]

The identification of intracellular targets directly mobilized or indirectly affected by PrP^C clearly provides a screening basis to assess the impact of PrP^{Sc} accumulation on PrP^C function. It may also guide the development of therapeutic strategies with the aim to both clear neurons from PrP^{Sc} and restore PrP^C normal function. Hopefully, the 1C11 cell line may be instrumental to tackle these issues since it combines functional PrP^C expression and the ability to propagate infectious prions (unpublished observations).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In summary, taking advantage of the 1C11 bioaminergic cell line has allowed us to unravel some unexpected modulatory role of the cellular prion protein on GPCR signaling. The bipotentiality of the 1C11 precursor offers the opportunity to probe whether PrP ligation also impacts on the signaling activity of the α_{1D}-adrenoceptor that is implemented during the noradrenergic differentiation program, and if so, whether this effect is restricted to $1C11^{NE}$ cells expressing a complete noradrenergic phenotype. The priorities for future research include identifying the physiological input signal that engages PrP^C and is mimicked by antibody ligation. Another important question to address deals with the mechanisms through which PrP^C interferes with GPCR signaling. Which component(s) of post-GPCR signaling mediate(s) the PrP^C-related modulation? The phenotypic response of differentiated cells to concomitant PrP^C ligation and 5-HT receptor agonist stimulation will also have to be quantified, with a view to draw functional links between these external inputs and the overall 5-HT associated functions. Last but not least, the challenge will be to assess the impact of prion infection on the overall signal transduction

events imparted by PrP^C in mature bioaminergic neuronal cells, with the aim to gain some insight into the cellular and molecular mechanisms sustaining prion pathogenesis.

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