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PRION, 2017
VOL. 0, NO. 0, 1–6
https://doi.org/10.1080/19336896.2017.1412912

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PERSPECTIVE

Neuronal pathophysiology featuring PrP^C and its control over Ca^{2+} metabolism

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ABSTRACT

Calcium (Ca^{2+}) is an intracellular second messenger that ubiquitously masters remarkably diverse biological processes, including cell death. Growing evidence substantiates an involvement of the prion protein (PrP^C) in regulating neuronal Ca^{2+} homeostasis, which could rationalize most of the wide range of functions ascribed to the protein. We have recently demonstrated that PrP^C controls extracellular Ca^{2+} fluxes, and mitochondrial Ca^{2+} uptake, in neurons stimulated with glutamate (De Mario et al., J Cell Sci 2017; 130:2736-46), suggesting that PrP^C protects neurons from threatening Ca^{2+} overloads and excitotoxicity. In light of these results and of recent reports in the literature, here we review the connection of PrP^C with Ca^{2+} metabolism and also provide some speculative hints on the physiologic outcomes of this link. In addition, because PrP^C is implicated in neurodegenerative diseases, including prion disorders and Alzheimer's disease, we will also discuss possible ways by which disruption of PrP^C-Ca^{2+} association could be mechanistically connected with these pathologies.

ARTICLE HISTORY
Received 13 October 2017
Revised 27 November 2017
Accepted 28 November 2017

KEYWORDS
Cellular prion protein; PrP^C; prions; Ca^{2+}; store-operated Ca^{2+} entry; glutamate receptors; excitotoxicity; knock-out mice; neurons; Aβ oligomers

Introduction

Undoubtedly, stringent precautions for safe animal nourishment have now strongly lowered the threat for prion-tainted food that pervaded the last decades of the past century. Based on sporadic events or genetic grounds, prions originate from misfolded b-enriched conformers of an ubiquitously expressed protein, the cellular prion protein (PrP^C), and cause neurodegenerative pathologies of humans and animals, named transmissible spongiform encephalopathies or prion diseases [1]. Much advance has been made on the pathological aspects of these diseases. To date, however, secure therapeutic interventions are not available, nor it is established whether neurodegeneration arises from prion toxicity, or lack of the still mysterious PrP^C function, or a combination of both. In this view, understanding PrP^C cellular behavior would solve a long-standing biological issue, but would concurrently serve as strategic prerequisite to the design of drugs capable to halt the progression of fatal prion disorders. Localization of PrP^C to the external cell surface has stimulated investigations in a plethora of experimental paradigms aimed at assessing a possible (co-)receptor function of the protein and its downstream signals. Also this field has witnessed important advances, by proposing a number of PrP^C partners that together could trigger beneficial effects by governing particular pathways, and/or the concentration or activity of intracellular signaling mediators (lengthily reviewed in recent publications) [2-4].

In addition to being recognized as the first protein to become infectious, PrP^C presents a structure that is *per se* extremely interesting, being composed of a highly compact (a helix-rich) C-terminus (that which undergoes the a-to-b pathogenic conversion), and an unstructured N-terminus that is typical of "intrinsically disordered proteins" (IDPs) [5,6]. Lack of stable secondary and/or tertiary folding under physiological conditions provides IDPs with the capacity to interact with multiple functional partners, and to serve as central hubs in the coordination and integration of signaling networks [6,7]. In line with this concept, the extended unstructured N-terminus could perfectly tailor to PrP^C to explaining its pleiotropicity, in terms of the high number of cell surface partners and regulated signaling pathways that have emerged from decade-long research on the issue. It is also to underline that IDPs are implicated in the pathogenesis of several human diseases, such as cancer, type II diabetes, cardiovascular diseases and – most importantly – different neurodegenerative disorders [8,9]. This aspect is not secondary to the (benign and toxic) dichotomic nature of PrP^C. Indeed, it was proposed that dysregulation of intrinsically disordered regions, or genetic mutations, reduce the capacity of IDPs to recognize correct binding partners, thus allowing formation of non-functional complexes and aggregates generating aberrant signalling.

