Prion Protein: Orchestrating Neurotrophic Activities

Vilma R. Martins[1][#]*, Flavio H. Beraldo[1][#], Glaucia N. Hajj[1][#], Marilene H. Lopes[1][#], Kil Sun Lee[2], Marco A. Prado[3,4] and Rafael Linden[5]

[1]Ludwig Institute for Cancer Research, Hospital Alemão Oswaldo Cruz, São Paulo, SP, Brazil
[2]Associação Fundo de Incentivo à Psicofarmacologia, São Paulo, SP, Brazil
[3]Robarts Research Institute, Departments of Anatomy and Cell Biology/Physiology and Pharmacology, University of Western Ontario, London, Ontario, Canada.
[4]Department of Pharmacology, Federal University of Minas Gerais, MG, Brazil.
[5]Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

Abstract

PrP[C] is highly expressed in both the central and peripheral nervous systems from early stages of development and in adulthood. Its major conformational change and conversion into an abnormal form (PrP[Sc]) has been associated with the generation of prions, the infectious agent of transmissible spongiform encephalopathies (TSEs). The massive neurodegeneration presented by individuals suffering from these diseases has been associated with the gain of neurotoxic activity of PrP[Sc]. On the other hand, major neurodegeneration is also observed in transgenic mice expressing PrP[C] molecules deleted of specific domains, which points to important functional domains within this molecule, and supports the hypothesis that loss-of PrP[C] function may contribute to the pathogenesis of TSEs. Furthermore, a large body of data demonstrates direct or indirect interaction of PrP[C] with extracellular matrix proteins, soluble factors, transmembrane proteins, G-protein coupled receptors and ions channels. The ability of PrP[C] to drive the assembly of multi-component complexes at the cell surface is likely the basis for its neurotrophic functions. These properties indicate that PrP[C] may be relevant for not only the spongiform encephalopathies, but also as an ancillary component of the pathogenesis of other neurodegenerative diseases, and therefore amenable to therapeutic targeting.

The prion protein and the neurotrophic theory

The neurotrophic theory originally referred to the idea that all body organs were dependent on nutritional factors secreted by the nervous system (Levine, 1992). However, and more akin with Cajal´s theories on the neurotrophic activity of the Schwann cell (Azmitia, 2002), the theory gained momentum upon the discovery of the properties of the nerve growth factor - NGF (Levi-Montalcini, 1987). Then, its central tenet became that the survival of neurons during development of the nervous system was determined by the limited availability of trophic molecules delivered by target organs to the nerve terminals (Oppenheim, 1989).

In parallel with the discoveries concerning a wide range of effects of NGF (Levi-Montalcini et al., 1996), work in the 1980s and 1990s greatly expanded the scope of the neurotrophic theory. Adding to the original emphasis on the role of individual target-derived neurotrophins (Thoenen et al., 1993), current neurotrophic theory allows for the operation of multiple neurotrophic systems upon single neurons (Davies, 1996), the production and physiological release of neurotrophic factors by glia (Sariola and Saarma, 2003), the role of afferent-derived molecules (Linden, 1994), trophic effects of both neurotransmitters and neuromodulators (Linden et al., 2005; Martins and Pearson, 2008), as well as cytokines (Krieglstein and Unsicker, 1996; Middleton et al., 2000) and hormones (Datson et al., 2008; Williams, 2008), and encompasses not only the survival but also the differentiation and functional integrity of components of the nervous system, among other complexities (Chao and Bothwell, 2002; Chao, 2003; Kalb, 2005; Levi-Montalcini and Calissano, 2006).

Neurotrophic interactions are based on cell-cell communication through either the release or the presentation at the cell surface, of specific molecules that bind to other molecules present in a target cell. The latter may be either the same cell, providing for autocrine effects, a neighboring cell or even reside at a distance, depending on both the neurotrophic factor and the structure and diffusion properties of the tissue environment. Both cell surface (Huang and Reichardt, 2003; Blochl and Blochl, 2007) and intracellular (Datson et al., 2008), including nuclear (Williams, 2008) receptors, serve as docking elements for the neurotrophic agents, and activate multiple signaling pathways leading to neurotrophic effects. Cell surface receptors constitute the majority of the targets of neurotrophic agents, and they can transfer signals through the plasma membrane either through their own intracellular domains, or via the activation of transmembrane partners (Yano and Chao, 2000; Runeberg-Roos and Saarma, 2007).

Evidence that the prion protein (PrP[C]) is involved in neurotrophic activity first appeared from studies in which the expression of PrP[C] prevented cell death triggered by serum deprivation of a hippocampal cell line (Kuwahara et al., 1999). It was subsequently shown that engagement of PrP[C] with antibodies triggered cell signaling (Mouillet-Richard et al., 2000), and that interaction of PrP[C] with a soluble binding partner induced neurotrophic-like effects mediated by the cAMP-protein kinase A and Mitogen activated protein kinase (MAPK) pathways (Chiarini et al., 2002; Zanata et al., 2002; Lopes et al., 2005). Evidence that PrP[C] mediates neurotrophic interactions is now both abundant and compelling (e.g. (Nishimura et al., 2004; McLennan et al., 2004; Spudich et al., 2005; Coulpier et al., 2006; Rangel et al., 2007; Nazor et al., 2007; Weise et al., 2008; Rambold et al., 2008).

The hypothesis has been put forward that the prion protein functions as a dynamic cell surface platform for the assembly of signaling modules, based on which selective interactions with many ligands and transmembrane signaling pathways translate into wide-range consequences

[#]These authors contributed equally to this work.
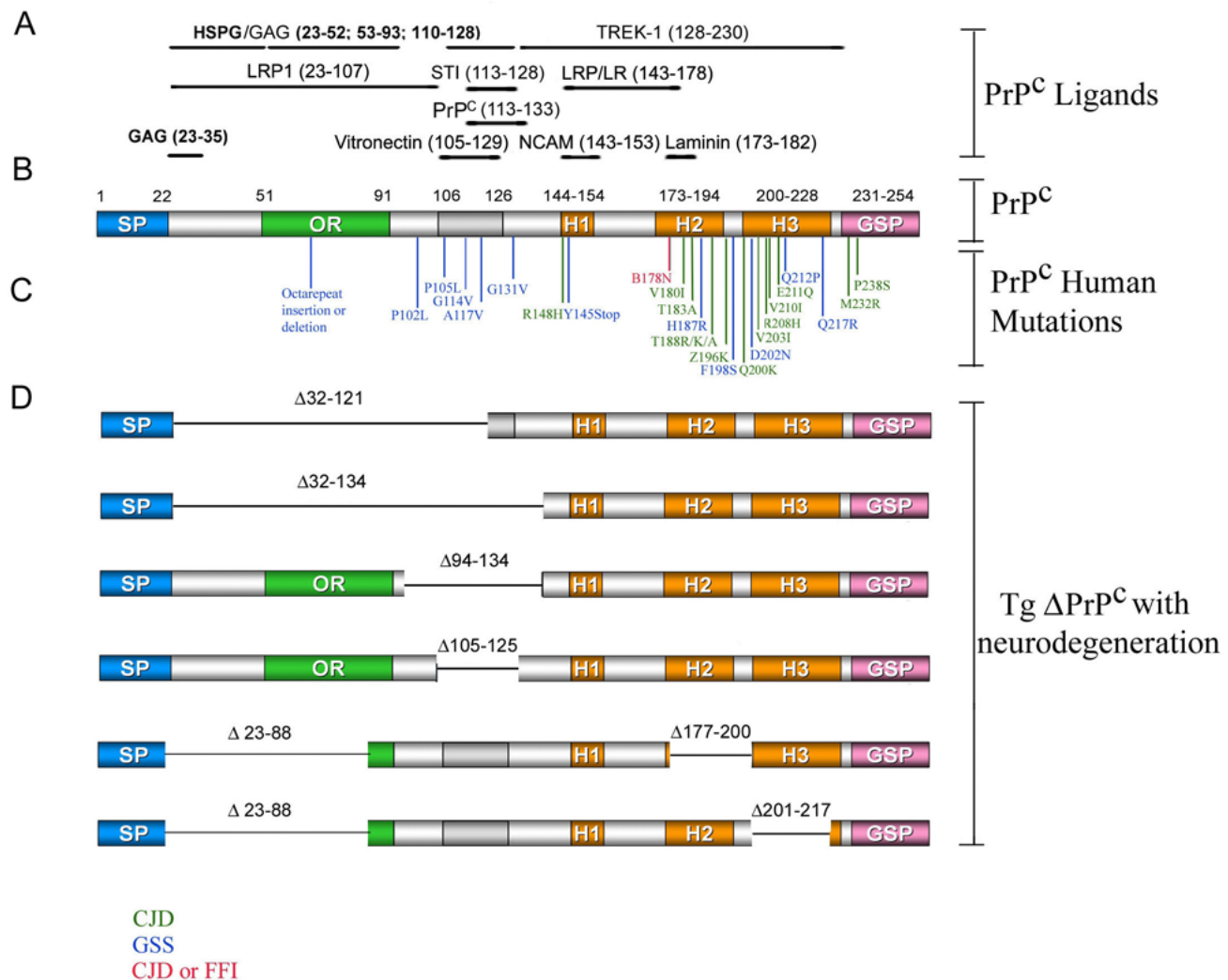*vmartins@ludwig.org.br

Figure 1. A schematic representation of PrP^C with ligand binding sites, human mutations leading to prion diseases, and transgenic mouse models presenting neurodegeneration.
A) Mouse PrP^C binding sites for ligands with neurotrophic activity. Numbers in parentheses indicate residue position. B) Schematic drawing of mouse PrP^C. During the post-translational modification, the N-terminal signal peptide (SP) and GPI sequence (GPS) are cleaved and a GPI anchor is attached to the C-terminal. The flexible N-terminal portion contains octapeptide repeats (OR) and a hydrophobic domain (dark grey). Orange boxes (H1, H2 and H3) represent regions of α-helical secondary structure. C) Mutations associated with inherited human prion diseases are indicated, CJD (green), GSS (Blue) and GSS/FFI (red). D) Deletions of PrP^C expression which cause neurodegeneration in transgenic mice, Δ numbers indicate the deleted domains.

upon both physiology and behavior (Linden et al., 2008). Thus, PrP^C may scaffold various sets of extracellular and transmembrane molecules, providing for distinct signaling events, depending on the cell type, developmental stage, level of expression of both PrP^C and its partners, the availability of ligands in the immediate environment of the target cells, and endocytic trafficking (Linden et al., 2008). The current review focuses upon the role of PrP^C as a mediator of neurotrophic interactions, in the context of the hypothesis of loss-of-function components of prion diseases.

Following the cleavage of a signal peptide, the mammalian prion protein is produced mostly in the form of an N-glycosylated, GPI-anchored protein of 208-209 aa (Riek et al., 1997; Lopez et al., 2000; Zahn et al., 2000; Hornemann et al., 2004; Calzolai et al., 2005; Lysek et al., 2005; Parkin et al., 2007), containing an N-terminal flexible, random coil sequence that spans approximately half of its aminoacid residues, plus a C-terminal globular domain that constitutes the other half. The globular domain of human PrP^C is arranged in 3 α-helices corresponding to aas 144-154, 173-194 and 200-228, interspersed with an antiparallel β-pleated sheet formed by β-strands at residues 128-131 and 161-164. A single disulfide bond is formed by cysteine residues 179 and 214. The N-terminal residues 23-124 constitute the flexible tail, while residues 229-230 form a short flexible C-terminal domain (Zahn et al., 2000). Major structural features of PrP^C are remarkably preserved

among both mammalian and non-mammalian species (Lopez et al., 2000; Hornemann et al., 2004; Calzolai et al., 2005; Lysek et al., 2005).

Full length PrP^C is found in non-, mono- or di-glycosylated forms, corresponding to the variable occupancy of residues Asn181 and Asn197 in human PrP^C - Asn180 and Asn196 in mice (Haraguchi et al., 1989). A rather large variety of N-glycans were found attached to both full-length and truncated PrP^C (Rudd et al., 1999), which may be differentially distributed in various areas of the central nervous system (CNS) (DeArmond et al., 1999; Beringue et al., 2003). Although neither the attached N-glycans nor most of the GPI anchor appear to significantly affect major structural features of the prion protein (Hornemann et al., 2004), both may interfere upon the binding of physiological ligands, which, in turn, may be relevant for its neurotrophic properties (Linden et al., 2008).

PrP^C deletions in mice and mutations in humans: a tip for neurotrophic functions?

