Rab Proteins and Alzheimer's: A Current Review of Their Involvement in Amyloid Beta Generation with Focus on Rab10 Expression in N2A-695 Cells

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Rab Proteins and Alzheimer’s: A Current Review of Their Involvement in Amyloid Beta Generation with Focus on Rab10 Expression in N2A-695 Cells

Ivan Arano Rodriguez

A thesis submitted to the faculty of Brigham Young University in partial fulfillment of the requirements for the degree of Master of Science

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March 2015

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This thesis work describes the role of Rab proteins in amyloid processing and clearance in different cell pathways. It also describes an experimental approach used to analyze the expression effects of Rab10 in amyloid beta production. Since the main theory behind neurodegeneration in Alzheimer’s disease claims that high levels of amyloid beta 42 (Aβ42) molecules trigger widespread neuronal death, control of Aβ42 has been a main target in Alzheimer’s disease research. In addition, several studies show increased levels of particular Rab proteins in Alzheimer’s pathogenesis. However, no review consolidates current findings in neurodegeneration of Alzheimer’s with Rab protein dysfunction. The first chapter of this thesis aims to address this need by providing a current review of Rab proteins associated with APP and neurodegeneration. The second chapter constitutes an experimental approach used to characterize the effects of Rab10 and Sar1A GTPases in APP and amyloid processing. We found that Rab10 expression does not affect APP production but significantly changes Aβ generation, particularly the toxic Aβ42 and Aβ42:40 ratio. On the other hand, we found no significant effect of Sar1A expression on either APP or amyloid beta generation. These findings partially confirm the work done by Kauwe et al (2015) and provide preliminary evidence for two potential targets for protective effects in neurodegeneration.

Keywords: Rab proteins, GTPases, Alzheimer’s disease, gene, genetic variants, 3’ UTR, amyloid beta, neurodegeneration, endocytosis, anterograde transport, autophagy, amyloid precursor protein, transient transfection, overexpression, knockdown
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CHAPTER 1: Rab GTPases in Alzheimer’s Disease: A Review on Main Players That Influence Amyloid Production

Alzheimer’s disease is the most common form of dementia and it is the sixth leading cause of death in the United States. In 2014, an estimated 5.2 million Americans developed Alzheimer’s disease and the cost for caregiving to be $214 billion (Plassman, Langa et al. 2007). Amyloid production resulting by processing of APP is the main hypothesis associated with triggering the development of this disease. Rab GTPases are small proteins belonging to the Ras superfamily. Due to their major role in intracellular and membrane trafficking, some Rab GTPases have been identified as important players in regulating APP transport, processing and clearance. Consistent with these roles, AD brains present expression changes of Rab proteins involved in these processes. This review aims to provide a framework to understand those Rab GTPases associated with APP and neurodegeneration and to propose targets for future Alzheimer’s disease research relating to this group of proteins.

APP Function and Amyloid Production

The most accepted theory for neurodegeneration in Alzheimer’s disease is the widespread neuronal death triggered by high levels of a molecule called amyloid beta 42 (Aβ-42), which is produced by the cleaving of trans membrane amyloid precursor protein (APP) (Masters, Multhaup et al. 1985, Glenner and Wong 2012). This is commonly known as the amyloid cascade hypothesis (Hardy and Higgins 1992). The human APP gene is located in chromosome 21 and produces a type I protein with a large extracellular N-terminal, transmembrane and short C-terminal cytoplasmic domain (Reinhard, Hebert et al. 2005). APP is highly expressed in the brain and produces eight isoforms that range from 365 – 770 amino acids. The 695 isoform is the most expressed in the central neural system while the other isoforms can be ubiquitously
expressed in all tissues (Placido, Pereira et al. 2014). Several studies have proposed a variety of functions for APP in the brain; the most supported is the trophic effect on formation and regulation of synapses as well as neuritic outgrowth (Turner, O'Connor et al. 2003, Lee, Moussa et al. 2010). Additional evidence suggests APP is involved in synaptic plasticity (Hung, Koo et al. 1992, Oh, Savonenko et al. 2009), cell adhesion (Yamazaki, Koo et al. 1997, Sosa, Bergman et al. 2013), migration (Young-Pearse, Bai et al. 2007), axonal transport (Sisodia 2002), cell surface receptor (Selkoe 2001, Hashimoto and Matsuoka 2014) among others.

