The Amyloid Precursor Protein (APP) binds the PIKfyve complex and modulates its function.

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Abstract

Phosphoinositides are important components of eukaryotic membranes that are required for multiple forms of membrane dynamics. Phosphoinositides are involved in defining membrane identity, mediate cell signalling and control membrane trafficking events. Due to their pivotal role in membrane dynamics, phosphoinositide deregulation contributes to various human diseases.

In this review we will focus on the newly emerging regulation of the PIKfyve complex, a phosphoinositide kinase that converts the endosomal phosphatidylinositol-3-phosphate (PI(3)P) to phosphatidylinositol-3,5-bisphosphate ($PI(3,5)P_2$), a low abundance phosphoinositide of outstanding importance for neuronal integrity and function. Loss of PIKfyve function is well known to result in neurodegeneration in both mouse models and human patients.

Our recent work has surprisingly identified the Amyloid Precursor Protein (APP), the central molecule in Alzheimer's disease aetiology, as a novel interaction partner of a subunit of the PIKfyve complex, Vac14. Furthermore, it has been shown that APP modulates PIKfyve function and $PI(3,5)P_2$ dynamics, suggesting that the APP gene family functions as regulator of $PI(3,5)P_2$ metabolism. The recent advances discussed in this review suggest a novel, unexpected, beta amyloid independent mechanism for neurodegeneration in Alzheimer's disease.

Phosphoinositide metabolism and the PIKfyve complex

Phosphoinositides are important components of cellular membranes. What sets them apart from other phospholipids is their potential for conversion into different species by phosphorylation or dephosphorylation of the inositol head group by various, organelle-specific kinases and phosphatases. This allows phosphoinositides to assume specific subcellular localisations and allows them to fulfil their functions in an organelle-specific manner.

Phosphoinositides fulfil a plethora of important roles. A key aspect is their ability to provide membrane attachment for phosphoinositide binding proteins (1). Their membrane specific localisation, combined with localised production or breakdown of phosphoinositide species can control membrane association of effectors or their activation in a temporally and spatially defined manner. These principles are well illustrated by membrane association of the protein kinase Akt/PKB by binding $PI(3,4,5)P_3$ upon activation of receptor tyrosine kinases (2). This generic mechanism has been adopted in a plethora of biological processes (3, 4).

This review will focus on the regulation of a kinase complex, known as the PIKfyve complex that produces the low abundance phosphoinositide $PI(3,5)P_2$ (reviewed in (5–8)). This species was shown to be of outstanding physiological significance for the maintenance of the central and peripheral nervous systems (CNS and PNS) (9–11). The regulation of the PIKfyve complex is currently beginning to emerge with fascinating implications for our understanding of neurodegenerative disorders such as Alzheimer's disease.

The PIKfyve kinase (also known as Fab1) converts endosomally localised PI(3)P to $PI(3,5)P_2$ by phosphorylation of the 5-hydroxyl group of the inositol ring (12). PIKfyve acts as part of a larger complex that in metazoa consists of PIKfyve, Vac14 (also known as

ArPIKfyve) and Fig4 (Sac3) (6, 13–15). PI(3,5)P_2 can be dephosphorylated to yield PI(3)P by Fig4 which has an interesting dual role as activator of PIKfyve as well as a PI(3,5)P_2 specific phosphatase (16). PI(3,5)P_2 can also be dephosphorylated by myotubularin 3 to yield PI(5)P and has been argued to represent the major source of PI(5)P (17). By consequence, inactivation of PIKfyve will have both PI(3,5)P_2 and PI(5)P dependent effects (11).

Work from the Weisman and Meisler labs has demonstrated that inactivation of any of the subunits of the PIKfyve complex leads to significant drops of PI(3,5)P_2 levels and pronounced neurodegeneration in mouse models and human patients (9–11). Specifically, loss of function mutations in Fig4 and Vac14 have been shown to lead to rare variants of Amyotrophic Lateral Sclerosis (ALS) and Marie-Charcot-Tooth syndrome in patients, necessitating to understand the functions of PI(3,5)P_2 and how it is regulated (10, 18).

