Role of Polyphosphate in Amyloidogenic Processes

Justine Lempart and Ursula Jakob

Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109

Correspondence: ujakob@umich.edu

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Polyphosphate (polyP), an extremely simple polyanion, has long been known to be involved in a variety of different cellular processes, ranging from stress resistance, biofilm formation, and virulence in bacteria to bone mineralization, blood clotting, and mammalian target of rapamycin (mTOR) signaling in mammalian organisms. Our laboratory recently discovered a completely unexpected role of polyP as a stabilizing scaffold for β-sheet-containing protein-folding intermediates. This realization led us to investigate the effects of polyP on amyloidogenic processes and the novel concept that polyP might play a role in neurodegenerative diseases. In this review, we will summarize recent results that show that polyP is a physiological modifier that accelerates amyloid fiber formation, alters fiber morphology, and protects cells against amyloid toxicity. We will review the current knowledge on the distribution, levels, and roles of polyP in the mammalian brain, and discuss potential mechanisms by which polyP might ameliorate amyloid toxicity.

Inorganic polyphosphate (polyP), a linear arrangement of orthophosphate units covalently linked via phosphoanhydride bonds, has been around for billions of years (Kulaev and Vagabov 1983; Wood and Clark 1988). First identified in 1888 by the German scientist Leo Liebermann, polyP initially received very little attention (Baev et al. 2016). Based on its chemical structure, it was presumed that polyP serves as an inorganic phosphate (P_i) reservoir, metal chelator, and/or buffer system in cells (Fig. 1). However, it took nearly 100 years until the real multifunctionality of polyP became known. Arthur Kornberg was, in large part, responsible for the renewed interest in polyP. Kornberg and his team not only showed that polyP is universally present in prokaryotic and eukaryotic species but, even more importantly, identified the enzymatic systems responsible for polyP biosynthesis and degradation in bacteria (Kornberg et al. 1956, 1999; Akiyama et al. 1993; Baldwin 2008). This discovery allowed first phenotypic studies with mutant strains lacking the polyP-synthesizing enzyme polyphosphate kinase PPK and revealed that polyP plays a multitude of different roles in bacteria, ranging from motility and biofilm formation to virulence and stress resistance (Fig. 1) (Kornberg et al. 1999; Rashid et al. 2000a; Rao et al. 2009; Docampo et al. 2011; Kulakovskaya et al. 2012). Although no polyP-synthesizing machinery has been identified in higher eukaryotes as of yet (Azevedo and Saiardi 2014), the

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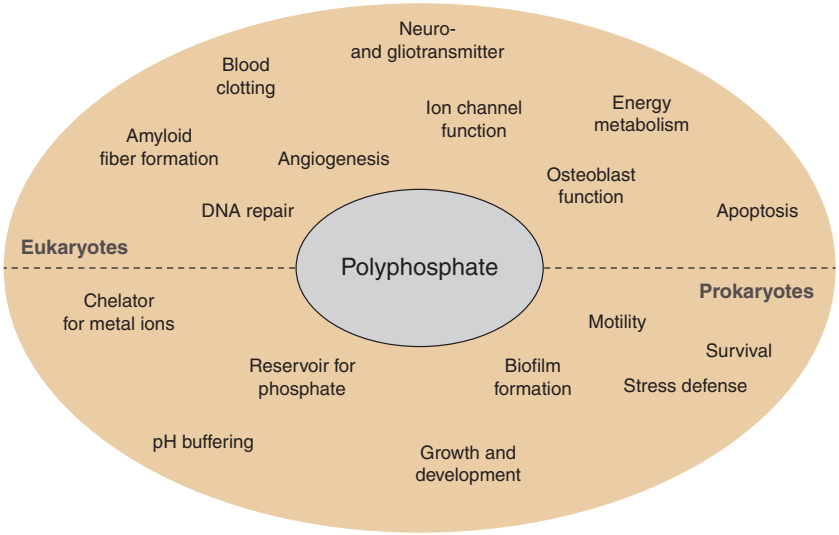