Since the early 1990s, knockout constructions for PrP gene (*Prnp$^{0/0}$* or *Prnp$^{-/-}$*) have emerged as an attempt to elucidate the PrP^C functions in vivo (Bueler et al., 1992; Manson et al., 1994). However, the first study in PrP^C-null mice did not reveal an obvious phenotype implying that PrP^C is either not essential for normal development, or could be compensated by other molecules (Bueler et al., 1992; Manson et al., 1994). However, when distinct features of these animals were investigated in further studies, subtle changes have been noticed in certain biological processes such as neurotransmission and synaptic plasticity (Collinge et al., 1994; Maglio et al., 2004; Prestori et al., 2008), hippocampal spatial memory (Criado et al., 2005) and aversive hippocampal memory in aged animals (Coitinho et al., 2003), circadian rhythms (Tobler et al., 1996; Tobler et al., 1997) and immune responses (de Almeida et al., 2005; Bainbridge and Walker, 2005). Remarkably, PrP^C-null mice also show higher sensitivity to various stress conditions which cause increased neuronal death (Walz et al., 1999; McLennan et al., 2004; Weise et al., 2004; Shyu et al., 2005; Weise et al., 2006; Rangel et al., 2007). Therefore, although compensatory mechanisms may balance the absence of PrP^C, they are not sufficient and the threshold for neuronal death is lower when PrP^C is deleted.

Large and diverse phenotypic abnormalities were observed when PrP^C deletion mutants were re-expressed in *Prnp$^{0/0}$* mice. PrP^C mutants deleted either aas 32-121, (PrPΔ32-121) or 32-134 (PrPΔ32-134), led to severe ataxia and apoptosis in the cerebellum (Shmerling et al., 1998). Severe demyelination and axon loss in both the spinal cord and cerebellar white matter were also observed in PrPΔ32-134 mice (Radovanovic et al., 2005) (Figure 1D). The leucodystrophy, but not the cerebellar granule cell degeneration, was rescued in these mice by oligodendrocyte-specific expression of PrP^C (Radovanovic et al., 2005). On the other hand, neuron-specific expression of PrP^C partially rescued cerebellar granule cell degeneration but not demyelination (Radovanovic et al., 2005). This indicates that these diseases are distinct, and that endogenous expression of PrP^C in both neurons and glia was required for complete reversion of the degenerative phenotype.

Transgenic mice expressing truncated PrPΔ94-134, but not those expressing a smaller deletion PrPΔ114-121,

also showed extensive central and peripheral myelin degeneration and early ataxia, which progressed to spastic paraparesis and full paraplegia (Baumann et al., 2007). This lethal phenotype was not associated with the presence of pathological aggregates, altered PrP^C glycosylation, subcellular mislocalization or inappropriate membrane topology (Baumann et al., 2007). Interestingly, the most prominent phenotype was observed in animals expressing an even smaller truncation, PrPΔ105-125. These mice developed an extremely severe illness within two weeks of birth, with decreased body size and weight, immobility, impaired righting reflexes, myoclonus and tremor, and died within one month. Histopathology demonstrated cerebellar atrophy, severe loss of cerebellar granule cells, gliosis and astrocytic hypertrophy (Li et al., 2007). It is important to point that the degenerative phenotype observed in mice expressing PrPΔ32-121, PrPΔ32-134, PrPΔ94-134 or PrPΔ105-125 was rescued by introducing the wild-type *Prnp* gene (Shmerling et al., 1998; Baumann et al., 2007; Li et al., 2007).

These deleted regions likely contain functional PrP^C domains relevant for trophic interactions. They lay within the N-terminal and hidrophobic regions, which are the less structured domains and therefore more accessible regions. In fact, this is the interface for PrP^C dimerization (Rambold et al., 2008) and the region where most of the binding sites for PrP^C ligands such as Heparan Sulphate Proteoglicans (HSPG), vitronectin, low-density lipoprotein receptor related protein 1 (LRP1) and Stress Inducible Protein 1 (STI1) were identified (Figure 1A).

Transgenic mice expressing truncated molecules at the C-terminal of PrP^C, also presented an altered phenotype. The expression of truncated PrP^C molecules, PrPΔ177-200 or PrPΔ201-217, associated with another deletion at aas 23 to 88, which is by itself innocuous, caused a neuronal storage disease associated with neurodegeneration and signals of cerebellar disorder (Muramoto et al., 1997). Both deletions caused PrP^C accumulation within cytoplasmatic inclusion bodies in enlarged neurons. Relative smaller number of axons in the white matter tracks were observed in PrPΔ177-200 while PrPΔ201-217 animals presented extensive nerve cells loss in the CA1 region of the hippocampus (Muramoto et al., 1997) (Figure 1D).

The PrP^C ligands at the C-terminal domain of PrP^C characterized until recently are laminin, 37 kDa Laminin Receptor Precursor/ 67 kDa Laminin Receptor (37LRP/67LR), and the K$^+$ channel, TREK1 (Figure 1A). All these ligands present neurotrophic properties, and particularly the association of laminin with PrP^C allows for neuritogenesis and maintenance of growth cones (see below).

In turn, a large spectrum of clinical symptoms and pathological features are found in patients presenting genetic prion diseases. A series of at least 55 pathogenic mutations are distributed along the PrP^C sequence, and respond for 3 distinct illnesses: Creutzfeldt - Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS) Syndrome, and Fatal Familial Insomnia (FFI) (Figure 1C).

Two octapeptide deletions and 29 insertions were described in the octarepeat region, with the former being associated with a CJD phenotype and later often linked with GSS (Kong Q et al., 2003). Seven point mutations are found in the N-terminal domain and Helix 1 (H1) of PrP^C and 6 of

them cause GSS (Figure 1C). On the other hand, 11 out of 17 mutations described in the C-terminal domain of PrP^C cause CJD and most of them are clustered in helices 2 and 3 (H2 and H3) (reviewed by Kong et al., 2003; Rodriguez et al., 2005; Ye et al., 2008). Thus, there is a preferential cluster of GSS phenotypes associated with the N-terminal and H1 regions and a preferential CJD phenotype linked to the C-terminal domain of PrP^C.

The human PrP^C domain comprehending aas 95 to 135 (equivalent to mouse 94 to 134) presents 5 point mutations (P102L, P105L, G114V, A117V and G131V) all of each associated with GSS. This is exactly the PrP^C domain the deletion of which causes neurodegeneration in mice, as discussed above. Remarkably, although brain homogenates obtained from GSS patient with P102L mutation induced spongiform encephalopathy in recipient animals (Masters et al., 1981), mice overexpressing the transgene P101L (corresponding to the human 102) showed spontaneous neurodegeneration without any detectable protease-resistant PrP^{Sc} (Telling et al., 1996). In addition, the mutation A117V which appears to induce the formation of a transmembrane form (Ctm) of PrP^C, produced neurodegenerative changes in mice but does not cause either PrP^{Sc} deposition or infection in rodents (Hegde et al., 1998). Conversely, the expression of the CJD-linked mutations T183A (DeArmond et al., 1997) or E199K (human E200K) (Telling et al., 1996) in PrPnull mice did not cause any pathological signals.

In cell cultures, the PrP^C hydrophobic domain 113-123 was required for the stress-protective activity of PrP^C (Rambold et al., 2008). In addition, some PrP^C mutants associated to genetic prion diseases and presenting codon 129M are unable (D178, V181I, E196K, V210I, M232R and P238S) or only partially effective (R208H, E211Q) to protect against Bax-mediated cell death (Jodoin et al., 2007). Interestingly, the presence of 129V renders mutated proteins into less active forms (A117V, V203I and T188A) or inactive (E200K, R208H, E211Q) for this phenptype (Jodoin et al., 2007).

The truncation of specific PrP^C domains in transgenic mice or the presence of *PRNP* mutations associated with human genetic prion diseases may alter the recruitment of specific proteins to a PrP^C-based multi-component complex (Martins et al., 2002; Linden et al., 2008) in particular those already known to interact with specific PrP^C domains and promote neurotrophic functions. In addition, the composition of these complexes may vary among neurons and glial cells which may explain, at least in part, the diverse degenerative phenotypes observed in transgenic mice and in patients expressing mutated PrP^C.

The PrP^C ligands mediating neurotrophic activity

Several dozen proteins have been described to bind PrP^C in various cellular compartments, but in most cases there is still no known associated biological function for these interactions (Linden et al., 2008). PrP^C is normally localized at the cell surface and traffics along the endocytic pathway (Prado et al., 2004), consistent with the idea that its association with other proteins in these compartments may be relevant for its neurotrophic activity.

Extracellular matrix (ECM) components, as well as their receptors at the cell surface, are known to create a milieu necessary for survival and differentiation in the developing nervous system (Pires Neto et al., 1999; De and Georges-Labouesse, 2000) and in the remodeling of adult brain in both normal (Grimpe and Silver, 2002) and pathological conditions (Rauch, 2004; Theodosis et al., 2004). PrP^C is able to associate with ECM proteins such as laminin (Graner et al., 2000a; Graner et al., 2000b) and vitronectin, but not to fibronectin or collagen (Hajj et al., 2007). PrP^C also binds to ECM glycosaminoglycans such as heparin and heparan sulfate (Warner et al., 2002) and to ECM receptors such as the 37 kDa Laminin Receptor Precursor/ 67 kDa Laminin Receptor (37LRP/67LR) (Rieger et al., 1997).

Laminins are large (400–900 kDa) extracellular heterotrimeric glycoproteins composed of various combinations of α, β, and γ chains. To date, five α, four β, and three γ chains have been identified, which may combine into sixteen known forms of laminins in mammals. The individual chains present diverse biological active domains and interact with a large number of receptors, such as at least eight different integrins, 37LRP/67LR, dystroglycan, 67 kDa elastin-laminin receptor, galactoside-binding lectin, galactosyltransferase, heparan sulfate proteoglycans and immunoglobulin-related basal cell adhesion molecule (Tzu and Marinkovich, 2008). These varied interactions are crucial to promote the diverse biological functions attributed to laminin in distinct cell types, particularly in the nervous system (Colognato and Yurchenco, 2000). For example, laminin is one of the most important ECM proteins responsible for axonal growth (Luckenbill-Edds, 1997). In addition, defects in both the peripheral and central nervous system frequently accompany mutations in laminin isoforms. Also, the loss of interneuronal laminin in the hippocampus due to proteolysis induced by excitotoxicity, leads to neuronal death (Colognato et al., 2005).

PrP^C binds to a laminin domain at the γ-1 chain (Graner et al., 2000a; Graner et al., 2000b), a region previously demonstrated to promote neuritogenesis (Liesi et al., 1989). The laminin binding site resides within amino acids 173-182 of the PrP^C molecule (Coitinho et al., 2006). Using primary hippocampal neuron cultures from either wild-type or PrP^C-null mice, it was shown that the interaction of PrP^C with the laminin γ-1 chain induces neuritogenesis (Graner et al., 2000a). Inactivation of PrP^C by chromophore-assisted laser inactivation (CALI) in PC-12 cells also impaired neurite extension induced by laminin. Furthermore, retraction of growth cones was observed when micro-CALI was used to inactivate PrP^C in these structures (Graner et al., 2000b).

The importance of the interaction of PrP^C and laminin was also demonstrated *in vivo*. Antibodies against either the entire PrP^C and laminin molecules, or against peptides representing the binding sites either in PrP^C or in laminin γ-1 chain, blocked memory consolidation when infused in the rat hippocampus. In addition, the γ-1 chain peptide competed with antibodies against the PrP^C 170-183 peptide, and reversed the latter's inhibitory activity upon memory consolidation (Coitinho et al., 2006). Thus, PrP^C-aminin interaction in the hippocampus is associated with neuritogenesis, with an impact upon neuronal plasticity and memory consolidation.

PrP^C also interacts with 37LRP/67LR, one of the first ligands of PrP^C identified by using a two-hybrid system (Rieger et al., 1997). The 37LRP/67 LR is a membrane associated protein, first discovered by laminin–sepharose affinity chromatography. At first, 37LRP/67LR interaction

with laminin was considered to be simply adhesive. However, it is now clear that activation of the 37LRP/67LR induces dynamic events (increased filopodia, directional motility and modulation of gene expression), as a consequence of hitherto poorly understood signal transduction. In addition to direct interactions with laminin, it has been proposed that the 67LR facilitates interactions between laminin and integrins (Nelson et al., 2008).