Cellular functions of the PIKfyve complex

On a cellular level the most noticeable defect upon impaired PIKfyve function is the formation of swollen ‘vacuoles’, easily visible at low magnification in living cells, presumed to stem from the endosomal system (19). Dysfunction of the endo- and lysosomal TRP-channel TRPML-1 (also known as mucolipin-1) significantly contributes to vacuole formation (20). PI(3,5)P_2 binds to this channel and controls its conductivity (20). Lack of PI(3,5)P_2 reduces TRPML-1 conductivity and appears to disrupt the fusion and fission cycle of endosomes and lysosomes (21). Interestingly, mutations of TRPML-1 lead to mucolipidosis type IV, a lysosomal storage disease resulting in childhood neurodegeneration (21).

Loss-of-PIKfyve function also disrupts endosomal receptor sorting, with a particularly strong effect on endosome to Golgi traffic (22–24). Other sorting events such as ESCRT dependent EGF receptor downregulation do not seem to be majorly affected (22).

PIKfyve can also modulate kinase activity. Recently the raptor subunit of the mTOR complex 1, a major signalling hub controlling catabolic and anabolic processes from the surface of the lysosome was shown to bind $PI(3,5)P_2$ and require PIKfyve activity for signalling to its downstream effector S6 kinase (25). However, in a study by Wang et al. PIKfyve inhibition using Apilimod failed to suppress mTOR signalling to S6 kinase (26).

The yeast homologue of PIKfyve (Fab1) has also been suggested to regulate the vacuolar H+-ATPase (V-ATPase) by controlling its assembly state (27). This enzyme controls acidification of the yeast vacuole, the functional equivalent of lysosomes in higher organisms. While Ho et al. did not find any major effect on vacuolar pH under steady state conditions, they confirmed that Fab1 is required for controlling vacuolar pH under osmo stress (28), known to boost PIKfyve activity and $PI(3,5)P_2$ levels (29). So both studies agree on the fact that Fab1 may affect vacuolar pH at least under conditions that require enhanced Fab1 activity. This is interesting as the V-ATPase is of great importance for endosomal sorting, receptor downregulation and the activity of acidic hydrolases in lysosomes (30). Unsurprisingly, loss of V-ATPase function also strongly disrupts autophagy (31).

The Amyloid Precursor Protein (APP) binds to and regulates PIKfyve function

The amyloid precursor protein (APP) is a type 1 transmembrane protein best known for its role in Alzheimer's disease. It belongs to the APP gene family, consisting of APP, APLP1 and APLP2, that exhibit a certain degree of functional redundancy (32). The APP gene lies on chromosome 21 and can be alternatively spliced to produce three isoforms. APP is ubiquitously expressed in mammalian cells with each isoform expressed at varying levels depending on tissue and cell type (32, 33).

APP is well known to be cleaved by secretases in Alzheimer's disease, first by the rate limiting β-secretase (known as BACE-1) in endosomes and lysosomes (34). The C-terminal C99 peptide, released upon β-secretase cleavage, is further cleaved by γ-secretase into Aβ and APP's intracellular domain (known as AICD) (34). APP can also be processed in a non-amyloidogenic way, first by cleavage of APP by α-secretase in the middle of the Aβ peptide sequence, followed by γ-secretase cleavage (34).

Despite APP's role in Alzheimer's disease its physiological role still remains largely unclear, as research has been focused heavily on the neurotoxic Aβ. APP has been linked to numerous physiological processes including cell adhesion, neurite outgrowth and synaptogenesis (reviewed in (35)). APP is thought to play a role in cell signalling as its proteolytic processing resembles that of the Notch receptor; AICD has also been proposed to translocate to the nucleus and alter gene expression (36).

The intracellular trafficking of APP is probably important for its physiological function and plays a role in APP's proteolytic processing. APP is produced in the endoplasmic reticulum followed by several post translational modifications in the Golgi, including glycosylation, phosphorylation and ubiquitination (34). APP is then trafficked to the plasma membrane via the constitutive secretory pathway (34). APP is also known to traffic from the Trans-Golgi-Network (TGN) to lysosomes in an AP-3 dependent manner (37). APP also traffics from the TGN to endosomes using AP-4 (38). Retrograde trafficking of APP from the endosomes to the TGN depends on its binding partner SorLA (39, 40), a member of the VPS10 receptor family, acting as an adaptor for retromer binding to APP (41). Interestingly, loss of VPS35 causes an increase of APP in endosomes and a decreased APP half-life (42). In a mouse model of Alzheimer's disease, the hemizygous deletion of VPS35 was shown to accelerate neuropathophysiology (43), suggesting that APP accumulation in the endosomal system may play a role in Alzheimer's disease. The transport

of the β-secretase BACE-1 from the endosomes to the TGN is also retromer dependent (44). Loss of VPS35 reduces the retrieval of both APP and BACE-1 back to the TGN, leading to an increase of aberrant APP processing (42). Endosome to TGN transport of a number of retromer-dependent and retromer-independent routes requires PIKfyve (22).