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Under the framework of such a multifaceted trait of PrP^C, the interest of our laboratory over the last few years has been focused in identifying a signaling factor with similar pleiotropic properties, i.e., serving as target of numerous upstream pathways, and, concurrently, regulating multiple downstream cell events. Our choice fell on calcium (Ca^{2+}), one of the most exploited cofactors and signaling mediators controlling all cell stages (from oocyte fertilization, to cell differentiation and death, to name a few), through rapid and transient concentration changes in restricted cell domains [10]. However, our choice considered also aspects common to PrP^C and Ca^{2+} in neurons; (i), the positive action of both that can switch into a danger for the cell life (aberrant conformation changes of PrP^C produce deadly prions; uncontrolled Ca^{2+} increases trigger cell death); (ii), synapses, whose dysfunction ultimately leads to loss of neurons [11], are the regions in which PrP^C is mainly expressed [12], and where the fundamental action of Ca^{2+} ensures correct synaptic transmissions by spatio-temporally coordinating electric signals and neurotransmitter release [10].

Here, we will briefly summarize our recent findings, along with evidence from the literature, connecting PrP^C pathophysiology with Ca^{2+} metabolism. Speculative functional consequences of such a still enigmatic liaison will also be proposed.

The pathophysiologic connection of PrPC with Ca^{2+} metabolism

The possible connection of PrP^C to Ca^{2+} metabolism is far from recent. It can be traced back to studies aimed at understanding the toxic mechanism of prions, and to initial attempts designed to identifying PrP^C physiology through the comparison of paradigms harboring, or not (PrP-knockout, PrP-KO), PrP^C. Although not always was Ca^{2+} the direct investigation target, altered Ca^{2+} homeostasis was observed, or could be envisaged, in prion-infected or PrP-KO model cells[13-15]. Ultimately, by proving a direct interaction of PrP^C with the N-methyl-D-aspartate (NMDA)-sensitive Na^+/Ca^{2+}-permeable glutamate receptor (NMDAR), combination of electrophysiological and biochemical approaches in hippocampal paradigms established that native PrP^C behaved as sentinel against Ca^{2+} overload [16]. This result provided a likely explanation for loss of synapse integrity caused by PrP^C misfolding.

Our approach to the field was mainly methodological, having used genetically-encoded Ca^{2+} indicators (aequorins, AEQs) targeting specific cell regions of primary cultures of neurons from co-isogenic mice expressing, or not, PrP^C [17,18]. In particular, we used AEQs sensing Ca^{2+} movements in microdomains of the cytosolic side of the plasma membrane (PM), in the cytosol, in the mitochondrial matrix and in the lumen of the endoplasmic reticulum (ER). Thus, as opposed to chemical dyes widely used for monitoring cytosolic Ca^{2+} fluctuations, AEQs allow to construct an integrated picture of how different cell compartments respond to stimuli promoting Ca^{2+} entry from the extracellular space, or release from intracellular Ca^{2+}-reservoirs (ve.g., the sarco-endoplasmic reticulum).

PrPC Governs several pathways for Ca^{2+} entry at the plasma membrane

Store-operated Ca^{2+} entry

Using mostly primary cerebellar granule neurons (CGN) expressing, or not, PrP^C, we first considered store-operated Ca^{2+} entry (SOCE), one major PM Ca^{2+} route distinct from PM receptor-channels. Becoming activated upon a decrease of ER Ca^{2+} content, SOCE serves to refill intracellular Ca^{2+} reservoirs and to regulate key cell parameters, from gene expression to tissue development and function [19]. Our work demonstrated that, also in the case of SOCE, PrP^C served to oppose dangerous Ca^{2+} overloads because Ca^{2+} accumulation in PM subdomains was attenuated compared to PrP-KO CGN, and this reflected in lower cytosolic and mitochondrial Ca^{2+} fluxes [20,21]. In addition, combined biochemical approaches allowed us to substantiating the alleged involvement of PrP^C in signal transduction related to the modulation of phosphorylation cascades, in particular that governed by the Src Tyr-kinase Fyn [2]. In fact, PrP^C was found to constitutively downregulate Fyn activation and the Tyr-phosphorylation of the stromal interaction molecule 1 [21], an ER transmembrane Ca^{2+} sensor which is key to the mechanism linking ER Ca^{2+} depletion to stimulation of the channel-forming proteins of SOCE [19].