After expression, newly formed APP undergoes the constitutive secretory pathway where is subsequently internalized to the cell via clathrin mediated endocytosis (here referred as endocytosis) for processing. As part of the secretory pathway, APP undergoes several post-translational modifications in the ER and in the trans-Golgi network (TGN) including N- and O-glycosylation, cytoplasmic phosphorylation and sulfation (Selkoe 2001, Bhattacharyya, Barren et al. 2013) before being trafficked to the plasma membrane. In neurons, APP is mainly transported to the cell membrane of axons and dendrites using the axonal transport system with kinesin-1 as the main motor protein (Kins, Lauther et al. 2006). Although APP is mainly processed via endocytosis, evidence suggests that nascent APP molecules can be processed by intracellular transport from the TGN to the endosome, resulting on increased Aβ-42 production in these organelles (Capell, Meyn et al. 2002, Tam, Seah et al. 2014)

Different studies estimate that about 10% of APP is present on the plasma membrane at all times while the rest is carried into the cell via endocytosis for processing (Thinakaran and Koo 2008). APP has three main proteolytic sites (alpha, beta & gamma) and different enzymes termed secretases are used to cleave APP on each site. APP is processed in two pathways: the non-amyloidogenic pathway and the toxic or amyloidogenic pathway. The non-amyloidogenic
pathway is carried in the plasma membrane where APP is first cleaved in the N-terminus by alpha-secretase to produce sAPPα. The C-terminal is then cleaved by gamma-secretase to produce a short p3 and APP intracellular domain fragments (Reinhard, Hebert et al. 2005, Dawkins and Small 2014). In the amyloidogenic pathway, APP molecules not cleaved by alpha-secretase are internalized and processed by beta-secretase (BACE1) and the gamma-secretase complex (PSEN1 or PSEN2, nicastrin, APH1 & PEN-2) to produce sAPPβ and AB fragments ranging from 38-42 amino acids (Kamenetz, Tomita et al. 2003, Dawkins and Small 2014). This process is carried by the secretory and recycling pathways, where some APP fragments can be recycled back to the cell membrane along with sAPP-B and AB fragments. Most of the secreted amyloid beta produced contains 40 amino acids (Aβ40), however, the smaller portion of amyloid beta that contains 42 amino acids (Aβ42) tends to form extracellular aggregates (Haass, Kaether et al. 2012). The processing, aggregation and subsequent neuronal death triggered by Aβ42 fibrils are the center of the amyloid cascade hypothesis. Extensive reviews of amyloid processing and function can be accessed in references (Kamenetz, Tomita et al. 2003, Dawkins and Small 2014). Since APP is internalized to produce Aβ42, the main steps in this process, which include endocytosis and proteolytic processing via recycling and autophagy pathways will be analyzed next.

Endocytosis Pathway in Alzheimer’s Pathogenesis

Endocytosis is a process by which many molecules enter the cytoplasm via cell membrane receptors. Specific cell membrane receptors contain signal domains that trigger the recruitment of clathrin molecules and the endocytic response, which engulfs the portion of the cell membrane containing the cell receptor along with other molecules around it. The intracellular and C-terminal domains of APP contain a YEMPTY motif that has been
demonstrated to be involved in the signal for endocytosis (Nordstedt, Caporaso et al. 1993, Ring, Weyer et al. 2007). Consistent with this observation, mutations on the YEMPTY domain inhibit APP internalization and Aβ42 generation, suggesting that endocytosis is critical for AB production (Perez, Soriano et al. 1999). The YEMPTY domain also serves as the binding site for other cytosolic adaptors including Mint1, Mint2, Mint3, Fe65, Dab1 and JNK protein members (Thinakaran and Koo 2008). Interestingly, Mint and Fe65 proteins also play an important role in APP endocytosis and Aβ42 regulation (Haass, Kaether et al. 2012, Perez, Soriano et al. 1999, Ring, Weyer et al. 2007). A more detailed review of the YEMPTY region of APP and predicted interactions can be accessed in these references (Kerr and Small 2005, Miller, McLoughlin et al. 2006, Schettini, Govoni et al. 2010).

In neurons, several regulatory proteins are involved in starting the endocytosis signal of APP; the most common are Rab5, Dynamin1 and, most recently, Mint1 and Mint2 proteins (Thomas, Lelos et al. 2011, Sullivan, Dillon et al. 2014). Newly formed APP vesicles are transported and fused to the first sorting station: the early endosome. Concurrently, BACE-1 is also trafficked to the early endosome from the plasma membrane where AB biogenesis occurs. Early endosomes are a major site for APP processing and Aβ42 production and gradually mature into late endosomes and subsequently into lysosomes (Cataldo, Peterhoff et al. 2000, Chia, Toh et al. 2013). Once on the early endosome, APP and AB containing vesicles can undergo different pathways: 1) early endosome to plasma membrane 2) early endosome to recycling endosome 3) early endosome to TGN or 4) early endosome to late endosome and lysosomes. Many studies show evidence of high activity of BACE-1 and gamma-secretases on these sorting pathways following endocytosis (Cataldo, Peterhoff et al. 2000, Chia, Toh et al. 2013, Lu and Hong 2014). In addition, newly formed APP molecules that are processed in intracellular fashion are also
incorporated into the TGN or into the lysosomes where are processed into AB peptides (Tam, Seah et al. 2014). Consequently, AB peptides can be measured in higher quantities along these pathways, particularly on the TGN and endosomes. Thus, keeping track of AB peptides have become a good indicator for APP processing cycle in the cell. Finally, the autophagy mechanism in the cell completes the APP processing cycle by recycling and secreting AB peptides to the extracellular space.