Figure 1. Polyphosphate (polyP)-associated processes in prokaryotes and eukaryotes.

function of polyP in eukaryotes appears to be similarly wide and seemingly mechanistically unrelated. polyP has been shown to be involved in processes related to cellular energy homeostasis (Abramov et al. 2007; Wang et al. 2016), blood clotting (Smith et al. 2006, 2010; Montilla et al. 2012; Morrissey et al. 2012), inflammation (Müller et al. 2009; Morrissey et al. 2012), apoptosis and permeability transition pore (PTP) formation (Abramov et al. 2007; Seidlmayer et al. 2012), osteoblast function (Kawazoe et al. 2004), DNA repair and nuclear transcription (Jimenez-Nunez et al. 2012; Bru et al. 2017), ion channel function (Kim and Cavanaugh 2007; Zakharian et al. 2009; Paulsen et al. 2015), and cell signaling in the mammalian brain (Holmström et al. 2013). Many excellent reviews have been dedicated to these various aspects of polyP function (Kornberg et al. 1999; Brown and Kornberg 2004; Kampinga 2014; Baev et al. 2016; Wang et al. 2016). In this review, we will therefore focus primarily on the most recent discovery that polyP serves as a physiologically relevant modifier of amyloidogenic processes and protects neurons against the toxic effects of disease-related amyloids (Cremers et al. 2016; Müller et al. 2017). These results, together with previous findings that polyP levels in the brain decrease with age (Lorenz et al. 1997), warrants the exploration of polyP as a novel player in age-related neurodegenerative diseases, such as Alzheimer's disease (AD) or Parkinson's disease (PD).

POLYPHOSPHATE—A NOVEL PROTEIN-STABILIZING SCAFFOLD

Interaction of polyP with Nonamyloidogenic Proteins

It is reasonable to assume that some of the reported polyP functions (Fig. 1) are either directly or indirectly linked to the energy-rich, metal-chelating, and/or pH-buffering nature of polyP. However, recent studies from our laboratory revealed an additional and quite unexpected activity of the polyanion that could not be directly explained by its physicochemical properties. We found that polyP, whose endogenous levels have long been known to increase with proteotoxic stress conditions, such as heat shock or oxidative stress (Akiyama et al. 1992), interacts with protein unfolding intermediates and acts as a stabilizing scaffold to protect them against irreversible protein aggregation both in vitro and in vivo (Gray et al. 2014). This activity put polyP into the same category as stress-induced, protein-based chaperones, which increase in levels or activity during stress conditions to prevent

irreversible protein aggregation (Feder and Hofmann 1999). In vitro studies, using a number of different purified proteins, showed that polyP binding to protein unfolding intermediates dramatically increases their thermal stability and maintains their solubility even on incubation at near-boiling temperatures (Yoo et al. 2018). Subsequent cooling in the presence of ATP-dependent chaperone systems promoted the refolding of the polyP client proteins, indicating that polyP maintains its bound proteins in a refolding-competent conformation. In vivo experiments confirmed these in vitro results and showed that Δ*ppk* strains show extensive stress-induced protein aggregation despite their attempts to compensate for the lack of polyP with the overexpression of common heat shock proteins (Gray et al. 2014; Yoo et al. 2018). These results helped to explain polyP's protective function under proteotoxic stress conditions, and suggested that polyP constitutes one of the earliest members of the cellular proteostasis network (Gray et al. 2014).