Using a recently developed methodology for analysing the interactome of the intracellular domains of transmembrane receptors using proteo-liposomes we surprisingly identified the PIKfyve complex as a putative interactor of the APP intracellular domain (45, 46). We analysed this interaction in detail and found that the highly conserved YENPTY motif of the APP intracellular domain is crucial for directly binding the Vac14 subunit of the PIKfyve complex (46). Using a novel $PI(3,5)P_2$ specific probe (47) we showed that overexpression of APP or ACID increased the number of $PI(3,5)P_2$ positive vesicles in both HeLa and SH-SY5Y neuroblastoma cells. Conversely, knock-down of APP or the APP paralogue APLP2 reduced the number of $PI(3,5)P_2$ positive vesicles in HeLa cells, suggesting that APP and its paralogues APLP2 are both able to stimulate PIKfyve function (46). RNAi Suppression of APP and APLP2 also increased the number of cells that display vacuoles, similar to the phenotype observed with PIKfyve RNAi, although to a lower extent (22, 46). Moreover, APP and APLP2 joint suppression sensitised cells for vacuole formation when PIKfyve was inhibited for a brief amount of time (46). While so far we have not been able to measure $PI(3,5)P_2$ levels biochemically, all our cell biological findings suggest that APP gene family members modulate PIKfyve function.

It is important to bear in mind that PIKfyve activity is necessary for both the formation of $PI(3,5)P_2$ and PI(5)P (11), a significant hurdle for defining which of the cellular effects of PIKfyve depend on $PI(3,5)P_2$ and which on PI(5)P.

We also tested whether the APP/PIKfyve interplay is conserved in evolution using the *C. elegans* genetic model. A genetic analysis of the function of the APP orthologue APL-1 revealed that the APL-1 C-terminus is required for PIKfyve (PPK-3 in *C. elegans*) function required for endosomal homeostasis and neuronal function (45), entirely consistent with the data obtained in mammals.

Together these studies showed that APP family members bind the PIKfyve complex and control its function, shedding light on the elusive functions of the APP gene family. Both studies suggest that the APP family members function as activators of the PIKfyve complex.

The fact that APP, a type I transmembrane receptor that traffics between the Golgi, plasma membrane and the endosomal system, can bind to and modify PIKfyve function has interesting implications. What is the purpose of a receptor, cycling through an organelle, being able to modify the phosphoinositide composition of this organelle? An interesting answer was provided when we noticed that PIKfyve inhibition led to the trapping of APP in EEA1 positive early endosomes (37), showing that APP trafficking requires PIKfyve activity. This would suggest that APP, upon arrival in early endosomes is able to bind the PIKfyve complex and stimulate $PI(3,5)P_2$ formation, promoting its own sorting and thus escaping from early endosomes. The most likely destination for APP sorted from endosomes is the trans-Golgi-network, as endosome to TGN transport has been shown to be one of the key functions of the PIKfyve complex (22).

This is an interesting example of a receptor that has the potential to actively contribute to its own sorting by an interaction with a phosphoinositide kinase complex. It remains to be explored whether this may be an example of a more widespread principle or whether the APP/PIKfyve interplay represents a truly special relationship.

The APP/PIKfyve interplay in Alzheimer's disease.

The fact that APP can modulate PIKfyve function has important implications for the molecular mechanism of neurodegeneration in Alzheimer's disease. Loss of PIKfyve function in mouse models has been demonstrated to result in serious neurodegeneration (9–11)□. For example, knock-out of Vac14 led to perinatal death of mouse pups. Closer analysis of brain sections highlighted extensive neurodegeneration, the appearance of aberrant vacuoles and apoptosis (9, 10). These observations were paralleled by the identification of a loss-of-function mutation in the Fig4 gene, which also resulted in significant, post-natal neurodegeneration in the CNS and PNS (10). More recently it was shown that a strong reduction of PIKfyve expression seriously affects the integrity of the CNS as well, but has additional deleterious effects on the heart, lungs, kidneys and other organs in mice, consistent with the ubiquitous expression of the PIKfyve complex subunits (11). These findings highlight that loss of function of the kinase subunit, e.g. PIKfyve causes more widespread defects than loss of its accessory subunits Vac14 and Fig4, suggesting that CNS and PNS are exquisitely sensitive to perturbations of $PI(3,5)P_2$ metabolism, more so than other organs and tissues.