Macroautophagy (here referred as autophagy) serves as the main clearance mechanism for APP processing and AB clearance. During autophagy, double membrane vesicles that contain waste components and hydrolases are fused into large vesicles called autophagosomes. Autophagosomes then fuse with late endosomes, which eventually turn into lysosomes to degrade the original autophagosome components (Nixon 2007). The resulting products can be either recycled back as an amino acid source or secreted via exocytosis. A complete review of autophagy and its involvement in amyloidogenesis can be found in reference (Nixon 2007). In neurons, autophagosomes and endosomes are formed near the synapses and require retrograde transport to the lysosomes for efficient degradation (Nixon 2007). Briefly, as APP is processed in the endocytosis and intracellular pathways, the resulting AB peptides are mostly degraded by lysosomes. Upon reaching to the lysosomes, large pools of gamma-secretase components and other hydrolases complete the degradation process of APP and AB peptides, in which some Aβ42 is secreted via exocytosis (LeBlanc and Goodyer 1999, Rajendran, Honsho et al. 2006)

Additional experiments show that Aβ42 was reduced when autophagy was suppressed, highlighting the role of autophagy on Aβ clearance (Lee, Yu et al. 2010).

Although endocytosis and autophagy are vital processes in APP turnover cycle, their disruption can also facilitate intracellular Aβ aggregation and apoptosis in the brain. In a model
by Yu et al. (2005), autophagy vacuoles containing full length APP and Aβ peptides accumulated in significant numbers in axons and neurites in Alzheimer’s disease brains. These vacuoles were enriched in gamma-secretase activity inducing intracellular Aβ accumulation (Yu, Cuervo et al. 2005). A different study by Lee et al (2010) showed that PSEN1 mutations in Familiar AD cases affected the autophagy turnover of proteins (Lee, Yu et al. 2010). In familiar AD, mutations along APP & gamma-secretase complex (PSEN1 & PSEN2) increase the ratio of Aβ40 to Aβ42 (Lee, Yu et al. 2010). In late-onset AD, multiple genes that regulate endocytosis or Aβ clearance have been, unsurprisingly associated with AD (Wu and Yao 2009, Ridge, Ebbert et al. 2013). Examples of these genes involve BIN1, PICALM, CR1, CDAP2, and some Rab GTPases. This paper will focus solely on the role of Rab GTPases involved in APP trafficking, processing and clearance.

Rab GTPases in Alzheimer’s Disease

Rab GTPases are small proteins member of the Ras superfamily of monomeric proteins. They are involved in every step of the intracellular vesicle transport in the cell. Approximately 60 human Rab GTPases have been identified, being this family the largest and more heterogeneous of the Ras superfamily (Stenmark and Olkkonen 2001, Zerial and McBride 2001). Rab proteins are present in all eukaryotic cells, ranging from the most evolutionary conserved which are ubiquitously expressed for normal vesicle transport to the most specialized types that are only found on certain mammalian cell types (Seabra, Mules et al. 2002, Kaddai, Le Marchand-Brustel et al. 2008). Rab proteins switch between an inactive (GDP-bound) cytosol state and active (GTP-bound) membrane bound state. To switch from the GDP to GTP state, Rabs require activator proteins called GEFs (Guanine nucleotide exchange factors). Once in their active state, Rab-GTP recruits effector proteins that enable them to control different steps in
vesicle trafficking including cargo selection, tethering, movement and docking (Stenmark and Olkkonen 2001, Seabra, Mules et al. 2002, Grosshans, Ortiz et al. 2006, Kaddai, Le Marchand-Brustel et al. 2008). Once vesicle transport is complete, Rab-GTP is hydrolyzed by GAPs (GTPase activating proteins) and return to its inactive GDP-bound state. At this point, Rab-GDP is recycled back to the membrane of origin by binding to RabGDIs (GDP disassociation inhibitors), which determine the proper localization and activity of Rab proteins (Alory and Balch 2001, Markgraf, Peplowska et al. 2007).

The high number of components of the Rab family reflects the complexity of the intracellular transport system, thus Rab proteins can be assigned to a single or multiple sites. For instance, Rab5 plays a substantial role in endocytosis by transporting cargoes to multiple organelles (Markgraf, Peplowska et al. 2007, Ishikura, Koshkina et al. 2008), whereas Rab23 is a negative regulator in the Sonic hedgehog-signaling pathway (Eggenschwiler, Bulgakov et al. 2006). Because of their key role in vesicle transport, endocytosis and autophagy, Rab proteins have also been studied as part of the amyloid hypothesis of AB deposition. Disruptions of these pathways have been extensively documented in Alzheimer’s disease (Perez, Squazzo et al. 1996, Nixon 2007, Wu and Yao 2009, Ridge, Ebbert et al. 2013, Tam, Seah et al. 2014). Specifically, this review will cover the main Rab proteins associated with APP and AB production: Rab3, Rab5, Rab6, Rab7, Rab11, Rab4 & Rab8.