Structural analysis of the complexes formed between polyP and thermally unfolding proteins, such as lactate dehydrogenase, revealed the formation of small, soluble microaggregates, highly enriched in β-sheet structures and able to bind the amyloid-interacting dye thioflavin T (Yoo et al. 2018). Given that many of the polyP-binding partners are predominantly α-helical in their native conformation, these results suggested that polyP binding either induces secondary structure changes or stabilizes emerging β-sheet structures. Because polyP does not noticeably interact with native proteins, we prefer the second scenario, and propose that polyP interacts with β-sheet structures that transiently accumulate on the protein unfolding pathway. Given the highly negative charge of polyP, it is tempting to assume that polyP binding increases the solubility of the proteins and the interactions are of ionic nature. However, it remains an outstanding question and will require high-resolution studies to figure out whether polyP interacts with proteins via their charged side chains or binds to the backbone of β-sheet structures. The latter would explain the promiscuity of interactions and the ability of polyP to stabilize a number of unrelated proteins in an amyloid-like β-sheet conformation (Gray et al. 2014; Yoo et al. 2018).

Interaction of polyP with Amyloidogenic Proteins In Vitro

The ability of polyP to bind to and stabilize β-sheet-rich secondary structures motivated us to study the effects of polyP on amyloid fibril formation. This was a particularly intriguing aspect because polyP has long been known to enhance fibrin clot structure during blood clotting in mammals (Smith and Morrissey 2008) and stimulate bacterial biofilm formation (Rashid et al. 2000b), processes that both involve the fiber formation of functional amyloids (Taglialegna et al. 2016).

Amyloid fiber formation is triggered by the conversion of soluble unstructured or α-helical proteins into association-competent β-rich monomers (Eichner and Radford 2011), which assemble into cross-β-sheet oligomers, protofibrils, and mature fibrils (Fig. 2) (Soto 2003). In bacteria, the amyloidogenic proteins involved in biofilm formation (e.g., CsgA in *Escherichia coli*) are typically unstructured until they reach the cell surface, where they associate into stable, detergent, and protease-resistant cross-β-sheet fibrils (i.e., Curli) and form an integral part of the biofilm matrix (Hufnagel et al. 2013). Kornberg's early phenotypical studies using Δ*ppk* strains revealed that polyP-deficient bacteria form biofilms significantly more slowly than do wild-type strains (Rashid et al. 2000b). This phenotype was thought to be a result of a defect in quorum sensing. However, our studies showed that polyP directly affects biofilm formation by accelerating the conformational rearrangement of the amyloidogenic protein CsgA into cross-β-sheet fibers (Cremers et al. 2016). Moreover, supplementation of polyP into the media of Δ*ppk* mutants rescued CsgA fibril formation and restored the biofilm defect (Cremers et al. 2016). Unresolved thus far is whether polyP binds to unstructured CsgA and induces formation of the association-competent β-sheet protein, or whether polyP captures and stabilizes β-sheet-containing CsgA, which transiently

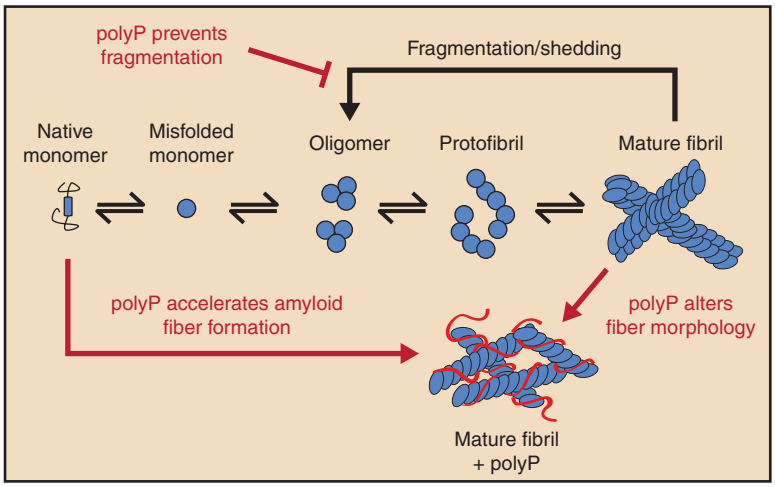

Figure 2. Influence of polyphosphate (polyP) on amyloid fiber formation. Soluble unfolded or α-helical amyloid monomers undergo structural rearrangements to adopt β-sheet-rich structures, which associate into oligomers and protofibrils. Eventually, mature fibrils form and are prone to fragmentation and shedding. polyP accelerates amyloid fiber formation, alters the fiber morphology, and stabilizes the mature fibers.

emerge from the unfolded state. In either case, these results explain at least one mechanism by which polyP increases the rate of biofilm formation in pathogenic bacteria (Rashid et al. 2000b; Chen et al. 2002), and makes the development of PPK inhibitors an important goal to treat biofilm-forming bacteria, which are involved in persistent infections (Dahl et al. 2017).