The interaction between PrP^C and the 37LRP/67LR was confirmed in mammalian cells, where it was shown that 37LRP/67LR co-localizes with PrP^C at the cell surface. Recombinant or purified PrP^C bind 37kDa LRP/ 67kDa LR at the cell surface, where the latter mediates the internalization of exogenous added PrP^C (Gauczynski et al., 2001). It should be noted, however, that this exogenous added PrP^C lacks the localization in rafts and is of course not GPI-anchored at the membrane. The binding residues for 37kDa LRP/ 67kDa LR have been identified between amino acids 144-179 in PrP^C (Hundt et al., 2001), which partially overlaps the binding site for laminin itself (Coitinho et al., 2006). Thus, it is likely that interaction of PrP^C either with the 37LRP/ 67LR or laminin may be mutually exclusive at this PrP^C domain. Although this competitive interaction has yet to be tested, such an overlap, together with the coincidence of binding sites in 37LRP/ 67LR for both laminin and PrP^C (Rieger et al, 1999), should have important consequences for the effects of PrP^C upon neurite growth and differentiation.

Binding of 37LRP/67LR to an additional binding site at PrP^C amino acids 53 to 93 is mediated by the presence of heparan sulfate proteoglycans at 37LRP/67LR. Interestingly, the 37LRP/67LR domain which contains heparan sulfate proteoglycans maps to between amino acids 101–160 or 180–295 (Hundt et al., 2001), the latter of which straddles a laminin binding region (205-229) (Nelson et al., 2008). Indeed, two out of three laminin binding sites at the 37LRP/67LR may compete by the latter's interaction with PrP^C.

Remarkably, barring steric hindrance constrains, laminin may be able to simultaneous bind the 37LRP/67LR (using its third available binding site located within the cysteine-rich EGF-like repeat in the β1 subunit) (Nelson et al., 2008), PrP^C (at the C-terminal portion of the γ1 chain), as well as other receptors, such as integrins (most of which bind to the α chain of laminin) (Tzu et al., 2008). These multiple interactions would lead to the assembly of a molecular cluster of cell membrane receptors, which may promote neurotrophic signaling by both integrin-mediated signal transduction, as well as through signals initiated by activation of PrP^C by laminin, followed by its possible internalization mediated by the 37LRP/67LR (Figure 2A). This is but one of the multicomponent signaling modules that may be assembled through molecular interactions mediated by the prion protein (Martins et al., 2002; Linden et al., 2008).

Interaction of PrP^C with glycosaminoglycans, particularly heparan sulfate, has been extensively documented (Priola and Caughey, 1994). In addition, in vitro binding studies demonstrated that PrP^C directly interacts with heparan sulfate through domains 23–52, 53–93, and 110–128 (Warner et al., 2002). Although no functional consequence of this interaction has been addressed so far, it may have important consequences especially for the binding and internalization of the 37LRP/ 67LR.

The PrP^C role as an ECM receptor/co-receptor is not limited to laminin. PrP^C also binds another ECM protein, vitronectin (Vn), through amino acids 106-116 (Hajj et al., 2007). This PrP^C-Vn interaction is important for the growth of axons in dorsal root ganglia during embryogenesis (Hajj et al., 2007) (Figure 2A). Interestingly, vitronectin has been described to have important roles in motor neuron differentiation in association with the morphogen Sonic Hedgehog (Pons and Marti, 2000; Pons et al., 2001). Also during development of the cerebellum, the migration of granular neurons is regulated by an ECM transition. When cells are in the external granule cell layer, the most predominant ECM molecule is laminin, which stimulates granule cells to proliferate. When cells start to migrate to the internal granule cell layer, they move towards an ECM region rich in vitronectin. Cells that reach vitronectin stop proliferating and differentiate, forming the layers of the fully differentiated cerebellum (Pons et al., 2001). Since PrP^C binds both laminin and vitronectin it may be involved in the development of the cerebellum, by regulating the precise moment of each association and thus influencing the fate of the cells.

Remarkably, knocking down PrP^C in cerebellar neural circuits and in particular on granule cells, compromises the function of these cells causing behavioral alterations such as low performance in rotarod and runway tests. It was demonstrated that cerebellar granule cells from wild-type mice stop their division by the second post-natal week, as expected, while PrP^C-null granule cells divide until post-natal week three. With older age, these differences disappear indicating that the absence of PrP^C can be compensated during the development (Prestori et al., 2008).

Perhaps the most extensively studied PrP^C-binding protein, so far, is Stress Inducible Protein 1 (STI1). Interaction of PrP^C with a 66kDa protein was discovered in 1997, and the latter was proposed as a PrP^C receptor, which might be responsible for mediating PrP^C internalization and inhibiting the toxicity of the PrP^C peptide 106-126 (Martins et al., 1997). It was subsequently found that the interaction between the 66kDa protein and PrP^C triggered protection against apoptosis in an in vitro retina model (Chiarini et al., 2002). Notwithstanding the lack of evidence for its functioning as a PrP^C receptor (Zanata et al., 2002), the role of STI1 as an important inducer of PrP^C-mediated neurotrophic effects was further supported by the demonstration that their binding promoted both protection against neuronal death as well as neuritogenesis, through the PKA and ERK1/2 signaling pathways, respectively (Lopes et al., 2005) (Figure 2B).

Recent work in astrocytes has shown that STI1 is a secreted PrP^C ligand. STI1 is released by cultured astrocytes in large amounts by still unknown mechanisms, and interacts with neuronal PrP^C, promoting protection against cell death (Lima et al., 2007). Effects of STI1 in vivo have also been demonstrated. Experiments with rats in an inhibitory avoidance task demonstrated that antibodies against STI1 and PrP^C, when injected directly into the hippocampus, impair memory formation and consolidation. Remarkably, the STI1 peptide that represents the PrP^C binding site improves memory formation and consolidation (Coitinho et al., 2007). It is, however, still unknown whether the effects upon memory are related to neurotrophic effects of the interaction of STI1 with PrP^C.
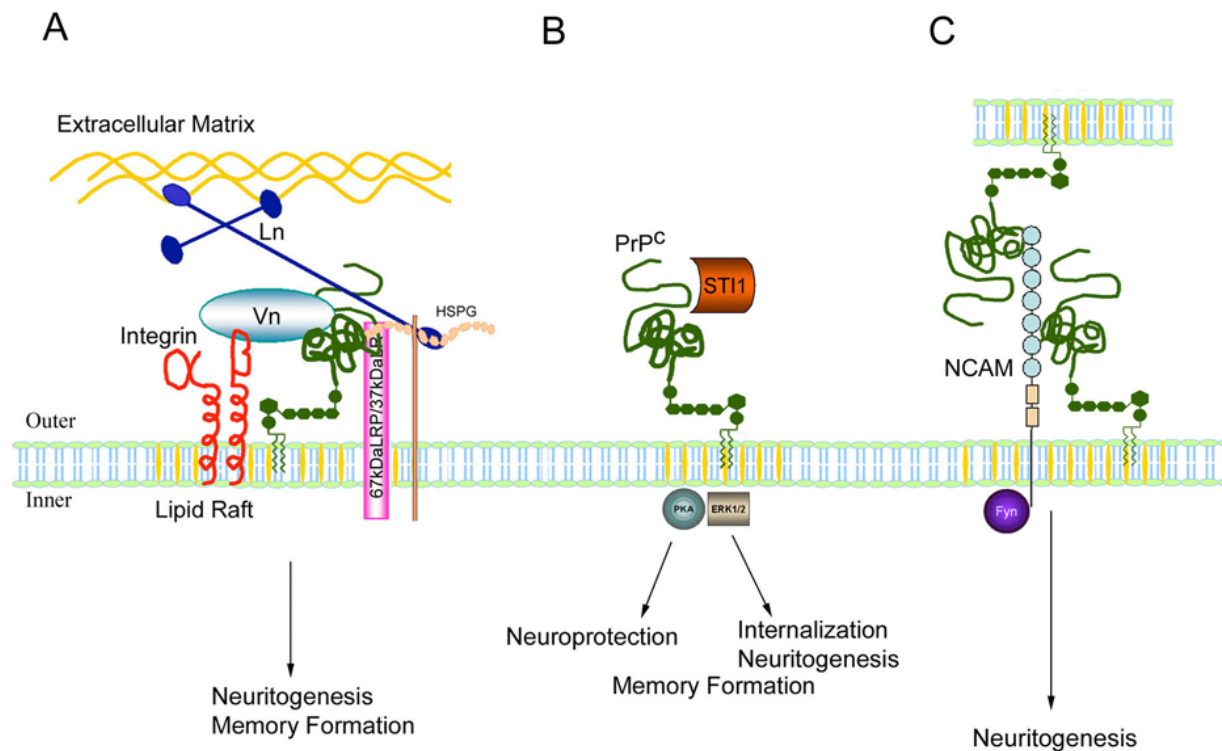
Figure 2. The PrP^C ligands mediating neurotrophic activity.
A) PrP^C directly interacts with the ECM proteins: Laminin (Ln) and Vitronectin (Vn), and with the laminin receptor (37LRP/67LR), possibly forming a large complex involved in neuritogenesis and memory formation. Interaction of the laminin receptor with PrP^C is also mediated by Heparan Sulfate Proteoglycans (HSPG). Integrins are ECM receptors, and may also take part in this complex. B) PrP^C interacts with the secreted Stress Inducible Protein 1 (STI1), which mediates neuroprotection and neuritogenesis through independent signaling pathways (PKA and ERK1/2). C) NCAM is a transmembrane protein that interacts with PrP^C either in *cis* or in *trans*, and promotes neuritogenesis through the activation of Fyn kinase.

PrP^C modulates expression and/or activity of transmembrane receptors associated with neurotrophic activities. Since so many ligands have been ascribed to PrP^C, the fundamental question remaining about PrP^C is how the signal is transduced through the membrane. PrP^C is a GPI-linked protein known to be preferentially localized in membrane domains enriched in sphingolipids and cholesterol (lipid rafts) (Fivaz et al., 2002). These raft domains contain several molecules which recruit specialized proteins for intracellular signaling (Gorodinsky and Harris, 1995) and it is possible that PrP^C can modulate the activity of these proteins by either direct or indirect interaction.

In fact, several transmembrane proteins with neuro-trophic activities were shown to be modulated by PrP^C, leading to the activation or inhibition of intracellular signal pathways. These proteins include the neural cell adhesion molecule (NCAM) (Santuccione et al., 2005), integrins (Hajj et al., 2007), G protein coupled receptors (Kristensson et al., 1993; Wong et al., 1996; Mouillet-Richard et al., 2000; Brini et al., 2005) and membrane ion channels (Whatley et al., 1995; Colling et al., 1996; Herms et al., 2001; Azzalin et al., 2006; Khosravani et al., 2008).

NCAM plays crucial roles in brain development, synaptic plasticity and regeneration and is considered an inducer of complex intracellular signaling cascades in response to extracellular cues triggered by either homophilic or heterophilic binding (Ditlevsen et al., 2008). PrP^C-NCAM interaction is direct (Schmitt-Ulms et al., 2001) and occurs in either *cis* or *trans*, i.e. both molecules in the same cell or in distinct cells, respectively. This interaction takes place in raft microdomains, where NCAM uses its PrP^C-guided enrichment in lipid rafts to activate p59fyn kinase and inducing neuritogenesis (Santuccione et al., 2005) (Figure 2C). These data are especially interesting, since it has been demonstrated that Fyn kinase pathway is the most relevant for NCAM-induced neurite outgrowth and synaptic functions (Ditlevsen et al., 2008). On the other hand, NCAM-induced signaling also engages other signaling molecules, such as focal adhesion kinase, growth-associated protein-43, the mitogen-activated protein kinase (MAPK), intracellular Ca^{2+}, and protein kinases A, C, and G (reviewed by (Ditlevsen et al., 2008). In fact, it has been recently demonstrated that PrP^C accumulates is focal adhesions, modulationg cell-substrate interactions both in cells from mammals and *Drosophila*. When PrP^C is highly expressed it modulates cell spreading and filopodia formation while in low levels of PrP^C lamellipodia is more abundant (Schrock et al., 2009).