Rab3

Rab3 is a small GTPase that is localized in synaptic vesicles in neural cells and thought to be highly associated with regulating vesicle fusion in exocytosis. Rab3 has four isoforms (Rab3A, Rab3B, Rab3C & Rab3D) that contain a 77-85% amino acid homology (Baldini, Hohl et al. 1992). Although deletion of Rab3 causes a lethal phenotype in mice, experiments suggest
that the role of the four isoforms might be redundant in synaptic vesicle transport (Li, Jahn et al. 1996). Rab3A is the most common of the four and localizes on the surface of synaptic vesicles of various type of neurons in the rat brain including motor, sensory, adrenegalic and cholinergic neurons. More specifically Rab3A has a key role in vesicle docking in the Ca\(^{2+}\) dependent exocytosis of neurotransmitters in synapses (Komuro, Sasaki et al. 1996, Coleman, Bill et al. 2007). In neurons, Rab3 protein associates with motor protein Kinesin-1 in anterograde transport of synaptic vesicles to the neuritic axons (Schluter, Schmitz et al. 2004, Coleman, Bill et al. 2007, Kimura, Okabayashi et al. 2012). Another role of Rab3A is the association with actively recycling vesicles carried to synapse terminals. Star et al (2007) demonstrated that vesicles that are actively recycling contain higher Rab3A than vesicles that are stationary (Star, Newton et al. 2005). Taken together, these studies suggest that Rab3 is involved in anterograde transport and exocytosis of synaptic vesicles in neurons.

Rab3A is required for anterograde transport of APP and is associated with regulating APP and AB peptide levels via the secretory pathway. A study by Szodorai et al (2009) showed that the complex required for anterograde transport of APP vesicles contained Rab3A and Kinesin-1C. Szodorai argues that Rab3A is more important for initial recruitment of APP and kinesin-1C rather than for the actual vesicle fusion to the axons (Szodorai, Kuan et al. 2009). A separate study showed that a disruption of the anterograde transport increases levels of Rab3 and it is correlated with age-dependent impairment of cognitive function (Kimura, Okabayashi et al. 2012). Rab3A was found to be significantly downregulated, among other presynaptic proteins in the frontal and parietal cortex of AD brains (Reddy, Mani et al. 2005). Consistent with these findings, a recent study by recent study by Tan et al (2014) showed that a Rab3A effector Rabphilin3A (RBP3A) is also reduced in AD post-mortem brains; this reduction was correlated
with dementia progression, cholinergic deafferentiation and high Aβ42 aggregation (Tan, Lee et al. 2014). In a different study, a Western Blot screening of lysosomal network proteins identified Rab3 and Rab7 as significantly increased in CSF of AD patients and was validated using a second cohort. This study suggested that both Rab3 and Rab7 are upregulated in the endosomal and autophagy pathways early in disease progression (Armstrong, Mattsson et al. 2014). These studies support the observation that Rab3 expression might be reduced in the brain and upregulated in the CSF of Alzheimer’s disease patients. Finally, a high-throughput RNAi screening of all human Rab GTPases confirmed the role of Rab3 in the trafficking and maintenance of APP levels in neurons. In this same study, knockdown of all isoforms of Rab3, except for Rab3C, decreased both Aβ42 and sAPP-β levels (Udayar, Buggia-Prevot et al. 2013).

Rab5

Rab5 is by far the most characterized member of the Rab GTPase proteins and the most associated with AD progression. Rab5 has three known isoforms (Rab5A, Rab5B & Rab5C), similar to Rab3; all isoforms have a similar function. Although Rab5 is widely expressed in the cell, it is mainly localized in the early endosome, cell membrane, clathrin-coated vesicles and in recycling endosomes (Gorvel, Chavrier et al. 1991, Bucci, Parton et al. 1992, Li and Stahl 1993, Somsel Rodman and Wandinger-Ness 2000). The most established functions for Rab5 are the fusion of endocytic vesicles to the early endosomes and the vesicle docking events in recycling and endocytosis. Reviews can be accessed in references (Gorvel, Chavrier et al. 1991, Somsel Rodman and Wandinger-Ness 2000, Woodman 2000). Additional functions for Rab5 include promoting cell migration and integrin signaling pathways (Mendoza, Diaz et al. 2014). Overexpression of Rab5 causes an increase of endocytosis as well as a formation of large early endosomes whereas the opposite effects applied upon down regulation of Rab5 (Stenmark,
Parton et al. 1994, Horiuchi, Lippe et al. 1997). Due to the critical role of Rab5 in endocytosis and recycling pathways, there is much evidence that links Rab5 and its effectors with neuronal degeneration and Alzheimer’s disease.