Curli fibrils in bacterial biofilm display all the characteristics of classical disease-associated amyloid fibrils (Hufnagel et al. 2013). Indeed, follow-up studies from our laboratory revealed that polyP significantly accelerates the fiber formation of α-synuclein, the peptide associated with the pathology of PD, as well as Aβ and Tau, amyloidogenic peptides associated with the pathology of AD (Cremers et al. 2016). The degree to which polyP accelerates formation of some of these fibrils is truly exceptional, for instance, TauK19, a shorter variant of Tau that forms fibrils more rapidly ($T_{1/2}$: 24–36 h) than full-length Tau ($T_{1/2} > 9$ mo) forms fibrils with a $T_{1/2} < 5$ min when incubated in the presence of physiological amounts and chain lengths of polyP (Cremers et al. 2016). Moreover, both full-length Tau, as well as very low, physiologically relevant concentrations of α-synuclein and Aβ, which do not fibrillate in a reasonable timeframe in vitro, were found to form fibrils within a couple of days in the presence of polyP (Cremers et al. 2016). This effect was shown to be chain-length dependent with longer polyP chains (>60P_i) being disproportionally more effective than shorter-chain polyPs (<14P_i). These results suggested that polyP chains are able to nucleate fibril formation, presumably by bringing together multiple monomers, whose increased local concentrations accelerate fibril formation. More detailed analysis of α-synuclein fibers formed in the presence of polyP revealed that the fibers are significantly less effective in seeding α-synuclein fibrillation, about 30% more stable toward molecular disassembly (i.e., shedding), and less protease resistant than fibers formed without polyP (Cremers et al. 2016). Finally, the morphology of fibrils formed in the presence of polyP was distinctly different. Whereas α-synuclein fibers formed in the absence of polyP consist of two single protofilaments twisted around each other in a distinct helix (Guerrero-Ferreira et al. 2018), fibers formed in the presence of polyP are significantly thinner and lack the characteristic twisted structure (Cremers et al. 2016). These results

also agree with recent studies of platelet-derived polyP, which showed that polyP elicits structural changes in fibrin that generate shorter protofibrils with reduced stiffness and produce a fibrin network with increased knotted regions, which are polyP and fibrin rich (Whyte et al. 2016).

POLYPHOSPHATE IN THE MAMMALIAN BRAIN

Levels of polyP in the Mammalian Brain

Arthur Kornberg was the first to study polyP levels in different mammalian tissues (Kumble and Kornberg 1995). According to his studies, the highest polyP concentrations in rats are found in the brain (25–120 μM per P_i) and the liver (22–42 μM per P_i) (Gabel and Thomas 1971; Kumble and Kornberg 1995). Although the mammalian polyP-synthesizing machinery itself has yet to be identified, good evidence exists that polyP levels are directly linked to cellular respiration (Baev et al. 2016; Wang et al. 2016). This conclusion is based on studies with both intact astrocytes and isolated mitochondria in which interference with either the F_1F_0-ATP-synthase, the membrane potential, or the electron transport chain was found to lead to dramatically reduced polyP levels in both mitochondria and the cytosol (Baev et al. 2016; Wang et al. 2016). The chain length of polyP in mammalian cells appears to be dependent on the cell type and physiological conditions, and ranges between 50 and 800 residues (Gabel and Thomas 1971; Kumble and Kornberg 1995). Studies on the specific polyP length distribution in the mammalian brain are slightly controversial, with some reports stating that brain cells contain predominantly long chains (800 mer) (Kumble and Kornberg 1995), whereas others found that the polyP chain lengths range between 10 and 100 phosphates (Lorenz et al. 1997; Stotz et al. 2014). These differences are likely because of variations in the experimental setups, and the challenges involved in selectively and quantitatively extracting different polyP chains from mammalian cells and tissues (Kornberg et al. 1999).