The major role of PrP^C in cell adhesion and cell-cell communication has also been elegantly demonstrated in zebrafish which presents two PrP^C gene orthologues (PrP1 and PrP2). Deletion of PrP1 caused impairment of embryonic cell adhesion and arrested gastrulation (Malaga-Trillo et al., 2009) while PrP2 removal affects latter stages of neuronal development possibly affecting proliferation and
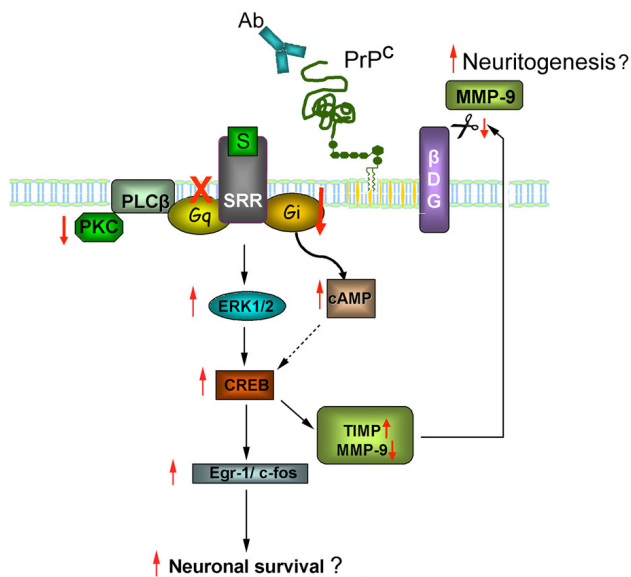
Figure 3. Engagement of PrP^C with specific antibodies modulates signal transduction by altering the activity of G protein-coupled serotonergic receptor. The serotonergic receptor (SRR) in the cell membrane is associated with a calcium stimulatory G protein (Gq), to the Phospholipase Cβ (PLCβ and with a cAMP inhibitory G protein (Gi). This complex responds to serotonin (S) by activating PKC, modulating the levels of cAMP/ERK1/2 (Extracellular Signal-Regulated Kinases 1/2) and CREB (cAMP response element binding protein) pathways. PrP^C binding to specific antibodies (Ab) impaired PLCβ/PKC signaling pathway and partially blocked the reduction of cAMP, up modulates ERK, CREB and Egr-1/c-Fos. These factors may contribute to neuronal survival. On the other hand, up modulation of CREB increases the transcription of TIMP-1 and decreases MMP-9 levels. MMP-9 is a secreted metalloproteinase that cleaves β-dystroglycan (BDG) reducing cell-matrix adhesion. Upon PrP^C ligation, MMP9 levels are diminished and β-dystroglycan interaction with the extracellular matrix is stabilized, what may increase neuritogenesis.

differentiation of developing neurons (Malaga-Trillo et al., 2009 and unpublished results). The alterations related to PrP1 are caused by deficient cell movements modulated by E-cadherin–based adhesion and signaling which are conserved across vertebrates.

As described above PrP^C is able to interact with vitronectin. The vitronectin peptide which represents the PrP^C binding site promotes axonal growth in wild-type but not in PrP^C-null neurons. Vitronectin, on the other hand, supports axonal growth in both wild-type and PrP^C-null neurons, suggesting that other vitronectin ligands can compensate for the absence of PrP^C. In fact, the conduction of functional assays showed that neurons derived from two different PrP^C-null mouse strains presented higher activity of the integrin αvβ3 (Hajj et al., 2007), a classical vitronectin transmembrane receptor. Although, a direct interaction of αvβ3 with PrP^C has never been demonstrated, β3 integrin immunoprecipitates with PrP^C (Hajj G and Martins VR, unpublished results) and β1 integrin is engaged by antibody

crosslinking-mediated neuritogenesis (Pantera et al., 2009), indicating that they can associate in the same protein complex (Figure 2A).

Integrins are a large group of heterodimeric surface membrane receptors widely expressed in the nervous system and have been shown to have diverse roles in axon extension, neural development and, in addition, they are key regulators of synaptic plasticity involved in memory and learning in the adult nervous system (reviewed by (Clegg et al., 2003). The negative control of integrin activity by PrP^C may represent an important regulation of neurotrophic functions.

In the neuroectodermal progenitor 1C11 cell line, that can be differentiated into either serotonergic (1C11^5-HT) or noradrenergic (1C11^NE) phenotype, the engagement of PrP^C with antibodies had a high impact on signal transduction (Mouillet-Richard et al., 2000). This effect was restricted to cell processes and most likely on varicosities of the neurites (Mouillet-Richard et al., 2000). PrP^C engagement with G-protein coupled serotonergic receptors (GPCR) 5-HT, 5HT2A, 5HT1B/D and 5-HT2B simultaneously with their stimulation by agonists, altered the intensities and/or dynamics of G protein activation (Mouillet-Richard et al., 2005). In particular, PrP^C binding with antibodies impaired phospholipase Cβ (PLCβ) activation by 5-HT2A receptors, partially blocked the reduction of cAMP promoted by agonist binding to 5-HT1B/D and potentiated the Phospholipase A2 response through 5-HT2A (Mouillet-Richard et al., 2005). In addition, the same procedure triggered transduction cascades controlling the cellular redox state and the ERK1/2 kinases (Mouillet-Richard et al., 2007), leading to CREB activation followed by transcription of Egr-1 and c-fos. These factors have been previouly associated with the control of neuronal survival (Zhang et al., 2002; Thiel and Cibelli, 2002). On the other hand, the transcription of the metalloproteinase TIMP-1 was enhanced by CREB while the transcription of MMP-9 was reduced, decreasing the processing of β-dystroglycan. Since MMP-9 process laminin, which is also a ligand for β-dystroglycan, it has been suggested that the PrP^C-mediated control of MMP-9 stabilizes the interaction between PrP^C and its cell/ECM partners, and may potentiate synaptic efficacy (Pradines et al., 2008) (Figure 3).

The transfection of the PrP^C gene into CHO cells affects intracellular calcium dynamics following activation of purinergic receptors (P2Y). In transfected cells treated with ATP, Ca^{2+} mobilization from endoplasmic reticulum and Ca^{2+} uptake by mitochondria were lower than in non-transfected cells (Brini et al., 2005). These alterations are known to reduce cellular sensitivity to Ca^{2+}-mediated apoptosis and therefore, are cytoprotective (Giacomello et al., 2007; Marchi et al., 2008). In addition, the expression of PrP^C in CHO cells also increased the the Ca^{2+} influx through the plasma membrane after ATP treatment (Brini et al., 2005). It was speculated that the latter effect was mediated by PrP^C activation of store operated calcium channels (SOCCs) (Brini et al., 2005). These channels are functionally coupled with the activation of adenyl cyclase (Fagan et al., 2000) which has been associated with PrP^C-mediated neuronal survival (Chiarini et al., 2002; Lopes et al., 2005) (Figure 4). Whether Ca^{2+} hadling of the main intracellular stores and entry from extracellular space depend on the direct interaction of PrP^C with P2Y receptors or SOCCs is still unknown.
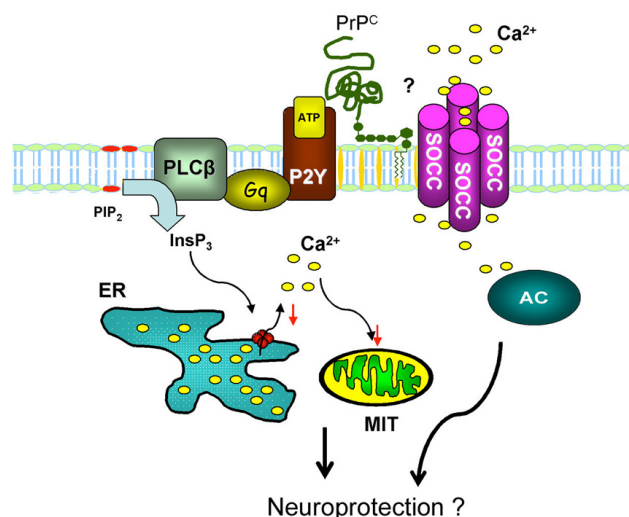
Figure 4. Expression of PrP^C modulates intracellular Ca^{2+} homeostasis and signaling transduction through G protein-coupled purinergic receptors. The purinergic receptors (P2Y) are transmembrane proteins coupled to calcium stimulatory G protein (Gq) and to phospholipase Cβ (PLCβ). P2Y receptors are activated by ATP, promoting cleavage of phosphatidylinositol-bisphosphate (PIP2) into Inositol-3-Phosphate (InsP3) by the PLCβ. InsP3 promotes a calcium release from the endoplasmic reticulum (ER), and released calcium can be sequestered by the mitochondria, leading to cell death. The expression of PrP^C leads to a reduction of Ca^{2+} mobilization from the ER and Ca^{2+} uptake by the mitochondria what can contribute to neuronal protection. In contrast, there is an increasing of calcium influx through the plasma membrane, probably by the activation of Store Operated Calcium Channels (SOCCs) and increase of the activity of adenylate cyclase. The latter has been associated with PrP^C-mediated neuroprotection.

Figure 5. Prion infection impairs mobilization of intracellular Ca^{2+} by bradykinin. The bradykinin receptor (BKR) is a cell membrane protein coupled to calcium stimulatory G protein (Gq) and to Phospholypase Cβ (PLCβ). When bradykinin (BK) binds to its receptor, it promotes the PLCβ-catalysed cleavage of phosphatidylinositolbisphosphate (PIP2) into Inositol-3-Phosphate (InsP3). InsP3 promotes calcium release from endoplasmic reticulum (ER), which has been attributed a role against ischemia. In cells infected with PrP^{Sc}, there is an increase in BKR expression. Paradoxically, there is a reduction of 90% in InsP3 production, resulting in a decrease of Ca^{2+} mobilization from the endoplasmic reticulum (red arrows). These effects are attributed to both reduced affinity of BK for its receptor in this condition, and to a modification of lipid composition in the plasma membrane, with diminished availability of PIP2.

Prion infection reduced the effect of bradykinin on the increase of intracellular Ca^{2+} (Kristensson et al., 1993). This alteration occurs by a reduced affinity of BK for its receptor and also by a modification of lipid composition in the plasma membrane with the diminished availability of phosphatidylinositol-bisphosphate (PIP2). Bradykinin is a 9-amino acid peptide with a wide range of biological actions mediated through B1 and B2 subtypes of G-protein-coupled receptors (reviewed by (Calixto et al., 2004). Activation of these receptors induces a transient increase in the cytosolic concentration of calcium caused by both its mobilization from intracellular stores and influx from extracellular environment (Kristensson et al., 1993 ; Martins et al., 2005). This has been associated with neuroprotection after ischemia (Ping et al., 2005; Yan-Feng et al., 2008; Danielisova et al., 2008). Whether PrP^C modulates the activity of bradykinin receptors, and the suppression of bradykinin activity in prion infected neurons is due to PrP^C-loss-of-function deserves further investigation (Figure 5).

PrP^C also interacts with ionic channels activated by conformational modification promoted by either voltage and/or binding to a specific liga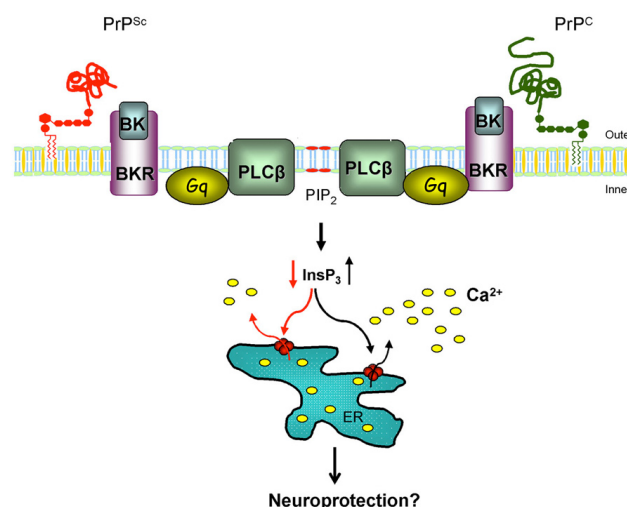nd. Once activated, these proteins promote changes in the concentrations of Na$^+$, K$^+$, Ca^{2+} or Cl$^-$, thus modulating a variety of electrophysiologically-dependent cellular responses. An interaction between PrP^C and a two-pore potassium channel protein, TREK (TWIK-1-related K$^+$ channel), has been demonstrated using a bacterial two-hybrid approach (Azzalin et al., 2006). TREK-1 (Fink et al., 1996) is a protein that forms a mechanically-gated channel activated by temperature, membrane stretch and internal acidosis, and involved in neuroprotection via activation of PKA (Patel and Honore, 2001; Franks and Honore, 2004). Immunoprecipitation assays confirmed the association between both proteins, and that the binding site for TREK localizes at the carboxy-terminal of PrP^C (aas 128-230). Confocal analysis demonstrated that fluorescent transfected PrP^C and TREK co-localized and, more importantly, that endogenous proteins exhibited co-localization in cerebellar Purkinje cells (Azzalin et al., 2006) (Figure 6A).