Characterization of Rab5 and its effectors in endocytosis and recycling have provided important insights towards APP processing and AD pathogenesis. Disruptions of the aforementioned pathways are one of the earliest changes observed in AD and could be one of the main mechanisms involved in the progression of this disease. Increased endocytosis markers and endosome enlargement are one of the first responses in AD, independently of Aβ42 accumulation (Cataldo, Rebeck et al. 2001, Grbovic, Mathews et al. 2003, Nixon 2005, Ginsberg, Mufson et al. 2011). Rab5 overexpression replicates many of these observations in early endosomes and dramatically increases secreted Aβ40 & Aβ42 (Cataldo, Peterhoff et al. 2000, Grbovic, Mathews et al. 2003, Nixon 2005, Ginsberg, Mufson et al. 2011). A possible mechanism is that APP functions as an endocytosis receptor for Rab5, which could trigger overexpression of Rab5 and its correspondent downstream effects in endosomal dysfunction. A study by Laifenfeld et al (2007) showed that Rab5 binds to APP-B1 and initiates a signaling pathway that leads to apoptosis in a FAD model (Laifenfeld, Patzek et al. 2007). In addition, microarray analyses show that Rab5 and Rab7 are selectively up regulated in cholinergic and hippocampal neurons of MCI and AD individuals (Ginsberg, Mufson et al. 2010, Ginsberg, Mufson et al. 2011). Along with these observations, the majority of Aβ production occurs in in Rab5-endocytic vesicles in the early endosome and lysosomes. Overexpression of Rab5 and Rab7 showed to increase Aβ42 trafficking and processing in lysosomes. This effect is further increased in APOEe4 when compared to APOEe3 carriers (Li, Kanekiyo et al. 2012).

Interestingly, Rab5B isoform is associated with neuroprotection by providing neuronal resistance
to exocytosis injury and synaptic plasticity (Baskys, Bayazitov et al. 2007). Similarly, silencing of only Rab5C isoform increased significantly APP, sAPPβ and Aβ42 cellular levels (Li and Stahl 1993). Together, these reports highlight the role of Rab5 in neurodegeneration and as a therapeutic candidate for AD progression.

Rab6

Rab6 is another well-characterized GTPase, which is involved in anterograde and retrograde transport of APP and Aβ peptides. Rab6 has four different isoforms: Rab6A, Rab6A’, Rab6B and Rab6C. Rab6A’ is a splice variant generated by alternative splicing on the Rab6A gene and only differs from Rab6A by three amino acids (Opdam, Echard et al. 2000). Rab6 GTP is commonly found in the TGN and regulates retrograde and anterograde intra-Golgi pathways (Martinez, Schmidt et al. 1994, Darchen and Goud 2000). In contrast to the previous Rab GTPases, Rab6 isoforms have differential tissue expressions and functions. RabA is a dynamic GTPase that regulates retrograde transport from the TGN to the ER in a COP-I independent mechanism and from endosomes (early endosomes and recycling endosomes) to the TGN or vice versa (Martinez, Schmidt et al. 1994, Young, Staubet et al. 2005). On the other hand, Rab6A’ regulates anterograde transport from the TGN to the plasma membrane (Siniossoglou and Pelham 2001, Mallard, Tang et al. 2002). Rab6B is also involved in retrograde transport in the TGN and ER but it is expressed specifically in brain cells such as neurons, microglia and Purkinje cells (Opdam, Echard et al. 2000, Wanschers, van de Vorstenbosch et al. 2007). Finally, Rab6C is the least characterized of the four isoforms and it has been associated with the cell cycle progression and drug resistance to particular cancer types (Shan, Mason et al. 2000, Young, Menetrey et al. 2010).
In neurons, Rab6B GTP requires Mint effector proteins to bind to APP. As described earlier, Mint proteins bind to the YENPTY motif of APP, facilitating anterograde transport. Studies show that Rab6 can bind to Mint1, Mint2 and Mint3 to form a complex with APP (Teber, Nagano et al. 2005, Thyrock, Ossendorf et al. 2013). Evidence suggests that Rab6 is involved in retrograde trafficking of APP from the endosomes to the TGN and from the TGN to the ER. However, there is not a consensus of the actual role of Rab6 in APP processing and AD. A study by Elfrink et al (2011) showed that Rab6 is increased in non-tangle bearing neurons. This increase might occur as a protective response to reduce ER stress neurotoxicity (Elfrink, Zwart et al. 2012). Failure to recover from ER stress also contributes to intracellular Aβ42 accumulation and neurodegeneration in early AD pathology (Soejima, Ohyagi et al. 2013). Rab6 can also promote sAPPa production and decrease intracellular Aβ42 accumulation. A recent study suggests that Rab6 selectively facilitates anterograde transport of APP to the plasma membrane to be processed by alpha-secretase (McConlogue, Castellano et al. 1996). Moreover, a recent RNAi screen showed that silencing of Rab6 decreased Aβ42 production; the authors concluded that this effect could be a result of an alteration of the Gamma-secretase cleavage or the secretion of Aβ (Udayar, Buggia-Prevot et al. 2013). This conclusion is in line with previous results in fibroblasts where Rab6 is dependent on PSEN1 and protein kinase C for membrane association (Scheper, Zwart et al. 2004). These observations suggest of a protective role of Rab6 for neurodegeneration, however, further experiments are needed to assess this specific connection.