Factors that Alter polyP Levels in the Mammalian Brain

Quantitative analysis of polyP in mammalian tissues is challenging because of its low abundance (Kumble and Kornberg 1995; Kornberg et al. 1999), limiting the number of reports that have studied effectors of endogenous polyP levels. However, one study conducted in rats provided evidence that although total liver polyP levels stayed constant over time, total brain polyP levels appear to significantly change over the lifetime. The investigators found that polyP levels peak at 12 mo of age and then steadily decrease to about 50% of the maximal polyP levels by age 28 mo of the animal (Lorenz et al. 1997). This decrease was attributed to a specific loss in long-chain polyPs ($150P_i$) and likely is the result of changes in endogenous exopolyphosphatase activity (Lorenz et al. 1997). If and how polyphosphatase activity or levels are regulated over the life span remains to be tested, as are other factors that could contribute to the loss of polyP. In addition, no information exists about the spatial and/or cell-type-specific distribution of polyP in the brain and whether all or only select regions undergo a loss in polyP over time. In addition to lifetime changes in brain polyP levels, certain pathological conditions appear to also alter polyP levels in the brain. For instance, it has been reported that the polyP levels in the brains of AD mice are significantly lower than the polyP levels in healthy, age-matched animals (Cremers et al. 2016). In a separate study, Angelova et al. (2014) detected increased amounts of polyP in cells with PD-related mutations. The fact that most familial forms of PD show mitochondrial dysfunction (Burchell et al. 2010a,b) correlates with previous findings that polyP levels are dependent on the energy status of mitochondria, and might explain the altered levels (Angelova et al. 2014).

Roles of polyP in the Mammalian Brain

polyP—A Structural and Functional Component of Channels and Pores

Early studies in bacteria revealed the unexpected finding that transformation-competent bacteria

accumulate high levels of a membrane-spanning complex, consisting of the polymer polyhydroxybutyrate (PHB), short-to-medium chains of polyP, and Ca^{2+} (Reusch and Sadoff 1988). The precise structure of these channels is unknown but it has been proposed that by solvating PHB, polyP might penetrate lipid bilayers and support the transport of divalent cations across the membrane (Reusch and Sadoff 1988; Castuma et al. 1995). The channels function as classical voltage-gated transporters on reconstitution into lipid bilayers (Reusch and Sadoff 1988; Castuma et al. 1995), and seem to play an important role in the transport of divalent metals such as Ca^{2+}, as well as phosphate and DNA across bacterial membranes (Reusch and Sadoff 1988; Castuma et al. 1995). Soon after the discovery of PHB-polyP-Ca^{2+} ion channels in bacteria, similar structures were identified in the rat liver mitochondria (Pavlov et al. 2005). These complexes, on their purification and reconstitution into lipid bilayers, were found to resemble the PTP, which is involved in Ca^{2+}-mediated cell death (Crompton 1999; Rasola and Bernardi 2011). Indeed, depletion of mitochondrial polyP by the targeted overexpression of the yeast polyphosphatase PPX, significantly decreased mPTP opening and prevented ionomycin-induced cell death (Abramov et al. 2007). Conversely, treatment of astrocytes with exogenous polyP led to increased mPTP opening and cell death, an effect that was prevented by blocking PTP opening with cyclosporine A (Angelova et al. 2016). It is of note that only long-chain polyPs (>120P^i) appear to effectively induce mitochondrial depolarization by inhibition of respiration, opening of the mPTP, Ca^{2+} efflux, and activation of apoptosis (Angelova et al. 2016). More recently, polyP was also found to serve as a crucial structural and/or functional component of the pain-sensing transient receptor potential A1 (TRPA1) ion channel, expressed in sensory neurons (Kim and Cavanaugh 2007).