It has been proposed that PrP^C acts as a cell surface binding partner for, and transduces the neurotoxic action of, b-enriched soluble protein aggregates such as prions and amyloid-b (Ab) oligomers related to Alzheimer's disease (AD) [22,23]. It was also reported that PrP^C-mediated Ab effects imply the engagement of Fyn [12]. On this, we added another important piece of information by showing that Ab oligomers subvert the tripartite connection between PrP^C, Fyn and SOCE in both CGN and cortical neurons [21]. This enabled us to suggest that disruption of SOCE-mediated Ca^{2+} signaling could contribute to PrP^C-dependent effects in AD.

Glutamate-sensitive receptors

Because hippocampal neurons are protected from excessive extracellular Ca^{2+} influx owing to PrP^C-NMDAR

interactions restricting the receptor Ca^{2+} permeability [16], we took advantage of AEQs to expand analysis of Ca^{2+} movements in different domains of ionotropic glutamate receptor (iGluR)-stimulated primary CGN and cortical neurons. In particular, we either activated each iGluR sub-type separately [by adding the specific agonist NMDA, or α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), or kainate], or all iGluRs together (with the physiologic agonist, glutamate). Our findings corroborated in these neurons what had been previously observed for hippocampal NMDAR activity. However, data showing that PrP^C equally downregulated AMPAR- and kainateR-mediated Ca^{2+} entry suggested a much wider action of PrP^C in diminishing neuronal vulnerability by glutamate over-stimulation [24], in accord with results obtained in PrP-KO animals exposed to different excitotoxic insults [25-29].

As to the mechanism allowing PrP^C to regulate AMPAR-dependent Ca^{2+} entry, we focused on AMPAR trafficking based on the notion that regulation of this process goes through Ser845 phosphorylation on the GluR1 subunit [30]. Surface biotinylation of CGN with the two PrP genotypes clearly demonstrated that the surface expression of GluR1 was more abundant (~70%) in PrP-KO neurons than in PrP^C-expressing counterparts, a result highly suggestive that the diminished presence of the receptor in PrP^C-expressing CGNs could have contributed to the restricted AMPAR-mediated Ca^{2+} entry [24]. Ser845 is phosphorylated by protein kinase A (PKA) [30], whose activity depends on the interplay between the formation and hydrolysis of cyclic adenosine monophosphate (cAMP). In line with our expectations, extracellular signal-regulated kinase 1 and 2, which potentiates PKA activity by its inhibitory phosphorylation of (cAMP-hydrolyzing) phosphodiesterase [31], was less active (~50%) in PrP^C-expressing than in PrP-KO CGN [24]. This finding adds a further molecular tile for explaining why the surface expression of AMPAR GluR1 was diminished in the presence of PrP^C.

PrPC governs movements of, and Ca^{2+} uptake by, mitochondria

Altogether, works from our and others' laboratory substantiate association of PrP^C with the cell apparatus deputed to maintain a correct Ca^{2+} homeostasis. Specifically, by controlling PM pathways PrP^C contributes to prevent potentially noxious high Ca^{2+} entry [27]. Intriguingly, however, we also found that, despite PrP^C sits on the cell surface, its beneficial action strategically extends beyond the PM by protecting mitochondria from an equally deleterious Ca^{2+} overload [24].

Mitochondria are crucial organelles for the cell life, which physiologically take up Ca^{2+} from different sources both in their Ca^{2+}-buffering task and to stimulate enzymes enhancing the production of ATP. Likewise, it is well established that excessive Ca^{2+} accumulation may alter the inner membrane permeability, which ultimately undermines mitochondrial integrity and triggers apoptosis [32]. During the morphological inspection of the two CGN types, somehow surprisingly we observed that, on average, mitochondria of PrP-KO CGN were around 30% more distant from the PM than in PrP^C-expressing neurons (an issue further discussed below). However, if this displacement protected PrP-KO mitochondria from increased Ca^{2+} uptake following the selective stimulation of a single iGluR sub-type, this behavior no longer held when neurons were exposed to glutamate. Such an apparent paradox was eventually resolved by demonstrating that the higher glutamate-mediated Ca^{2+} influx in PrP-KO neurons was capable to better stimulate the process of Ca^{2+}-induced Ca^{2+} release via ryanodine receptor channels [10], which, in turn, remarkably increased mitochondrial Ca^{2+} uptake [24]. This set of data suggests that (at least in CGN) the protection by PrP^C against Ca^{2+} overload is integrated towards a few regions of the cell, which may have a crucial relevance under pathologic settings.