Rab7

Rab7 is a GTPase required in the late stages of the recycling and autophagy pathways. Rab7 is ubiquitously expressed and it comes in one isoform. However, a new Rab GTPase that
contains 65% identity with Rab7 has been named Rab7b. This GTPase is not coded by the same gene and seems to have different functions than Rab7 (Progida, Cogli et al. 2010). Rab7 has a critical role in regulating the recycling pathway, specifically in late steps in endocytosis and the maturation of endosomes and autophagosomes (Feng, Press et al. 1995, Gutierrez, Munafo et al. 2004, Jager, Bucci et al. 2004, Rink, Ghigo et al. 2005, Hyttinen, Niittykoski et al. 2013). In late endocytic trafficking, Rab7 acts downstream of Rab5 in transporting cargoes from early to late endosomes (Feng, Press et al. 1995). In the process of maturation of early endosomes to late endosomes, a crucial step is the replacement of Rab5 compartments with Rab7 via GEF effectors and other vesicle fusion events (Rink, Ghigo et al. 2005, Hyttinen, Niittykoski et al. 2013). After late-endosome maturation, cytoplasmic vesicles or other late-endosomes containing Rab7 are fused together and aid to the formation of lysosomes according to the “kiss and run model” (Storrie and Desjardins 1996, Bucci, Thomsen et al. 2000). Lastly, Rab7 is also required for the final maturation of autophagic vacuoles and their final step in fusing with lysosomes. Rab7 deficient cells experience mayor failures on lysosome function and autophagy (Gutierrez, Munafo et al. 2004, Jager, Bucci et al. 2004). The role of Rab7 in recycling and autophagy makes it an attractive target in connection to the AB aggregation hypothesis in Alzheimer’s disease.

As explained earlier, changes in the endocytosis and recycling pathways are one of the earliest clinical changes in Alzheimer’s. In these pathways, Rab5 and Rab7 are critical in maintaining normal cellular functioning. Alzheimer’s disease brains exhibit increased endocytosis and vesicle fusion to produce enlarged early endosomes, which will later turn into enlarged late endosomes and lysosomes with impaired functions. Rab5 and Rab7 are common markers used to visualize each step on these events (Cataldo, Mathews et al. 2008, Ginsberg,
Alldred et al. 2010) and consequently, these seem to be upregulated in mild cognitive impaired and Alzheimer’s disease patients (Yuyama and Yanagisawa 2009, Ginsberg, Mufson et al. 2010, Ginsberg, Mufson et al. 2011). A recent study by Armstrong et al (2014) identified Rab7 as one of six lysosomal proteins that could be used for potential Alzheimer’s disease biomarkers in cerebrospinal fluid (Armstrong, Mattsson et al. 2014). Indeed, in Alzheimer’s disease brains, an excessive number of autophagic vacuoles accumulate in neurites and synaptic terminals as a result of disruption of autophagy and lysosome pathways (Nixon, Wegiel et al. 2005). Moreover, autophagic vacuoles in axons fuse with Rab7 vesicles to form lysosomes via retrograde transport to the cell for degradation. Lee et al (2011) demonstrated that disruption of this pathway in mouse cortical neurons produced AD-like axonal dystrophy (Lee, Sato et al. 2011). An interesting study concluded that amyloid fibrils are formed as a result of Rab7 suppression, whereas Rab5 knockdown did not seem to affect amyloid aggregation. The authors concluded that dysfunction of the late endocytosis pathway; controlled by Rab7, contribute to amyloid aggregation in neuronal cells (Yuyama and Yanagisawa 2009). A follow-up study showed that inhibiting Aβ42 endocytosis reversed the increased levels of Rab7 and Rab5 and prevented neurodegeneration in mice neurons treated with Aβ42 (Song, Baker et al. 2011). Overall, the role Rab7 in the late endocytosis pathway provides important insights into Aβ42 production and could be a useful therapeutic target in Alzheimer’s disease.

Rab11

Rab11 is a very dynamic player in different cell functions ranging from cell adhesion to endocytosis. Rab11 comes in three isoforms: Rab11A is ubiquitously expressed in mammalian cells, Rab11B which is mainly expressed in brain cells and Rab11C, which is known as Rab25 (Goldenring, Shen et al. 1993, Lai, Stubbs et al. 1994, Hales, Griner et al. 2001). Rab11 has a
fundamental role in the recycling endosome by controlling trafficking of proteins to and from the recycling endosome to other organelles in the recycling pathway (Urbe, Huber et al. 1993, Ullrich, Reinsch et al. 1996). Rab11 is also localized in compartments of the TGN, post-TGN vesicles and in vesicles for exocytosis, suggesting Rab11 as an important link between endocytosis and exocytosis (Urbe, Huber et al. 1993, Chen, Feng et al. 1998, Wilcke, Johannes et al. 2000). Recent studies also show that Rab11 promotes ciliogenesis, cell adhesion and cytokinesis functions by binding to several cell receptors and adhesion proteins (Knodler, Feng et al. 2010, Kelly, Horgan et al. 2012). Finally, Rab11 and its effector proteins associate with motor proteins such as myosin VA and dynein-1 in regulating transport along microtubule tracks (Welz, Wellbourne-Wood et al. 2014). Due to its importance in cell adhesion, Rab11C (Rab25) has been more characterized in Cancer biology; however, several new studies provide important insights towards the relation of Rab11 with neurodegeneration in AD.