polyP—A Glio- and Neurotransmitter

In vivo studies indicated that polyP plays a central role in key homeostatic physiological activities, such as breathing, central sympathetic outflow, and the arterial blood pressure, suggesting that it plays a role as signaling molecule (Holmström et al. 2013). Although polyP has been reported to be primarily located in mitochondria, the nucleus, and lysosomes (Griffin et al. 1965; Pisoni and Lindley 1992; Kornberg 1995; Kumble and Kornberg 1995), astrocytes show a significant amount of polyP in cytoplasmic vesicle-like structures, which appear to be secreted through Ca^{2+}-dependent exocytosis (Holmström et al. 2013; Stotz et al. 2014; Angelova et al. 2018) in a fashion similar to platelets and, potentially, bacteria (Müller et al. 2009; Sakatani et al. 2016). The secreted polyP can then be taken up by other astrocytes or neurons in which it has been shown to function as both a glio- and neurotransmitter, respectively (Holmström et al. 2013; Angelova et al. 2014). As a gliotransmitter, polyP appears to mediate communication between astrocytes through the binding to the P2Y1 purinergic receptors, which leads to the activation of phospholipase C, the activation of PIP2, and the induction of Ca^{2+} influx via IP3 from the endoplasmic reticulum (ER) (Dinarvand et al. 2014; Baev et al. 2016). This causes a depolarization of the cell, a further release of polyP from astrocytes, and the propagation of the signaling wave (Fig. 3) (Holmström et al. 2013; Dinarvand et al. 2014; Baev et al. 2016). This activity was shown irrespectively of the brain region or the chain length of polyP (Holmström et al. 2013). As a neurotransmitter, polyP was found to induce action potentials in both peripheral nervous system and central nervous system neurons by differentially modulating the function of neuronal voltage-dependent Na^+, K^+, and Ca^{2+} channels (Fig. 3) (Stotz et al. 2014; Baev et al. 2016). The finding that polyP serves as a neuroactive compound is supported by its presence in synaptosomes, in which it can be released on depolarization of the membrane potential by the addition of potassium chloride (Stotz et al. 2014).

POLYPHOSPHATE—A GUARDIAN AGAINST AMYLOID CYTOTOXICITY

Extensive work on elucidating the underlying mechanism of amyloid toxicity suggested that

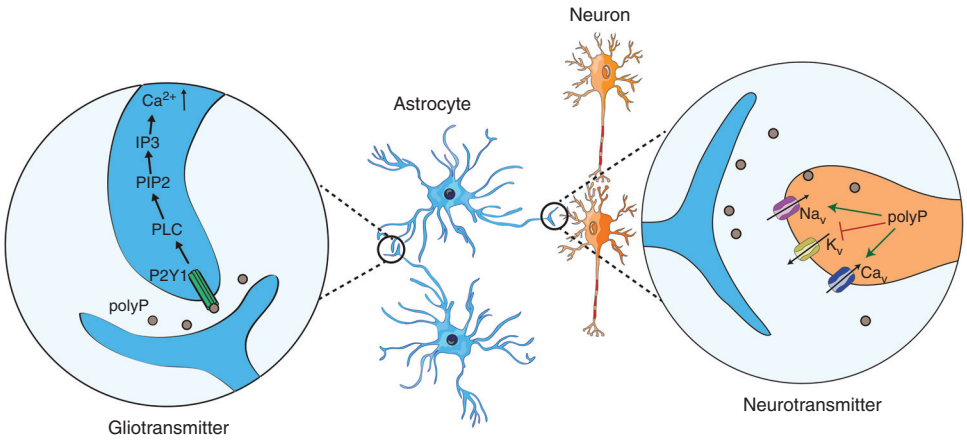

Figure 3. Polyphosphate (polyP) acts as a glio- and neurotransmitter: Astrocytes release polyP that can be taken up either by other astrocytes or by neurons. In astrocytes (blue), polyP binds to and activates the P2Y1 purinergic receptor, causing, ultimately, an increase in intracellular Ca^{2+} levels. In neurons (orange), polyP increases action potential generation by influencing voltage-gated ion channels, blocking K_v channels, sensitizing N_{av} channels, and activating C_{av} channels.