Open questions on prpc role in neuronal pathophysiology

If what hitherto reported clearly underlines the capacity of PrP^C to regulate multiple aspects of Ca^{2+} metabolism and to protect neurons from abnormal Ca^{2+} accumulations, these actions need further investigations as exemplified in the following open questions.

(I) How can PrP^C perform such a complex task, i.e., which are the events engaging the protein in regulating multiple Ca^{2+}-mobilizing systems?

With regards to Ca^{2+}-permeable pathways at the neuronal PM, data are now available pointing to the capacity of PrP^C to reducing Ca^{2+} entry by acting at different levels. On the one hand, PrP^C prevents glutamate potential excitotoxicity because the protein downregulates the channel activity of the NMDAR by interacting directly with one of its subunits [16], and by reducing the affinity for the co-agonist glycine in a copper-dependent manner [33]. On the other hand, in line with suggestions that PrP^C affects phosphorylation-based signaling pathways [2,34], we have also shown that PrP^C downregulates both SOCE and AMPAR by controlling specific phosphorylation events on the respective molecular machineries (see above) [21,24]. Yet, if the phenomenology of the PrP^C-Ca^{2+} connection in the above instances is quite

clear, mechanistic clues of these links still warrant a better definition.

(II) Which are the downstream events of the PrP^C-Ca^{2+} liaison?

Because Ca^{2+} is a key player in almost all aspects of cell physiology, particularly in excitable cells, it is hard to draw a unique picture rationalizing the various reports of PrP^C control over Ca^{2+} homeostasis and the entire range of outcomes of these regulations. It thus appears more sensible to envision that the protein acts in a context-dependent manner – in terms of type and actual status of the cell, and kind of stimulus (or combination of stimuli) the cell is exposed to – and/or in PrP^C capacity to integrating diverse signaling pathways. This view stems from the previously introduced concept, proposing that the intrinsically disordered PrP^C N-terminus could interact with multiple functional partners, and behave as a central hub in the coordination and integration of signaling networks [6,7]. A few speculative consequences of the PrP^C-Ca^{2+} interplay, for which we have now collected some evidence, may fall in the following scenarios.

(a) One of the primary Ca^{2+}-regulated cell functions is gene transcription [35]. On this, we have provided evidence that PrP^C could regulate the expression of different neuronal membrane proteins, following a large-scale proteomic study of membrane proteins in primary CGN with the two PrP genotypes [36]. We have also reported that, compared to control neurons, cultured PrP-KO CGN display a significant downregulation of PM and sarco-endoplasmic reticulum Ca^{2+} ATPases [20], two major cytosolic Ca^{2+} removal systems [10]. More recently, analysis of the whole proteome has shown a significant downregulation in PrP-KO CGN of a set of proteins functionally related to vesicular trafficking (among which some members of the Rab family of monomeric GTPases), resulting in reduced glutamate release and impaired synaptic vesicle re-uptake (Peggion et al., unpublished observations). These results are very intriguing although, here also, molecular details of a likely PrP^C-Ca^{2+}-gene transcription axis need a more refined characterization.

(b) Another possible outcome of PrP^C control over Ca^{2+} refers to SOCE. Following the notion that SOCE controls the differentiation of non-neuronal cell types [37], and neurogenesis [19], we assessed whether this was applicable to CGN differentiation. To this end, we analyzed the impact of SOCE on the *in-vitro* maturation of wild-type CGN using specific inhibitors, and post-transcriptional silencing of molecular constituents, of SOCE. Preliminary data collected in this study indicate that such a Ca^{2+}-mobilizing mechanism regulates the differentiation of CGN cultured under basal conditions (Peggion et al., unpublished observations). In light of this information, and the alleged role of PrP^C in (neuronal) cell differentiation [2,38], and in SOCE modulation [20,21], it seems plausible that PrP^C regulates neurogenesis via SOCE. Although we did not observe any apparent alteration of PrP-KO CGN differentiation compared to the PrP-expressing counterpart in our experimental constraints, still an involvement of the PrP^C-SOCE connection in neuronal development under different *in vitro* and/or *in vivo* settings cannot yet be excluded.