The involvement of PrP^C with L-type voltage-gated calcium channels (VGCC) has been first claimed some years ago (Whatley et al., 1995). Reduction of calcium influx, through VGCC, was observed in both cerebellar granule cells and hippocampal neurons slices of PrP^C-null
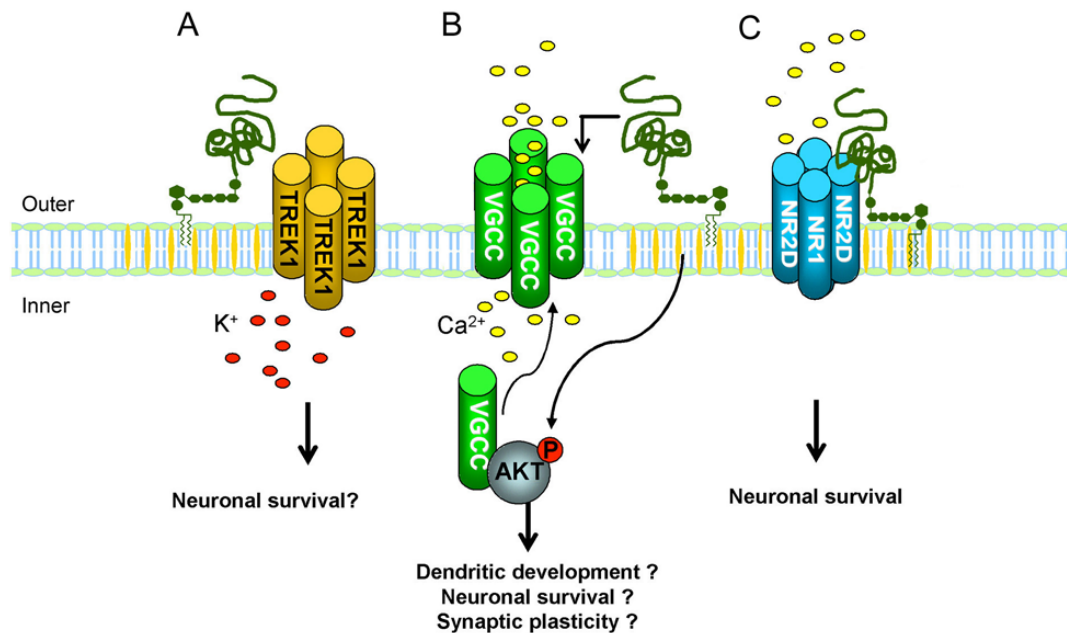
Figure 6. PrP^C interacts with or modulates the activity of ionic channels.
A) PrP^C interacts with a two-pore potassium channel protein (TREK-1), which forms a mechanically-gated K$^+$ channel, and reportedly promotes neuroprotection via PKA. B) Voltage gated calcium channels (VGCCs) promote an increase in a cytoplasmic calcium. Cells from PrP^C-null mice present a reduction in calcium influx by VGCCs, probably caused by the impairment in AKT activity and phosphorylation of the VGCC subunits which is an essential step for their insertion in the membrane. C) PrP^C interacts with the NR2D subunit of NMDA (N-methyl-D-aspartic acid) receptors, which are ionotropic glutamate receptors permeable to Ca^{2+}. When PrP^C is absent, the NMDA channel is more sensitive to NMDA, which promotes an increase of calcium influx leding to neuronal cell death.

mice when compared to wild-type neurons (Herms et al., 2000; Fuhrmann et al., 2006). In fact, it is known that VGCC are modulated by PI3K/AKT (Viard et al., 2004), whose activation is fundamental to increase VGCC insertion or prolong their expression at the plasma membrane. There is no evidence of a direct interaction of PrP^C with VGCC. However, it is known that activity of PI3K/AKT is diminished PrP^C-null mice (Weise et al., 2006), which may contribute either to a lower insertion or to abbreviate the surface expression of these receptors. One of the most relevant roles of VGCCs is to regulate mechanisms of calcium ions delivery into specific intracellular compartments for a variety of calcium-dependent processes; including dendritic development, neuronal survival, and synaptic plasticity (reviewed by (Moreno, 1999). PrP^C could then modulate neurotrophic functions indirectly through its positive effect on the activation on VGCC (Figure 6B).

Several studies demonstrated that hippocampal neurons from PrP^C-null mice presented increased excitability when compared to wild-type animals (Collinge et al., 1994; Colling et al., 1996; Mallucci et al., 2002). *In vivo* studies also showed that, when compared to wild-type mice, PrP^C-null animals show a lower seizure threshold and higher mortality after treatment with convulsant drugs such as the ionotropic glutamate analog, kainic acid (Walz et al., 1999). Interestingly, PrP^C-null mice presented an elevated susceptibility to neuronal damage induced by kainic acid which is inhibited by the N-methyl-D-aspartate (NMDA) receptor blocker MK-801 (Rangel et al., 2007). NMDA receptors are formed by a tetrameric channel (two NR1 and two NR2 subunits), which are activated by glutamate and are permeable to both sodium and calcium ions (Meldrum, 2000). The expression of NMDA subtypes can be regulated by PrP^C (Maglio et al., 2004). Remarkably, PrP^C associates with NMDA receptors containing NR2D and downregulates their function, preventing depolarization and calcium influx, thus playing a neuroprotective role (Khosravani et al., 2008) (Figure 6C).

PrP^C shedding and secretion: PrP^C as a neurotrophic factor

Despite the preferential PrP^C localization at the cell surface, as a glycosylphosphatidylinositol (GPI)-anchored protein, this molecule presents alternative topologies that may have inherent physiological functions. Soluble forms of PrP^C have been identified not only in the medium of cultured cells (Borchelt et al., 1993; Harris et al., 1993; Parizek et al., 2001; Mattei et al., 2009) but also in human cerebrospinal fluid (Tagliavini et al., 1992), human and murine serum (Parizek et al., 2001; Mattei et al., 2009), and released by human platelets (Perini et al., 1996). At least four mechanisms have been associated to "soluble" forms of PrP^C: 1) release to the extracellular space after removal of its GPI-anchor by post-translational modifications possibly caused by escape of glycolipidation (Borchelt et al., 1993; Harris et al., 1993) (Figure 7A); 2) shedding from the cell surface via phospholipase C cleavage of the GPI anchor (Parkin et al., 2004) (Figure 7A); 3) secretion by exosomes (Fevrier et al., 2004) (Figure 7B) and 4) endoproteolytic cleavage by ADAMs (A Disintegrin And Metalloproteases) (Vincent et al., 2001; Cisse et al., 2005) (Figure 7C).
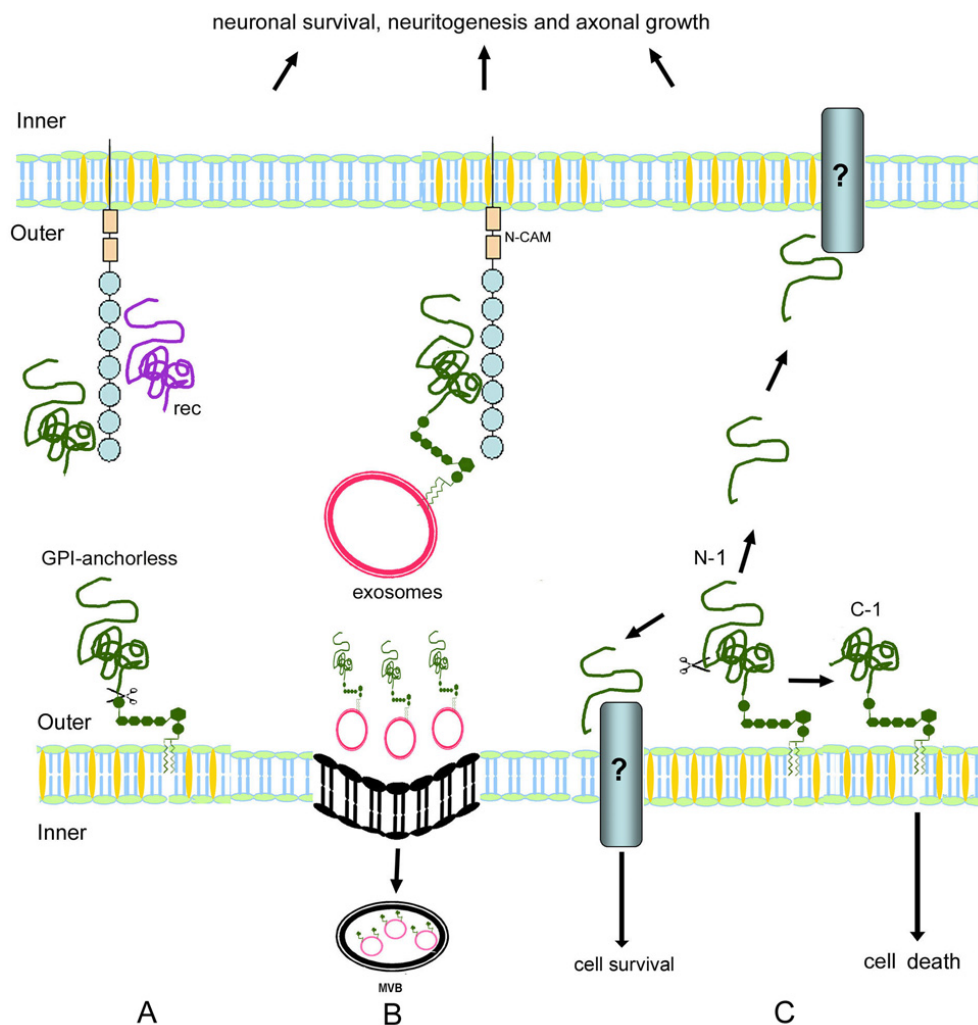
Figure 7. PrP^C can reach the extracellular space either in exosomes or in soluble forms, and mediate neurotrophic activities. PrP^C can be released from cells through distinct mechanisms. A) The PrP^C GPI-anchor can be removed by posttranslational modification or cleaved by phospholipase C. The anchorless PrP^C found in the extracellular space, as well as exogenous recombinant PrP^C, can bind NCAM and modulate neurotrophic functions. B) PrP^C is associated with exosomes, which are released by cells upon fusion of multivesicular bodies (MVB) with the plasma membrane. PrP^C in exosomos can interact with transmembrane proteins, i.e. NCAM, to transduce neuroprotective effects. C) Metaloproteases cleave PrP^C at residues 110/111 generating a soluble N-terminal fragment (N-1) and a C-terminal fragment (C-1) tethered to the plasma membrane. The soluble N-1 fragment induces neuronal survival probably by interacting in either *cis* or *trans* with an unidentified cell surface receptor. The C-1 fragment tethered to the plasma membrane has pronounced pro-apoptotic activity.

Soluble PrP^C can act as a ligand triggering neurotrophic functions, and these functions were mimicked in cell cultures by recombinant protein. When mouse primary cerebellar granule neurons were grown in the presence of recombinant PrP^C, signal transduction components such as PKA, Src-related tyrosin kinases, phosphatidylinositol3-kinaseAkt, and MAPK/Erk kinases were activated, promoting neurite outgrowth and neural survival (Chen et al., 2003). In addition, *in vitro* experiments demonstrated that recombinant PrP^C induces both neuronal survival in mouse cortical neurons (Lima et al., 2007), and rapid neuronal polarization and development of synapses in embryonic rat hippocampal neurons (Kanaani et al., 2005).

Although secreted forms of PrP^C were identified in exosomes and microvesicles from neuronal cells, (Fevrier

et al., 2004; Mattei et al., 2009), platelets (Robertson et al., 2006) and ovine cerebral spinal fluid (Vella et al., 2008) their biological functions are unknown. Remarkably, PrP^C secreted from astrocytes has been associated with neuronal survival (Lima et al., 2007), and vesicular structures containing PrP^C were identified by biochemical methods and ultrastructural criteria following immunohistochemistry of astrocyte conditioned media (C. Arantes, G. Hajj, M. H. Lopes, I. Porto-Carreiro, R. Linden and V.R. Martins, unpublished observations) (Figure 7B).