oligomers and/or protofibrils that accumulate in the pathway of fiber formation are the predominant species that causes neurotoxicity (Chen et al. 2015; Tipping et al. 2015). This is thought to be because of their ability to increase membrane permeability, alter mitochondrial function, and/or disrupt the cytoskeleton (Roberts and Brown 2015). Based on our observations that polyP substantially accelerates fiber formation in vitro, alters fiber morphology, and affects seeding and shedding behavior of the fibers, we examined the effect of polyP on amyloid toxicity. Indeed, we discovered that polyP protects differentiated SH-SY5Y cells against both α-synuclein and $A\beta_{1\text{-}40/42}$ toxicity, and AD mutants of *Caenorhabditis elegans*, through uptake of exogenous polyP, show a polyP-dependent delay in $A\beta_{1\text{-}42}$-induced paralysis (Cremers et al. 2016). At this point, it is unclear how polyP affects the toxicity and how much of polyP action happens inside or outside of the cell (Holmström et al. 2013). A recent study confirmed these results using a different neuronal cell line, as well as primary cortical neurons, and showed that polyP protects cells against the neurotoxic effect of the $A\beta_{25\text{-}35}$ peptide (Müller et al. 2017). Because the administration of polyP was found to cause an increase in intracellular ATP levels, the investigators concluded that the presence of polyP reverses the β-amyloid-induced compromised energy status in neuronal cells and, in turn, abrogates the neurotoxic effect of $A\beta_{25\text{-}35}$ (Müller et al. 2017). Moreover, elimination of polyP from mitochondria via insertion of yeast PPX into the mitochondrial genome was found to protect brain cells from β-amyloid peptide toxicity, potentially, by increasing mitochondrial Ca^{2+} capacity and lowering the susceptibility of these cells to the PTP opening (Abramov et al. 2007). Independent of the precise mechanism of action, the evidence is convincing that polyP plays a role in amyloidogenic processes and protects cells against amyloid toxicity. Together with the observation that brain polyP levels decrease with age, as well as age-related pathological conditions, these results raise the exciting possibility that polyP acts as a physiological modifier of amyloidogenic processes.

CONCLUDING REMARKS

In 2006, 26.6 million people were affected by AD worldwide, and as a result of the dramatic increase in life expectancy, the prevalence is expected to quadruple by 2050 (Wimo et al. 2006; Brookmeyer et al. 2007). PD constitutes the

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second most common neurodegenerative disease affecting 1%–2% of the population above 60 years of age (Tanner and Goldman 1996). Current therapies are limited to treating the symptoms but not the cause of the diseases, and despite significant advances in the research field, many aspects of disease development remain unknown. In this review, we summarized recent findings that define polyP as a novel modulator of amyloidogenic processes and as a guardian against amyloid toxicity in cell culture. At this point, however, we do not know by what mechanism(s) polyP protects cells against amyloid toxicity or the extent to which these protective effects translate into the context of neurodegenerative diseases. Yet, the fact that age is the largest risk factor for neurodegenerative diseases, together with the finding that polyP levels in the brain decrease with age, prompts us to speculate about the potential roles of polyP in neurodegenerative diseases (Fig. 4).