Rab11 regulates Aβ42 production by associating with the beta- and gamma- secretases but not directly to APP. Ubayar et al (2013) performed RNAi screening of all human Rab GTPases and identified Rab11 as a key regulator of sAPP-B and Aβ42 production by controlling trafficking of BACE-1 in endocytic compartments (Udayar, Buggia-Prevot et al. 2013). This observation is supported by other studies that confirm the trafficking of BACE-1 from axons and dendrites to the early endosomes in a Rab11-dependent manner (Chia, Toh et al. 2013, Buggia-Prevot, Fernandez et al. 2014). After internalized in endocytosis, APP and BACE-1 converge in the early endosome where Aβ42 production is initiated. Rab11 impairment decreases BACE-1 endocytosis and has the same effect in sAPP-β and secreted Aβ42 (Udayar, Buggia-Prevot et al. 2013, Buggia-Prevot, Fernandez et al. 2014). However, other studies used HEK293 cells expressing the Swedish mutation to show that sAPP-β and Aβ42 are not affected by Rab11
expression (Lopez-Perez, Dumanchin et al. 2000, Bulloj, Leal et al. 2010). Whether these contrasting observations are obtained because of the different cell type used remains to be elucidated. In the gamma-secretase complex, hydrophilic loops in PSEN1 and PSEN2 proteins interact with Rab11; however, this association did not seem to influence APP transport or processing in a negative way (Dumanchin, Czech et al. 1999, Lopez-Perez, Dumanchin et al. 2000). Altogether, Rab11 is a newly identified target that is associated with increased LOAD risk likely by the regulation of BACE-1 and Aβ42. Further studies of Rab11 regulation could provide important insights into therapeutic mechanisms in neurodegeneration.

The Rab GTPases reviewed above are the most associated with amyloid processing and neurodegeneration in AD. However, there are other Rab proteins which association with neurodegeneration is less documented. These Rab proteins are often associated with some of the Rabs reviewed here either by localizing to the same organelle or by regulating the same pathways. A quick overview of such less documented Rab proteins follows:

Rab4

Rab4 is localized to the early endosome along with Rab5 and Rab11 (Sonnichsen, De Renzis et al. 2000). However, Rab4 only affects trafficking to recycling and autophagy pathways and facilitates the formation of late endosomes and recycling endosomes (Vandersluijs, Hull et al. 1991, Vandersluijs, Hull et al. 1992, McCaffrey, Bielli et al. 2001). Rab4 initiates a small cascade of adaptor proteins that direct trafficking to other organelles and that also triggers the formation of endosomes (D’Souza, Semus et al. 2014). Rab4 overexpression does not influence endocytosis but changes the accumulation of recycling markers (TfRs) that could have an effect on the recycling pathway (Vandersluijs, Hull et al. 1992). Due to its involvement in the
endocytic and recycling pathways, Rab4 has also been associated with APP processing in neurons.

Rab4 is upregulated in forebrain neurons from mild cognitive impairment and AD patients, which suggests a role with AB accumulation (Ginsberg, Mufson et al. 2011, Soejima, Ohyagi et al. 2013). Indeed, independent studies found accumulation of exogenous Aβ42 in Rab4 compartments in the early endosome (Arriagada, Astorga et al. 2007, Arriagada, Bustamante et al. 2010). Enlargement of early endosomes, which is a hallmark of Rab5 overexpression, was also associated with increase of Rab4; suggesting that Rab4 and Rab5 might have similar functions in early endosomes and endocytosis. Finally, Udayar et al (2013) reports that silencing of Rab4 decreased production of Aβ42 and sAPP-B (Udayar, Buggia-Prevot et al. 2013). These studies agree that Rab4 and Rab5 are affected in early AD pathology and that Rab4 and Aβ42 accumulation in the early endosome leads to endocytosis and recycling dysfunction. However, further studies are needed to confirm this association.