GPI-anchored PrP^C is not a partner for soluble PrP^C, since similar effects were observed when the latter was used to treat either wild-type or PrP^C-null neurons (Chen et al., 2003; Kanaani et al., 2005; Santuccione et al., 2005; Lima et al., 2007). Conversely, NCAM-null neurons

were unable to respond to soluble PrP^C (Santuccione et al., 2005), thus indicating that NCAM is required for the neurotrophic functions mediated by physiologically secreted PrP^C molecules or recombinant PrP^C (Figures 7A and B).

PrP^C is endo-proteolytically cleaved at the N-terminal side, at the 110/111 residues, thus producing a 17-kDa C-terminal fragment C1 tethered to the plasma membrane (Harris et al., 1993; Chen et al., 1995; Laffont-Proust et al., 2005). The 9-kDa soluble N-terminal counterpart referred as N-1 is constitutively released in the extracellular medium depending on protein kinase C (Vincent et al., 2000). Three disintegrins contribute to this processing, either directly such as ADAM-10 and -17 (Vincent et al., 2001) or indirectly such as ADAM-9 (Cisse et al., 2005). The activation of muscarinic receptor increases the physiological processing of PrP^C through upregulation of the phosphorylation and activity of ADAM-17 (Alfa et al., 2007).

The C-terminal fragment that remains attached to the cell membrane (C-1) displays pro-apoptotic functions and regulates p53 mRNA transcription and activity (Sunyach et al., 2007) (Figure 7C). Conversely, a recombinant N-1 fragment protected cells from staurosporine-induced apoptosis, by reducing caspase-3 activity, p53 promoter trans-activation and activity as well as p53 mRNA levels (Sunyach in preparation, cited in (Vincent et al., 2008) (Figure 7C). These data indicate that opposite effects of C-1 and N-1 domains may maintain an appropriate balance between cell survival and death.

The mechanisms associated to the pro- and anti-apoptotic effects of the C-1 and N-1 PrP^C fragments remain unknown, and deserve further investigation. These effects may be due to the inability of the membrane-bound C-1 to bind and/or to modulate certain PrP^C partners such as GAG, vitronectin, ligands involved in the neurotrophic role of PrP^C, or LRP1, which binds PrP^C between amino acids 23-107 and is involved with internalization of PrP^C (Parkyn et al., 2008). However, the anti-apoptotic role of PrP^C has been ascribed to the cAMP/PKA pathway (Lopes et al., 2005), and neither the activation of this pathway nor its survival-promoting role appear to be dependent on endocytosis (Caetano et al., 2008). On the other hand, the protective effects of N-1 fragment can occur after its interaction in either *cis* or *trans* with unidentified cell surface proteins (Figure 7C).

Therefore, both GPI-anchored and soluble PrP^C, present overlapping neurotrophic properties. This is consistent with the hypothesis that PrP^C functions as a cell surface scaffolding protein, since the relative preservation of the structure of PrP^C irrespective of its anchoring into the plasma membrane would allow similar molecular interactions of either the membrane-bound or the released forms (Linden et al., 2008). Nonetheless, small structural changes of PrP^C upon interaction with lipids (Morillas et al., 1999; Eberl et al., 2004; Hicks et al., 2006) may affect interaction of PrP^C with its ligands. It is possible that neurotrophic effects of the GPI-anchored PrP^C may be complemented by specific regulation of PrP^C secretion, shedding and cleavage.

PrP^C localization in specific membrane microdomains and PrP^C cellular traffic modulate neurotrophic functions

Lipid rafts form an ordered phase in membranes rich in cholesterol and sphingolipids and are likely to be the sites where GPI-anchored proteins are preferentially partitioned.

It has long been recognized that PrP^C, as other GPI-anchored proteins (Fivaz et al., 2002), is present in lipid rafts (Naslavsky et al., 1997). Early work indicated that PrP^{Sc} was present in caveole-like domains in brain tissue (Vey et al., 1996), however pioneer studies using chicken PrP^C showed that the protein is sequestered from the cell surface in clathrin-coated pits (Shyng et al., 1994). This work received some initial criticism, due to the perceived default pathway of internalization of GPI-anchored proteins by caveolae and other non-clathrin mediated pathways (Vey et al., 1996). However, recent work has shown that PrP^C in neurons and neuronal cells is indeed predominantly sequestered by a clathrin-mediated pathway (Sunyach et al., 2003). Both dynamin K44A, a dominant-negative inhibitor of dynamin activity (Magalhaes et al., 2002), and AP-180C, the C-terminal fragment of the endocytic protein AP180, when overexpressed in cells block the internalization of PrP^C (Taylor and Hooper, 2007). Both these treatments have been suggested to block selectively clathrin-mediated endocytosis, albeit dynamin K44A also appears to interfere with caveolae-dependent endocytosis (Prado et al., 2004). Co-localization experiments in neurons shows that mammalian PrP^C is found in clathrin-coated vesicles that also internalize transferrin (Sunyach et al., 2003). In addition, a motif in the N-terminus of cellular prion (^{23}KKRPKP^{28}) appears to be critical for clathrin-mediated endocytosis (Sunyach et al., 2003). These results agree with earlier assessment that found GFP-PrP^C in Rab5-positive endocytic vesicles (Magalhaes et al., 2002).

These observations raise the possibility that PrP^C uses alternative mechanisms other than its GPI-anchor for internalization. A minimum GPI-anchored protein GFP-GPI, utilizes non-clathrin mediated endocytosis as a pathway for internalization (Nichols et al., 2001). Hence, it appears that the GPI-anchor of PrP^C does not contribute majorly for its sequestration from the cell surface. Alternative mechanisms of internalization for GPI-anchored proteins have been suggested. For example, the urokinase plasminogen activator receptor (uPAR, a GPI-anchored protein) piggybacks onto the low-density lipoprotein receptor-related protein (LRP1, a transmembrane scavenger protein that can interact with intracellular adaptor proteins) to be internalized (Nykjaer et al., 1992; Conese et al., 1995; Czekay et al., 2001). Manipulations that alter LRP1 expression or its interaction with ligands (using the chaperone RAP for example) decrease the internalization of PrP^C, suggesting that PrP^C may follow a similar internalization pathway by piggybacking with LRP1 (Taylor et al., 2007; Parkyn et al., 2008). Since PrP^C is found in clathrin-coated vesicles (Sunyach et al., 2003) it might be possible that this is the patway used to internalize PrP^C-LRP1 (Figure 8A). Moreover, LRP1 appears to have additional roles in PrP^C trafficking, as this scavenger receptor appears to interact with PrP^C during its biosynthesis, perhaps acting like a chaperone for PrP^C (Parkyn et al., 2008).

The 37LRP/67LR also promotes PrP^C internalization, even though this has been observed only for the recombinant protein and no internalization pathway has been described (Gauczynski et al., 2001) (Figure 8B).

Recent work from our laboratories suggested that at least for one PrP^C ligand, STI1, their binding triggers endocytosis of the prion protein (Caetano et al., 2008). Copper has been previously shown to interact with PrP^C and to trigger
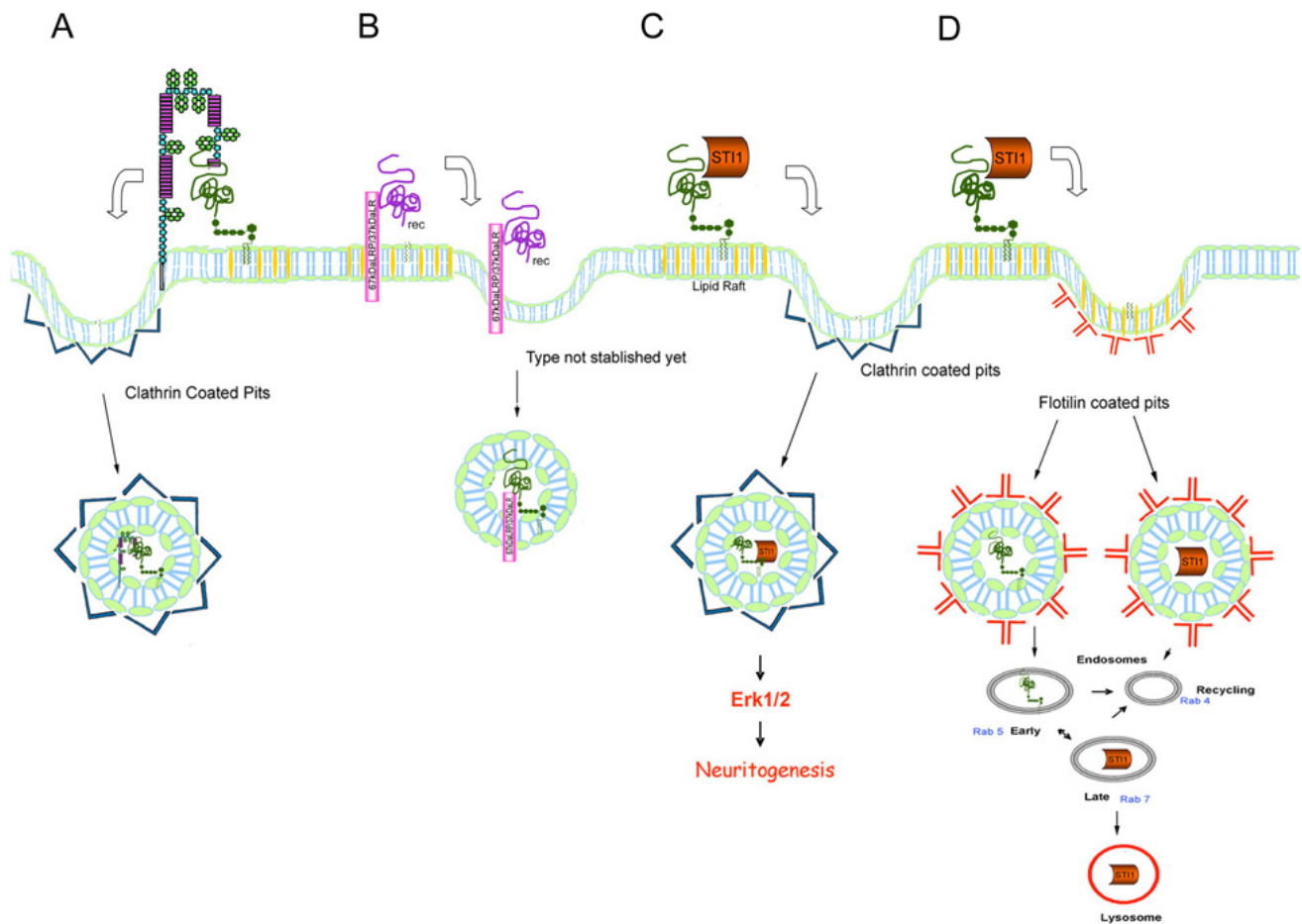
Figure 8. Distribution of PrP^C in specific membrane microdomains and endocytic vesicles mediates neurotrophic functions. A) LRP interacts with PrP^C located in lipid rafts and triggers the latter's internalization. B) 37LRP/67LR promotes the internalization of recombinant PrP^C through an unknown mechanism. C) STI1 induces PrP^C internalization by clathrin coated pits. ERK1/2 activation depends of PrP^C internalization. D) PrP^C and STI1 were also found in flotillin/reggie1 vesicles, recycling vesicles and lysosomes.

its endocytosis (Pauly and Harris, 1998; Lee et al., 2001; Perera and Hooper, 2001). Hemin (iron protoporphyrin IX) added to culture media can also evoke PrP^C internalization and recycling (Lee et al., 2007b). However, to the best of our knowledge, STI1 is the first ligand, which triggers cellular signaling by interacting with PrP^C that can also evoke sequestration of this protein. Internalization of cell surface receptors is usually considered a mechanism for modulation of cellular responses (Ferguson, 2001), suggesting the possibility that endocytosis of PrP^C triggered by STI1 may be involved in cellular signaling.

This possibility received initial support when it was found that treatments known to decrease endocytic activity in cells diminished STI1 mediated signaling (Americo et al., 2007). Recent work showed that a PrP^C mutant ([23]KKRPKP[28] to [23]KQHPSP[28]), that fails to undergo clathrin-mediated endocytosis (Sunyach et al., 2003), can reconstitute PrP^C mediated activation of PKA in response to STI1, but not activation of ERK1/2 activation in the same condition (Caetano et al., 2008). The most parsimonious explanation for these results is that activation of ERK1/2, but not PKA triggerd by STI1-PrP^C interaction depends on the

internalization of PrP^C into clathrin-coated vesicles (Figure 8C).

