Disordered structure and flexible roles: using the prion protein N1 fragment for neuroprotective and regenerative therapy

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The cellular prion protein (PrP^C) is a truly remarkable cell surface glycoprotein. With (i) its broad expression pattern and (ii) particularly high levels in the nervous system, (iii) its critical involvement in fatal neurodegenerative diseases affecting different mammalian species, (iv) its structurally diverging bipartite buildup, (v) its high degree of evolutionary conservation and (vi) a variety of –at least suggested– functions despite (vii) a surprising lack of major phenotypic deficits when absent (as in respective knock-out animals), PrP^C has raised considerable research interest over the last four decades. While most of these aspects have been reviewed extensively in the past (Linsenmeier et al., 2017), this perspective will focus exclusively on a soluble peptide, termed N1, which is constitutively generated by the main proteolytic cleavage event occurring on PrP^C (**Figure 1B**). In fact, considering that particular fragments of PrP^C account for intrinsic functions, may help to explain the multitude of physiological roles so far mostly –and maybe in part mistakenly– attributed to full-length PrP^C as the 'precursor'. The N1 fragment basically consists of the flexible N-terminal half of PrP^C (after removal of the signal peptide) ranging from residue 23 to ~110, contains several sites for coordinative binding of divalent cations and interaction with other binding partners, and represents a prime example of an intrinsically disordered peptide (Gonsberg et al., 2017). Physiologically it results from the α-cleavage of PrP^C which may take place at or en route to the cell surface or after re-internalization in endosomal compartments. It is eventually released into the extracellular space and tissue/body fluids where it is expected to exert its functions. Of note, while candidates have been suggested and controversially discussed, the responsible protease has not been convincingly identified yet, thus precluding any pharmacological manipulation at present. It would not even be surprising if different proteases could orchestrate and ensure this important cleavage in a redundant fashion (Linsenmeier et al., 2017).

Regarding physiological functions of N1, there is evidence for a (neuro)protective role in cellular stress conditions (Guillot-Sestier et al., 2009) and regulatory effects on neural stem cell quiescence (Collins et al., 2018), suggestive of an involvement in regenerative processes of the brain during aging or after injury. These effects are likely dependent on N1 acting as a ligand for currently ill-defined surface receptors (with GPI-anchored PrP^C possibly being one of them) on recipient cells and induction of receptor- and context-dependent signaling pathways (**Figure 1B**). Though mechanistic details and consequences clearly deserve further investigation, a picture arises with N1 being a relevant factor in intercellular communication. This is also supported by a recent study showing that N1 increases cell viability and supports interaction of microglia with other co-cultured brain cell types (Carroll et al., 2020). Notably, the well-established role of PrP^C in maintaining the myelin sheath around axons in the peripheral nervous system could in fact be executed by physiologically released N1 only (Kuffer et al., 2016). These few examples already highlight the valuable therapeutic potential of this interesting peptide.

But there is more to it than that. Roughly a decade ago, it was shown that toxic conformers associated with neurodegenerative diseases, such as amyloid-β (Aβ) oligomers in Alzheimer`s disease, bind with high affinity to cell surface PrP^C initiating neurotoxic signaling cascades (Lauren et al., 2009; Resenberger et al., 2011) (**Figure 1B**). Soon after formal demonstration that respective binding sites are located within the flexible N-terminal half of PrP^C, several *in vitro* studies convincingly showed that recombinant N1 or closely related derivatives are able to bind and neutralize toxic Aβ oligomers in the extracellular space, thereby reducing Aβ-associated neuronal impairment (Resenberger et al., 2011; Guillot-Sestier et al., 2012; Nieznanski et al., 2012; Fluharty et al., 2013; Nieznanska et al., 2018). Protective effects of N1 have also been observed in mice exposed to acute Aβ toxicity (Fluharty et al., 2013). Fittingly, the finding of increased α-cleavage rates in brains of Alzheimer`s disease patients may indicate a protective feedback attempt of the progressively damaged brain (Beland et al., 2014). However, mechanistic insight and analysis of N1-associated effects over the long-term course of neurodegenerative diseases remain scarce and, importantly, no analogue studies investigating similarly protective effects of N1 against misfolded prions in transmissible spongiform encephalopathies (prion diseases; such as Creutzfeldt-Jakob disease) have been reported. One major hurdle for insightful *in vivo* studies surely lies in the relatively low biostability of N1 and, consequently, in the challenge of reliable and protracted administration in respective animal models. In fact, in a recent study we could demonstrate that –once secreted from cells– N1 soon undergoes a proteolytic 'trimming' event starting from its new C-terminus and causing partial fragmentation of the peptide, which was blocked by C-terminal antibody binding (Mohammadi et al., 2020) (**Figure 1A**).