A key event in Alzheimer's disease is the proteolytic destruction of APP by beta and gamma secretase cleavage mainly in the endosomal system (34). Mutations in the APP gene or the gamma secretase complex identified in patients suffering from familial (also known as early onset) Alzheimer's disease document that aberrant APP cleavage by gamma secretase is sufficient to cause the disease (48, 49). Importantly, the intracellular domain of APP, released by gamma secretase cleavage is rapidly broken down, resulting in a very short lifetime of this fragment (50). As cleavage of APP by secretases is crucial for the development of Alzheimer's disease and as one cleavage product, beta-amyloid, accumulates in patients' brains, this led to the conclusion that beta amyloid production is the driving force in Alzheimer's disease (51). This view seemed to be strengthened by the fact that patients with

trisomy 21, on which the APP gene is located, almost invariably develop signs of neurodegeneration and accumulate beta amyloid (52). This was presumed to stem from a dosage effect of APP overproduction due to the extra copy of chromosome 21. However, a closer analysis of APP metabolism showed that in Down syndrome patients BACE1 activity is increased while the APP protein level appeared unchanged, suggesting that enhanced APP cleavage rather than overproduction of APP drives neurodegeneration in Down's syndrome (53). So data from both familial Alzheimer's disease and Down syndrome patients suggest that enhanced APP cleavage is the central event in Alzheimer's disease. It is currently widely assumed that the overproduction of beta amyloid initiates a cascade of deleterious events that ultimately results in neurodegeneration.

However, the fact that APP can bind to the PIKfyve complex and stimulate its function may suggest an entirely different explanation for neurodegeneration in Alzheimer's disease: Cleavage of APP by the gamma secretase releases APP's intracellular domain that is necessary for binding to and activating the PIKfyve complex (46). APP's intracellular domain, after cleavage, is followed by its rapid degradation (50). Thus enhanced gamma secretase cleavage would prevent APP from binding to and stimulating PIKfyve function, disrupting endosomal sorting and homeostasis. It is worth noting that endosomal abnormalities, especially an enlargement of EEA1 positive early endosomes, is an early pathological hallmark observed in Alzheimer's disease (54). Reduced PIKfyve function is known to result in defective neuronal function and neurodegeneration, as demonstrated by mouse models and human patients suffering from rare forms of Charcot-Marie-Tooth syndrome and ALS (18, 55). How PIKfyve dysfunction exactly causes neurodegeneration is still not fully understood, however, with the identification of additional $PI(3,5)P_2$ effectors this is becoming clearer. The previously mentioned $PI(3,5)P_2$/TRPML-1 interplay required for the fusion/fission cycle of endosomes with lysosomes and for successful completion of

autophagy is most likely to contribute to the neurodegenerative phenotype (56). Impairment of autophagy is well documented to result in neurodegeneration (57). $PI(3,5)P_2$ may also control V-ATPase function and endo/lysosomal acidification (18) which is crucial for the lysosomal breakdown of multiple substrates and for the successful completion of autophagy, and could therefore contribute to the neurodegenerative phenotype as well.

Disruption of endo/lysosome and autophagosome function caused by the lack of activation of multiple $PI(3,5)P_2$ effectors may also explain the toxic built up of molecules such as beta amyloid and hyperphosphorylated Tau, both characteristic of Alzheimer's disease (58). This hypothesis is illustrated in Figure 1.

This alternative neurodegeneration model raises the question whether the accumulation of beta amyloid is in fact a consequence resulting from an underlying endo/lyso/autophagosomal pathology rather than its cause. If this is the case then targeting beta amyloid production or aggregation may be of limited therapeutic effect. This is consistent with the failure of all published clinical drug trials that aim to block aggregation or increase solubility of beta amyloid to stop the progression of late onset Alzheimer's disease (59).