Interestingly, the activation of ERK1/2 induced by STI1 is fast and transient, lasting no more than a few minutes (Lopes et al., 2005; Caetano et al., 2008). This observation has led us to investigate if STI1 trafficking in cells could explain the short activation time of ERK1/2. Indeed, STI1 is also internalized by cells in a PrP^C independent-way. A fraction of STI1 appears to be internalized with PrP^C and, in the initial moments of internalization part of STI1 is found in clathrin-coated vesicles. However, shortly after internalization STI1 and PrP^C appear to be sorted to distinct intracellular paths, and upon prolonged chase they distribute to distinct intracellular compartments (Caetano et al., 2008). Remarkably, most internalized STI1 is eventually found in flotillin/reggie-positive vesicles (Figure 8C). We have speculated that the divergence in intracellular roads taken by the two proteins may function to attenuate ERK1/2 signalling (Caetano et al., 2008).

PrP^C was also found in flotillin/reggie-1 lipid rafts in non-neuronal cells, suggesting that, at least in certain circumstances PrP^C and STI1 could share the same

internalization pathway. PrP^C was targeted to these domains through antibody cross-linking, leading to actin polymerization, ERK1/2 phosphorylation and a distinct brief Ca^{2+} signal (Stuermer et al., 2004). These results also imply that PrP^C sequestration and activity are coupled events. Reggie/flotilins are cytoplasmic proteins involved in the capping of raft derived vesicles, and can also be found in the nervous system. Their ablation induces morphological defects in the nervous system during embryogenesis (Stuermer and Plattner, 2005), and they are involved in the secretion of morphogens such as Hedgehog and Wnt in *Drosophila* (Katanaev et al., 2008). Since both PrP^C and STI1 can be secreted, the interaction of these proteins with reggie/flotillin-positive organelles may have important implications for the availability of secreted PrP^C or STI1 (Figure 8D).

The possibility that PrP^C endocytosis is a triggered event related to cellular signaling merits further investigation. For example, as demonstrated in Figure 6C, PrP^C at the cell surface has been shown recently to constitutive silence the NR2D receptor subunit in hippocampal neurons (Khosravani et al., 2008). This subunit, when incorporated to functional NMDA receptors generates currents with long decay kinetics, which may be responsible for increased sensitivity to excitotoxicity in PrP^C-null neurons (Khosravani et al., 2008). It could be envisioned that sequestration of cell surface PrP^C may be a mechanism to transiently increase NMDA currents in hippocampal neurons. Another potential site for regulation is the β-secretase pathway involved in APP processing that has been recently shown to be regulated by the presence of PrP^C in lipid rafts (Parkin et al., 2007). Future experiments aimed at dissecting signaling complexes involved in PrP^C function, should also target the mechanisms through which these complexes regulate and are regulated by cellular prion trafficking.

PrP^C in astrocytes promoting neuronal survival and differentiation

Astrocytes and neurons interact in various neurophysiological events and represent an integral unit in brain function. Astrocytes are key elements in both the production of neurotrophic molecules and controlling the levels of neurotoxic substances. They can also modulate the production of extracellular matrix that supports neuronal development and differentiation (Theodosis et al., 2008).

The functions of PrP^C in astrocytes are largely unknown. It was shown that PrP^C associates with both the GluR2 subunit of AMPA receptors and 2α/β2 Na$^+$/K$^+$ATPase. The latter binds to basigin, a cell adhesion molecule (Kleene et al., 2007), which is tightly associated with the lactate monocarboxylate transporter 1 (MCT1). In the absence of glutamate, the 2α/β2 Na$^+$/K$^+$ATPase is less active, and MCT1 does not transport lactate. Conversely, when glutamate is present, MCT1 is active, leading to either increased release or uptake of lactate, depending on the concentration of the latter inside and outside of the astrocyte. Intense synaptic activity implies an increased energy demand, and lactate provided by astrocytes may be a necessary alternative substrate for balancing the energetics of neuronal activity. Deletion of PrP^C is followed by inactivation of 2α/β2 Na$^+$/K$^+$ATPase, and an interruption on the control of MCT1, which becomes permanently active even in the absence of glutamate. It was suggested that the deletion of PrP^C may result in increased levels of extracellular lactate, and lactate-induced acidosis leading to neuronal damage (Kleene et al., 2007). The PrP^C-dependent protein complex involved in lactate transport may therefore play an important role upon synaptic function (Figure 9A).

The role of PrP^C in neurotrophic interactions between astrocytes and neurons has also been addressed (Lima et al., 2007). Prominent neurite outgrowth was observed when wild-type neurons were co-cultured with wild-type astrocytes, while poor differentiation was present when both cells were derived from PrP^C-null mice. It is possible that PrP^C in astrocytes contributes to neurite outgrowth by trans-activating a ligand such as NCAM in neurons (Figure 9B). In addition, astrocytes produce and secrete laminin, among other ECM proteins. Laminin secreted and deposited at the ECM by wild-type astrocytes showed a fibrilar organization (Figure 9B), while that secreted from PrP^C-null astrocytes has a punctate pattern (Figure 9C). The pattern of laminin matrix has been shown to influence neurite outgrowth (Freire et al., 2002), either through modulation of its binding to PrP^C (Graner et al., 2000a) or to other cell surface receptors (Denda and Reichardt, 2007; Nelson et al., 2008).

Astrocytes also release soluble neurotrophic factors and proteins such as STI1 and PrP^C (Lima et al., 2007) and these proteins can bind to neuronal PrP^C and NCAM respectively, mediating neuronal survival and differentiation (Lima et al., 2007). Neuronal survival was higher when cells where treated with conditioned medium from wild-type astrocytes, as compared with medium from PrP^C-null astrocytes. These data clearly show that PrP^C in astrocytes is critical to sustain cell-cell interaction, organization of extracellular matrix and secretion of soluble factors, thus helping to maintain a healthy environment for neuronal survival and differentiation (Figures 9B and C).

Therefore, PrP^C not only works as a neurotrophic factor for neuronal cells, but also modulates astrocyte performance on neuronal survival and differentiation.

PrP^C as a therapeutic target for degenerative illnesses

Given that PrP^C interacts with several classes of molecules triggering neurotrophic functions, and the high expression of PrP^C in the nervous system, it is conceivable that the modulation of PrP^C activity and its interaction with specific ligands may constitute therapeutic targets not only for prion diseases, but also for other chronic degenerative diseases.

The pathogenesis of prion diseases can be explained, at least in part, by "loss-of-function" mechanism, which consists in the depletion of the neuroprotective function of PrP^C, when converted to PrP^{Sc}. However, this mecanism is still debatable, due to some noteworthy results. For example, the deletion of neuronal PrP^C in adult mice causes changes in some hippocampal properties, but no neurodegeneration (Mallucci et al., 2002). Spongiosis, neuron loss, cognitive and behavioral deficits, as well as impaired neurophysiological function, were reverted upon deletion of PrP^C in early stages of the disease in mice (Mallucci et al., 2003; Mallucci et al., 2007). Remarkably, these results were reproduced when the expression of PrP^C was inhibited by a lentiviral vector containing an anti-PrP^C shRNA in prion infected mice (White et al., 2008). This treatment also significantly prolonged the survival of those animals (White et al., 2008). Thus, despite limitations such as treatment in early stages of prion disease, the need
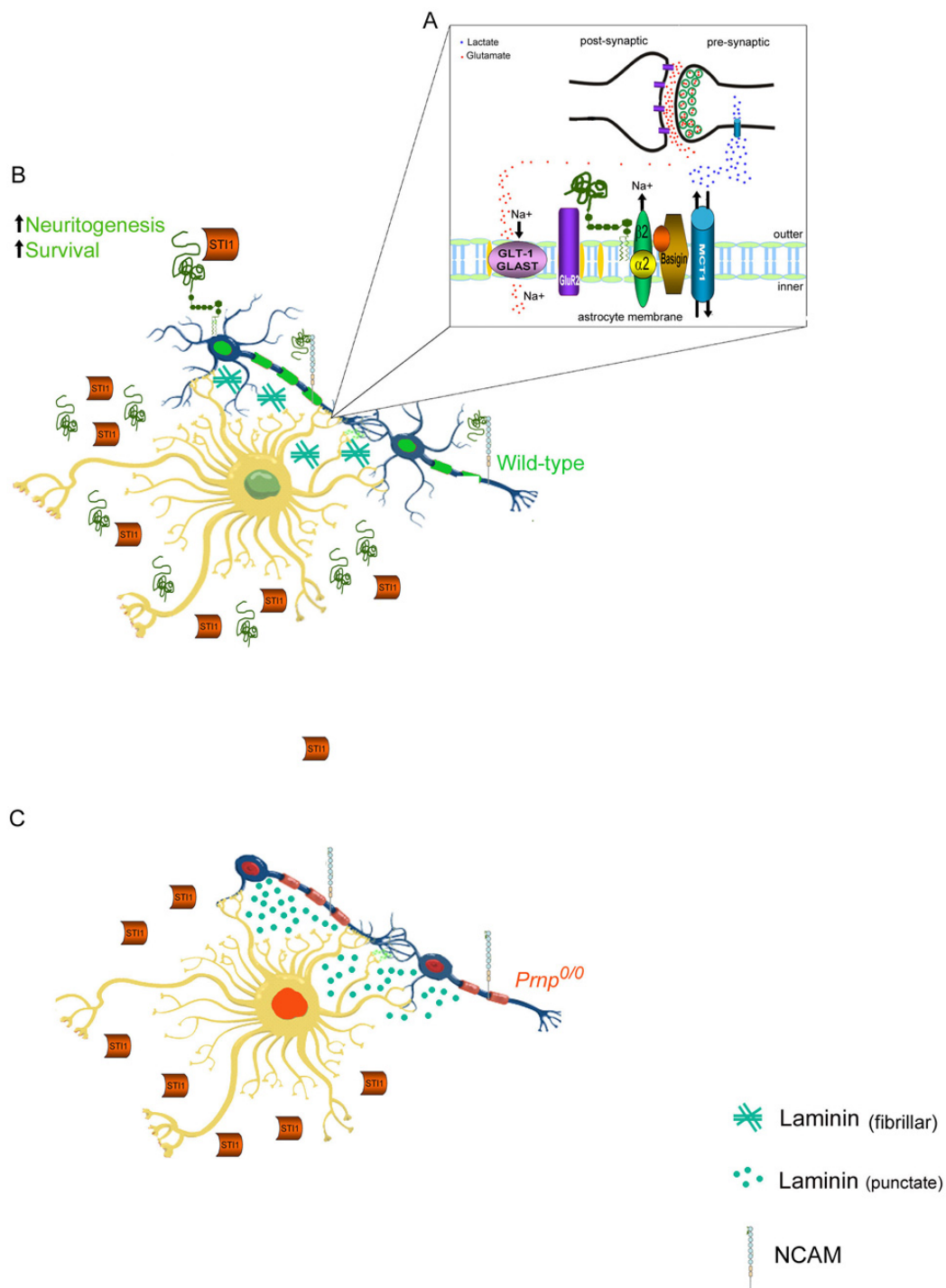
Figure 9. PrP^C modulates neurotrophic properties in astrocytes. A) Glutamate released in synapses is taken up by astrocytes via glial glutamate transporters (GLAST/GLT-1). Na$^+$ dependent transport of glutamate causes a massive Na$^+$ influx into the cell, leading to the activation of the Na$^+$/K$^+$ ATPase. In order to maintain a high glycolitic flux, lactate is released from astrocytes into the extracellular space via the monocarboxylate trasporter (MCT-1). Lactate is taken up by neurons. PrP^C associates with a protein-complex involved in lactate transport in the astrocytic membrane. PrP^C can directly interact with both, the GluR2 subunit of AMPA receptors and 2α/β2 Na$^+$/K $^+$ATPase. The latter binds a cell adhesion molecule, basigin, required as an auxiliary protein to maintain the catalytic activity of the lactate receptors. The lack of PrP^C reduces the 2α/β2 Na$^+$/K $^+$ATPase pump activity and activates basigin/MCT1associate lactate transport. B and C) PrP^C and STI1 as neurotrophic factors in neuron-glia interaction. A prominent neuritogenesis is observed when wild-type neurons are co-cultured with wild-type astrocytes (B) neurite outgrowth is impaired when both cell types are derived from PrP^C-null mice (C). Laminin secreted and deposited at the ECM by wild-type astrocytes has a fibrillar organization and promotes more intense neurite outgrowth than the puctated laminin secreted from PrP^C-null astrocytes. Astrocytes also release STI1 and PrP^C, which induce neuronal survival and differentiation.

for transport across the blood-brain barrier, and off-target effects, anti-PrP^C siRNA seems to be a promising approach to treat prion diseases. Notwithstanding, its effect regarding PrP^C loss-of-function in humans need to be considered. Still, short lived side effects may be acceptable, due to the devastating characteristics of prion diseases.