Given the urgent need for an *in vivo* model with a constitutive production of N1 to study its physiological roles and, in particular, its neuroprotective effects against degenerative conditions of the brain, we generated transgenic mice overexpressing this fragment (TgN1; (Mohammadi et al., 2020)). Unfortunately (yet not completely unexpectedly), another severe limitation became apparent: As suggested by *in vitro* studies (Gonsberg et al., 2017), it turned out that the N-terminal fragment alone, due to its lack of structural elements in the growing nascent peptide chain, is not properly translocated into the ER lumen co-translationally and, hence, is not secreted into the extracellular space, its physiological 'destination'. In contrast, it is retained with the uncleaved N-terminal signal peptide in the cytosol (**Figure 1B**). Accordingly, no protection was observed when these mice were inoculated with prions or when respective primary neurons were challenged with toxic Aβ. Despite confirmed overexpression, transgenic N1 was simply located in the wrong, non-physiological place (Mohammadi et al., 2020). While this model may represent the first *in vivo* proof-of-principle for the impaired endoplasmic reticulum translocation of intrinsically disordered peptides and could thus serve for respective studies with likely implications for a better understanding of basic protein synthesis and cell biology, an improved model is obviously required to study functions of N1 when present extracellularly. In that regard, we and others have shown that N1 secretion is supported by fusion with structured C-terminal tags (Gonsberg et al., 2017; Mohammadi et al., 2020) and generated a novel mouse model that is currently undergoing detailed characterization. Interestingly, α-cleavage or –to employ a more careful wording– an α-cleavage-like event still seems to occur on a relevant fraction of these fusion proteins as increased levels of N1 are also observed. We are optimistic that this new model, together with the currently gained knowledge on potential obstacles and pitfalls when working with N1, will allow for important insight into protective effects of this fragment.

For instance, while the blocking activity of N1 towards Aβ oligomers is widely accepted, it is less clear if and how N1 helps to sequester those problematic conformers into (possibly less toxic) deposits, such as amyloid plaques (Beland et al., 2014). Though only notional at the moment, N1 might 'opsonize' endogenously produced toxic protein oligomers, similar to what antibodies and complement factors do in the immunological defense against exogenous pathogens. Along that line, it would be interesting to study if, analogue to –for instance– Fc receptors, cellular 'N1 receptors' exist that could mediate uptake and degradation of N1 complexes with toxic conformers or initiate other protective responses in the nervous system. The recently described role of N1 in inducing interaction of microglia with other cells may point to this direction (Carroll et al., 2020).

Further exploration of N1`s role(s) in neuroprotective and regenerative processes, and especially its 'anti-proteopathic' mode(s) of action, ultimately requires meaningful animal models. Once the therapeutic potential of N1 has been convincingly demonstrated *in vivo*, molecular design could pave the way for the generation of 'improved' N1 derivatives for therapeutic administration (**Figure 1C**). Considering potential constraints regarding affinity and numbers of binding sites, distances between them, and total sequence length of such modified N1 versions (Fluharty et al., 2013; Nieznanska et al., 2018) may allow for even enhanced blocking and neutralization capacity directed against toxic conformers. Moreover, fusion of certain tags or structured domains may stimulate potential phagocytosis of N1-Aβ complexes and/or increase the