It will be extremely interesting to test whether evidence of PIKfyve dysfunction can be detected in early or late onset Alzheimer's disease and whether this could explain aspects of the neurodegenerative phenotype. If so this would make the PIKfyve pathway an interesting drug target not only in Alzheimer's disease but also in a other neurodegenerative diseases with strong endo/lyso/autophagosomal pathology.

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Figure 1

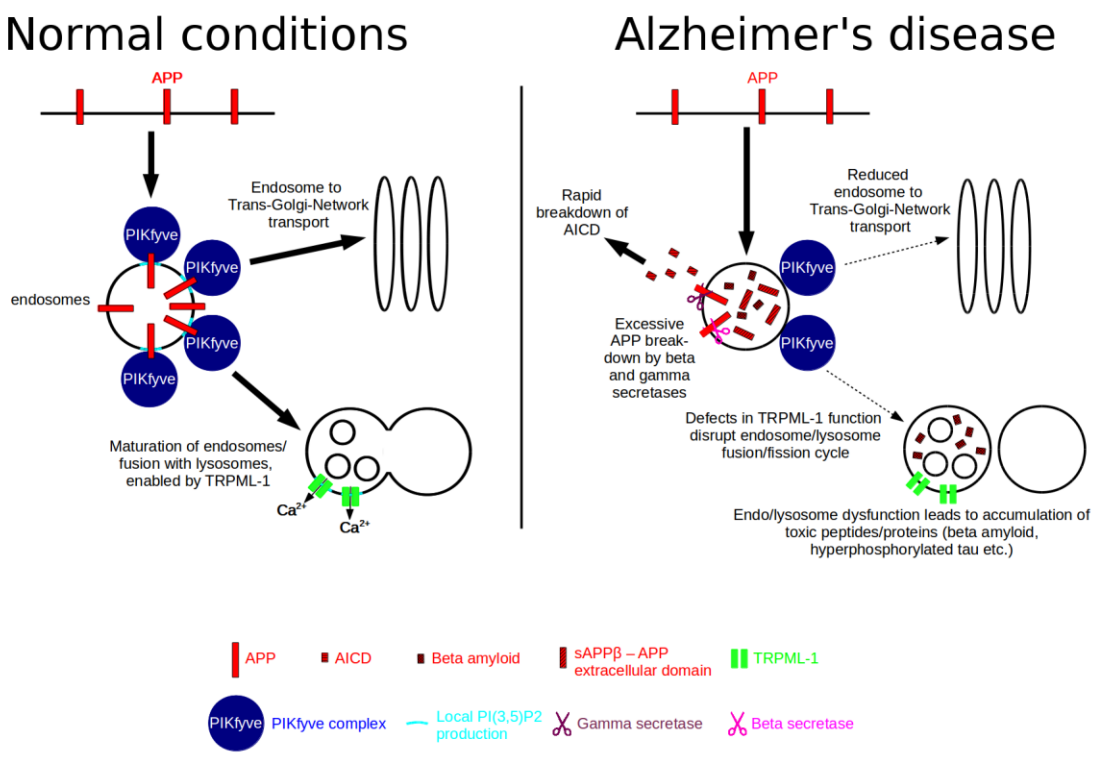

Figure 1) Working model exploring what role the APP/PIKfyve interplay may play in Alzheimer's disease

Under normal conditions, APP binds to and activates the PIKfyve complex to enable sorting events such as endosome to TGN transport as well as maturation of endosomes and fusion with lysosomes, allowing lysosomal breakdown of proteins destined for degradation. However, in Alzheimer's disease APP falls prey to excessive cleavage by beta and gamma secretases, thereby releasing the cytoplasmic domain of APP (necessary for binding the PIKfyve complex) from the endosomal membrane, followed by its rapid breakdown (50). This will reduce PIKfyve activity which is known to result in neurodegeneration by mechanisms that are currently not entirely clear (9–11). Reduced PIKfyve activity will impair endosome to TGN transport and the fusion/fission cycle of late endosomes and lysosomes controlled by TRPML-1 (22, 60). Interestingly, the relevance of retromer

mediated endosome to TGN transport in Alzheimer's disease has been highlighted previously (43). Disruption of endo/lysosomal fusion will reduce the capability of neurons to degrade toxic proteins such as beta amyloid and hyperphosphoryalted tau, resulting in their accumulation. These will be released from neurons either when neurons are dying or by fusion of late endosomes with the plasma membrane, for example during the release of exosomes (60).