The use of anti-PrP^C antibodies to block prion infection *in vivo* has also been addressed, but some of the data are contradictory, particulary regarding the toxicity of the treatment. The expression of a transgenic anti-PrP^C IgMa μ chain prevented pathogenesis of prions directly inoculated into brain or spleen (Heppner et al., 2001). In addition, peripheral infection was markedly reduced by treatment with anti-PrP^C antibodies (White et al., 2003). Extracerebrally infected CD-1 mice also presented effective suppression of PrP^{Sc} replication in the lymphoreticular system, lengthening of the incubation period and a significant decrease in CNS pathology after treatment with anti-PrP^C antibodies (Sadowski et al., 2009). However, important concerns about intracerebral treatment with antibodies to PrP^C were raised (Tayebi and Hawke, 2006). The cross-linking of PrP^C *in vivo* with specific monoclonal antibodies triggers rapid and extensive apoptosis in hippocampal and cerebellar neurons (Solforosi et al., 2004). Prolonged treatment with PrP^C antibodies also causes extensive neuronal loss, strong astrogliosis and microglial activation, even when IgG Fab fragments were applied (Lefebvre-Roque et al., 2007). In addition, this treatment was not protective against the development of BSE in transgenic mice overexpressing PrP^C (Lefebvre-Roque et al., 2007).

Blockade of PrP^C-PrP^{Sc} conversion without altering PrP^C function is also an interesting alternative for prion diseases. This has been approached through the targeting of physiological ligands already characterized as inhibitors of PrP^C conversion into PrP^{Sc}. A trans-dominant negative 37LRP/67LR mutant reportedly had an inhibitory effect on PrP^C-PrP^{Sc} conversion *in vitro* (Vana and Weiss, 2006). Preclinical studies demonstrated that passive immunotransfer of single chain Fv antibodies directed against the 37LRP/67LR reduced PrP^{Sc} levels in the spleen of prion infected mice (Zuber et al., 2008). Stereotatic intracerebral microinjection of adeno-associated virus (AAV) vectors expressing single chain Fv antibodies against this receptor leads to a reduction of peripheral PrP^{Sc}, however without a significant lenghtening of incubation times and survival (Zuber et al., 2008). In this line, other candidate targets may be glycosaminoglycans (GAGs), which have both high affinity for PrP^C and demonstrated anti-scrapie activities (Caughey et al., 1994; Warner et al., 2002; Schonberger et al., 2003; Kocisko et al., 2006; Caughey et al., 2006; Silva et al., 2008). Further studies are required to test how the modulation of these molecules for the treatment of prion diseases may affect physiological functions of PrP^C.

PrP^C may also be approached as a candidate neuroprotective agent in Alzheimer's disease, due to its effect of downmodulating the activity of β-secretase, therefore decreasing the production of the toxic amyloid-β (Aβ) peptide, the main component of senile plaques (Parkin et al., 2007). On the other hand, PrP^C was also found to be the major receptor for Aβ oligomers in neurons and their interaction is important for the effects of Aβ on synaptic disfunction (Lauren et al., 2009). The possible use of PrP^C as a therapeutic target in Alzheimer diseases has been explored (Parkin et al., 2007; Lauren et al., 2009), and the development of reagents that increase β-secretase inhibition by PrP^C and impair PrP^C-Aβ oligomers binding would be desirable.

Although exogenous GAGs may constitute lead compounds for the development of therapeutic methods for prion diseases (Schonberger et al., 2003), the same can be detrimental for Alzheimer´s disease. Whereas PrP^C interacts with BACE1 therefore inhibiting its β-secretase activity, the interaction between PrP^C and BACE1 involves GAGs, and the exogenous GAGs appear to disrupt this interaction, thus restoring the production of Aβ peptides (Parkin et al., 2007). Still, exploring PrP^C–GAGs interactions would potentially generate new and improved therapeutic strategies for both prion and Alzheimer´s diseases.

Both the differentiation of dorsal root ganglia (Hajj et al., 2007) as well as motor neuron survival (Barbeito A., Hajj G, Martins VR, Barbeito L unpublished results) can be modulated by PrP^C. Although previous results suggested downregulation of PrP^C in a transgenic mouse model for amyotrophic lateral sclerosis (ALS) (Dupuis et al., 2002) our data demonstrated that PrP^C expression in tissues from similar animals are comparable to their normal counterpart (Barbeito A., Hajj G, Martins VR, Barbeito L unpublished results). Therefore, protective effects of PrP^C in motor neurons can also become a therapeutic target for ALS, and this hypothesis deserves to be explored.

It has been reported that PrP^C depletion causes a higher aggregation of huntingtin (Htt), similar to what is observed when cells are treated with a proteasome inhibitor. Conversely, overexpression of PrP^C in cells expressing Htt caused a decrease in both the number of cells with Htt granules and the number of apoptotic cells, indicating a protective activity of PrP^C against pathological protein aggregation (Lee et al., 2007a). Histone deacetylase (HDAC) inhibitors have been proposed as a novel therapeutic approach for the Huntington's disease (Sadri-Vakili and Cha, 2006) and PrP^C is upregulated by such inhibitors (Cabral et al., 2002). Altogether, these data suggest that PrP^C should be considered in attempts to develop new therapeutic approaches for Huntington´s disease.

The protective function of PrP^C extends to other neurological disorders such as epilepsy. Two independent studies demonstrated that PrP^C-null mice are more susceptible to seizures induced by convulsant agents (Walz et al., 1999; Rangel et al., 2007). After injection of kainate or pentylenetetrazol, these animals presented higher grade seizures reaching elevated mortality and increased neuronal death when compared with control mice (Walz et al., 1999; Rangel et al., 2007). Moreover, several brain regions of PrP^C-null mice showed increased neuronal death upon injections of kainate (Rangel et al., 2007). The absence of PrP^C decreased the activity of GABAergic receptors (Collinge et al., 1994) and increased the expression and activity of NMDA receptors (Maglio et al., 2004; Khosravani et al., 2008). Thus, the neuroprotective function of PrP^C against seizures may be a consequence of its ability to balance inhibitory and excitatory synaptic transmission (Khosravani et al., 2008).

The expression of PrP^C is also important for the maintenance of the circadian rhythm and sleep homeostasis. Animals that do not express PrP^C presented longer period of activity in free-running conditions compared to normal mice

and this phenotype was rescued with re-expression of PrP[C] (Tobler et al., 1996). Moreover, the lack of PrP[C] expression increased the sleep fragmentation indicating that PrP[C] is required for sleep continuity (Tobler et al., 1996; Tobler et al., 1997). Consistently, patients with prion diseases also present altered sleep patterns that can extend to the case of patients carrying a mutation Asp178 Asn that leads to fatal familial insomnia (Landolt et al., 2006; Provini et al., 2008). Since sleep is a physiological process tightly controlled by various neurotransmitter systems, these data corroborate the view that PrP[C] can be a target to modulate synaptic transmission and for the treatment of sleep disorders.

Finally, PrP[C] can also be a target for the treatment of acute injuries. The induction of ischemic stroke in the animal model generates complex pathophysiological conditions in the brain that can culminate with cell death. This experimental approach was used by several groups to evaluate the neuroprotective role of PrP[C] *in vivo*. All findings unanimously demonstrated that ischemic lesions increased the local expression of PrP[C] and that PrP[C]-null mice were more vulnerable to the ischemic stroke presenting larger brain injury (McLennan et al., 2004; Weise et al., 2004; Shyu et al., 2005; Weise et al., 2006). The up-regulation of PrP[C] due to the ischemic stroke was also observed in human post-mortem brain tissue. Pyramidal neurons, inflammatory cells and micro vessels of the peri-infarcted area expressed higher level of PrP[C] compared to contralateral tissues (Mitsios et al., 2007). Moreover, a higher level of PrP[C] was also present in the plasma (Mitsios et al., 2007). The up-regulation of PrP[C] expression in the ischemic tissue may represent a protective mechanism against the injury and might conceivably be explored therapeutically.

Conclusion and future directions

Studies of neurotrophic signal transduction have evolved from the search for simple pathways controlling cell survival to complex, non-linear systems, including interactions of classic neurotrophic factors (Sharma, 2007; Sanford et al., 2008; Hossain et al., 2008), cross-talk and transactivation among differing neuroactive molecules and membrane receptors (Lee et al., 2002; Wiese et al., 2007; Jeanneteau et al., 2008; Iwakura et al., 2008), selective, and even opposite, responses either of individual receptors or to single neurotrophic molecules (Chao et al., 2002; Iwakura et al., 2008), modulation of receptor expression and exposure (Nagappan and Lu, 2005), as well as subcellular traffic-dependent signaling (Howe and Mobley, 2005; Ibanez, 2007; Cosker et al., 2008).

Scaffolding proteins provide for both spatial coordination and kinetics of molecular interactions, which are critical for the selective activation of signaling cascades within the intracellular environment (Pawson and Scott, 1997; Bhattacharyya et al., 2006; Murrin and Talbot, 2007; Jarnaess and Tasken, 2007; Bezman and Koretzky, 2007; Dohlman, 2008). A number of intracellular scaffolding proteins are required for signaling downstream of membrane receptors and for interaction of known neurotrophic molecules (Nakaoka et al., 2003; Hisata et al., 2007; Iida et al., 2007; Kurisaki et al., 2008).

We have proposed that the main function of the prion protein is to serve as a dynamic cell surface scaffolding protein, based on its ability to bind a large number of ligands, and to travel across boundaries of distinct plasma membrane domains, as well as to move bidirectionally between the cell surface and intracellular endocytic compartments. These properties allow for a role of PrP[C] upon both the selection and the kinetics of extracellular and cell surface components of signaling modules, as well as the recruiting of transmembrane components able to convey extracellular signals through the plasma membrane. Such properties are likely to underlie both the varied and often contradictory functions attributed to PrP[C], and the multiple phenotypes that follow modulation of the expression of PrP[C] (Linden et al., 2008).

The current review underscores the importance of PrP[C] in the coordination of signaling related to neuronal survival and differentiation. The ability of PrP[C] to mediate the assembly of multi-component complexes at the cell surface is likely the basis for its neurotrophic properties, and may be relevant for loss-of-function components of prion diseases (Samaia and Brentani, 1998), in particular those associated with neuronal death. In addition, the wide range of molecular interactions described for PrP[C] suggests that this protein may be relevant for not only the spongiform encephalophaties, but also as an ancillary component of the pathogenesis of other neurodegenerative diseases, and therefore amenable to therapeutic targeting. Further studies should be directed at the unraveling of additional signaling complexes scaffolded by PrP[C], as well as to the role of these molecular assemblies upon both physiological and pathological properties of both the nervous system and other organs (Linden et al., 2008).

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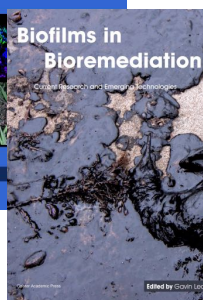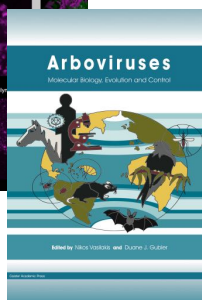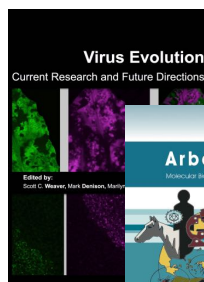
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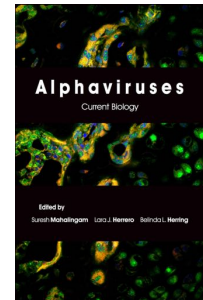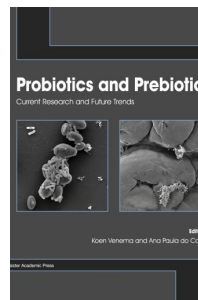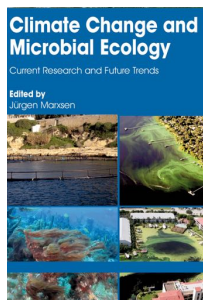
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