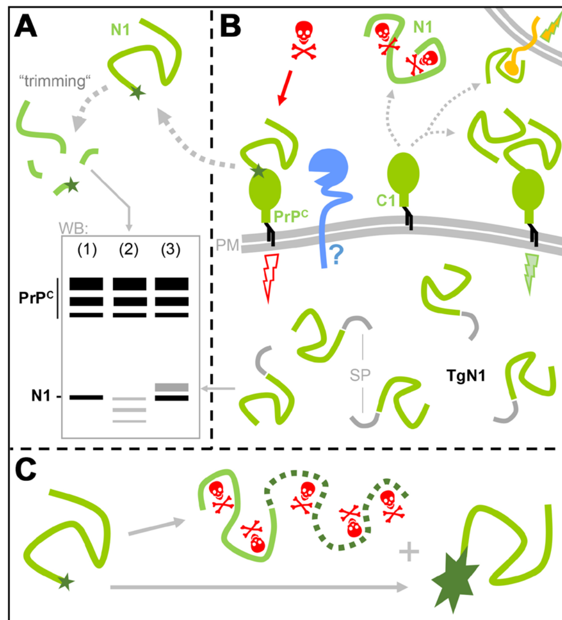

Figure 1 | Scheme summarizing important aspects related to the PrP-N1 fragment.
The prion protein (PrP^C, green, center upper part) is GPI-anchored to the outer leaflet of the plasma membrane (PM) and acts as a receptor for harmful misfolded protein conformers (red skulls) associated with different neurodegenerative diseases and mediates toxic signaling (red thunderbolt in B). In prion diseases, PrP^C additionally serves as a substrate for templated pathogenic misfolding. The α-cleavage in the middle of PrP^C (green asterisks) by an unknown protease (blue) protects from these processes as it produces a membrane-attached globular C1 and a released unstructured N1 fragment. The latter may bind to certain receptors in trans (yellow) or in cis (note that PrP^C itself might be such a receptor) to modulate or induce diverse physiological effects (green/yellow thunderbolts) in B. Importantly, N1 also blocks toxic conformers in the extracellular space (B). Note that details, such as the N-gycans of PrP^C or additional proteins involved in receptor complex formation, are neglected here to simplify matters. A: Upon release, N1 has a low biostability and undergoes proteolytic trimming from its new C-terminus (green asterisks). A schematic drawing of a typical western blot of brain homogenates (when detected with an antibody against the N-terminal half of PrP^C) below shows the three bands for different full-length PrP^C glycoforms and the N1 fragment (running at ~10 kDa) in A. The proteolytic fragmentation (lane 2) is compared to the expected pattern (lane 1) found in fresh biological samples. (Over)expression of N1 alone (i.e., lacking any structured C-terminal domains) in cells and mice (such as our recent TgN1 model) results in cytosolic retention with an uncleaved signal peptide (SP), since IDPs are not translocated into the ER (center). This results in the appearance of a double band in western blots (lane 3). C: Molecular design may enable generation of improved N1-based proteins for therapeutic purposes. Those could, for instance, possess more high-affinity binding sites (dotted extension) for toxic conformers and/or tags for improved secretion, biostability or receptor binding (green star symbol).

biostability of such N1 forms. The latter seems especially important in view of the C-terminal trimming event mentioned earlier (Mohammadi et al., 2020).

Pharmacological administration of such 'engineered' PrP fragments could have another important advantage: A very promising strategy to combat prion diseases (and potentially other neurodegenerative conditions as well) aims at reducing the overall expression of PrP^C (Raymond et al., 2019). While some of PrP's physiological functions may well be compensated by other molecules, others – and in particular the beneficial ones (e.g., its above-mentioned role in myelin maintenance (Kuffer et al., 2016) and protection in hypoxic conditions (Guillot-Sestier et al., 2009)– may get lost. Thus, lowering cell surface PrP^C is a reasonable approach against neurodegenerative diseases, but additional exogenous administration of modified PrP fragments (with no risk of misfolding or any toxic effects) may preserve some important functions and additionally block formation and/or toxicity of harmful conformers causally linked with neurodegeneration.

Although a huge amount of research on physiological functions and therapeutic applicability of soluble PrP fragments, such as N1 and related derivatives, is still required, recent insights and the development of reliable *in vivo* models will promote important and therapy-relevant progress in this field. The current view of N1 as a powerful 'multimodal' mediator in nervous system physiology, especially in neuroprotection and regeneration, clearly justifies and even calls for such efforts.

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