(c) The final framework refers to our finding that mitochondria are displaced from the PM in cultured PrP-KO CGN (see above) [24]. The cytoskeletal-mediated movement of mitochondria is of fundamental importance for cells, and for neurons in particular, since it couples the biogenesis of mitochondria to their transport to specific locations such as synapses. This complex and energy-dependent process, which is not yet entirely defined, is based on molecular motors involving several proteins, including those belonging to the Miro family that contains both GTPase and Ca^{2+}-binding EF-hand domains [39]. The suggestion that mitochondria transport along axons may depend on local variations of Ca^{2+} concentration, and the by now-established role of PrP^C in Ca^{2+} homeostasis, should therefore stimulate investigations on the possible connection between PrP^C-Ca^{2+} coupling and the neuronal transport of mitochondria.

(III) Is the PrP^C-Ca^{2+} relationship important in neuropathology?

It is good to underline that the "gold standard" of our experimental paradigms to tackle the issue of PrP^C physiology was the use of PrP-KO mouse models, and primary neuronal cultures thereof. It may thus be questionable if and how our findings can be directly translated into neuropathological contexts, e.g., prion diseases, primarily because all generated PrP-KO mouse lines do not display overt phenotypes, with the exception of a chronic peripheral demyelination associated to late-onset polyneuropathy [2,4]. On the other hand, one cannot rule out that under specific circumstances, such as those in which neurons are subjected to neurotoxic challenges altering PrP^C structure and function, the Ca^{2+}-related phenotypes reported by us in *in-vitro* PrP-KO models may participate in relevant pathogenic routes. This concept has been significantly reinforced by the finding that PrP^C acts as a high affinity receptor for neurotoxic b-rich protein aggregates, such as prions and Ab

oligomers [22,23], opening the possibility that these interactions may (at least) exacerbate oligomers' neurotoxic effects by ultimately displacing PrP^C from its native protective functions, including control of local Ca^{2+} homeostasis.

Various mechanisms can be hypothesized relating an impaired control of PrP^C on Ca^{2+} metabolism and alterations of neuronal function and survival, the most obvious of which is harmful Ca^{2+} overload associated to glutamate excitotoxicity [27]. Excessive mitochondrial Ca^{2+} uptake can also be a fatal conveyor of doom for neurons by leading to increased mitochondrial permeability, loss of mitochondrial functions and release of pro-apoptotic factors [32]. Our suggestion that PrP^C defends against perilous Ca^{2+} transients not only in the cytosol but also in the mitochondrial matrix, supports the possibility that loss of this function of PrP^C contributes to neuronal demise under neuropathological conditions. The latter line of reasoning may apply as well to alterations of neurotransmitter release, already recognized as central in different neurodegenerative disorders and model systems [11], which we observed in PrP-KO CGN as a consequence of the perturbed protein expression pattern and defective synaptic vesicle trafficking (see above). Within the same hypothesis, our observation that mitochondria in PrP-KO neurons are displaced from the PM [24], in a likely Ca^{2+}-dependent way, could also be pathologically relevant, since mitochondria localization near the synaptic membrane is undoubtedly strategic to satisfying the high energy demand necessary to ensure intact synaptic functions.

Disclosure of potential conflicts of interest

Authors declare no potential conflict of interest.

Acknowledgments and funding

Our gratitude goes to all collaborators whose work over the years has been essential to shed light on the PrP^C-Ca^{2+} connection. This work was supported by grants from the University of Padova (PRAT CPDA121988/12 to M.C.S.; PRAT CPDA158035/15 to A.B.).

Funding

Q6 Università degli Studi di Padova, PRAT CPDA158035/15.
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