It is possible that polyP protects against amyloid toxicity directly by modulating amyloid fibril formation, shedding, and seeding (Cremers et al. 2016). polyP present in the extracellular space might prevent the uptake of amyloid fibrils into and the transmission of amyloids between cells, thereby reducing toxicity and spreading of the disease (Brundin et al. 2010; Masuda-Suzukake et al. 2013; Steiner et al. 2018). Alternatively, endogenous polyP might segregate mature fibrils into inert compartments and/or increase the turnover of amyloids in the cell, thereby preventing dangerous concentra-

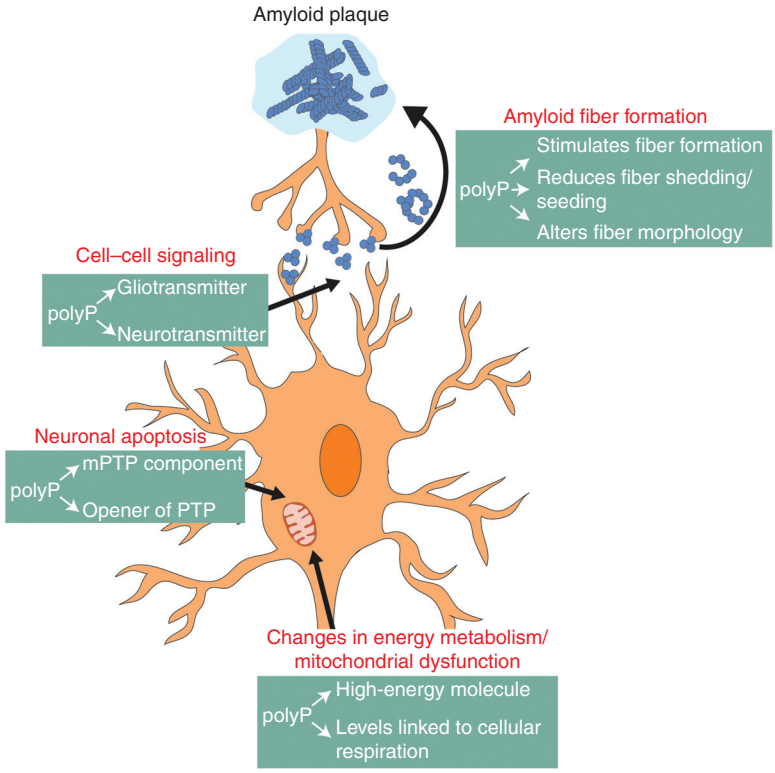

Figure 4. Processes that are directly or indirectly affected by amyloid fiber formation and contribute to neurodegeneration. Toxic oligomers and protofibrils, which form on the pathway from soluble amyloid monomers to insoluble fibers, have been shown to accumulate in the extracellular space, such as synapses in which they influence cell–cell communication or in cells in which they alter energy metabolism, cause mitochondrial dysfunction, and trigger apoptosis. Polyphosphate (polyP) has been shown to play critical roles in all of these processes.

tions of toxic oligomers. It is also conceivable that polyP protects against amyloid toxicity through its roles in energy homeostasis and/or mitochondrial function (Baev et al. 2016; Wang et al. 2016). It is well known that AD is characterized by an impaired energy homeostasis of brain tissue (Müller et al. 2017) and most familiar forms of PD display mitochondrial dysfunction and changes in signal transition (Mounsey and Teismann 2010). Age- and/or disease-induced changes in polyP levels could make cells more vulnerable to mitochondrial dysfunction and organisms more prone to undergo neurodegeneration (Hernandez-Ruiz et al. 2006; Holmström et al. 2013; Stotz et al. 2014; Baev et al. 2016). Until we have identified the system(s) responsible for polyP synthesis in mammalian cells, many of these ideas will remain speculations. However, as a physiological polymer used as a food additive and in cosmetics (Smith and Hong-Shum 2007; Müller et al. 2015a), polyP is considered to be safe for clinical applications (Tsutsumi et al. 2014; Müller et al. 2015b). Therefore, it is of great interest to further investigate the role of polyP in the development of neurodegenerative diseases, and identify whether the restoration of reduced polyP levels in aged brains would delay disease onset or progression.

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