Rab8

Rab8 is ubiquitously expressed in most cells and localizes to the TGN, vesicular structures and basolateral plasma membrane. In trafficking mechanisms, Rab8 regulates retrograde and anterograde transport between these structures (Huber, Pimplikar et al. 1993). Interestingly, Rab8 has a key role in regulating cell shape by the interaction of actin and microtubules with membrane trafficking. Rab8 also plays an important role in other cell processes such as ciliogenesis, cell migration, exocytosis and receptor exchange in the basal body (Peranen, Auvinen et al. 1996, Sato, Mushiake et al. 2007, Knodler, Feng et al. 2010, Peränen 2011).
In neurons, Rab8 regulates anterograde trafficking to the dendrites but not to the axons and it is required for neurite outgrowth (Huber, Dupree et al. 1995). This is supported by the observation that Rab8 depletion inhibits neuritic growth and mediates polarized membrane transport (Hattula and Peranen 2005). It is possible that Rab8 serves a similar function as Rab6 and Rab3 in anterograde transport of APP to the dendrites. This is supported by the observation that mutations in PSEN1 disturb APP transport and down regulates Rab8 levels in PC12 cells (Kametani, Usami et al. 2004). Analysis of small G proteins (Ras, Ral & Rab) from AD brains identified a significant increase of Rab8 only in the cell membrane whereas other Ras and Rab GTPases decreased in the cytoplasmic region (Shimohama, Kamiya et al. 1999). A similar observation showed that a PSEN1 mutation increased Aβ42 levels in vesicles containing Rab8. These vesicles accumulated in the late stages of the secretory pathway between the TGN and cell membrane (Petanceska, Seeger et al. 2000). Interestingly, previous studies presented here do not show the same observation in upregulated Rab GTPases in AD brains (Ginsberg, Alldred et al. 2010, Ginsberg, Mufson et al. 2010). Finally, RNAi screening for GTPases showed that silencing of Rab8 increased intracellular Aβ42 levels, indicating a possible neuroprotective effect of Rab8 (Udayar, Buggia-Prevot et al. 2013). These reports suggest a likely interaction of Rab8 with PSEN1 and APP via the secretory pathway as well as a possible protective effect on Alzheimer’s disease pathogenesis. Further studies need to confirm this association and establish a more relevant role of Rab8 in neurodegeneration.

Rab10

Rab10 is another dynamic GTPase involved mainly in anterograde transport and in the recycling pathway. Rab10 belongs to a Rab subfamily that includes Rab8 and Rab13 and shares about 66% homology with Rab8; however, both localize to different organelles in the cell (Chen,
Holcomb et al. 1993). Similar to Rab8, Rab10 is involved in numerous cell processes and functions including endocytosis (Babbey, Ahktar et al. 2006), ciliary transport (Babbey, Bacallao et al. 2010), phagosome maturation (Cardoso, Jordao et al. 2010), cell polarization (Schuck, Gerl et al. 2007) and insulin GLUT4 transport (Sano, Eguez et al. 2007, Chen, Wang et al. 2012). Even though Rab10 shares similar homology and functions to Rab8, most literature suggests a complimentary role to Rab11 in the recycling pathway. In *C elegans*, Rab10 is required for proper recycling function and trafficking from the early endosome to the recycling endosome and from there to the basolateral membrane (Chen, Schweinsberg et al. 2006, Schuck, Gerl et al. 2007). Finally, Rab10 seems to be a main regulator of organelle shape and morphology by associating with microtubules. A recent study shows that Rab10 has given a key role in the regulation of ER dynamic growth and morphology by associating with microtubules (Chang and Blackstone 2013, English and Voeltz 2013). A separate study shows that Rab10 is necessary for microtubule network growth in the basolateral recycling pathway in endosomes (Chen, Li et al. 2014).

In neurons, Rab10 is associated with anterograde transport of receptors that promote axon development and neuronal polarization. Rab10 binds to a specific number of effectors including Myosyn Vb, Lgl1, JIP1 and MARCKS in post-TGN vesicles that carry axonal receptors responsive to axonal growth factors (Wang, Liu et al. 2011, Liu, Xu et al. 2013, Deng, Lei et al. 2014, Xu, Deng et al. 2014). Despite its involvement in anterograde and recycling mechanisms, only one study links Rab10 with APP and amyloid processing. In the previously mentioned RNAi expression screen, silencing of Rab10 decreased Aβ42 production without affecting sAPP-B levels. This study also suggested of an interaction of Rab10, Rab23 & Rab25 in regulating the same trafficking pathways (Udayar, Buggia-Prevot et al. 2013). In adipocytes, Rab10
knockdown resulted in an attenuation of Glut4 redistribution to the plasma membrane and a two-fold decrease of Glut4 exocytosis rate (Sano, Eguez et al. 2007). Together, these studies underscore a role of Rab10 in anterograde transport of important cell receptors and exocytosis of recycled peptides. It is possible that Rab10 regulates transport of APP molecules and Aβ42 to the cell membrane via the constitutive secretory and recycling pathways. However, further studies are needed to confirm this association, making Rab10 a promising marker in AD pathogenesis.

Conclusion

Rab GTPases are involved in most intracellular trafficking, endocytosis, recycling and exocytosis in the cell. They are also involved in numerous processes such as organelle formation, response to stimuli, cell migration, etc. Due to their important role in these processes, Rab GTPases provide important insight into mechanisms involved in disease progression and signaling such as Cancer, diabetes, anemia and neurological diseases. In this review, we focused on Rab GTPases that have been mainly associated with Alzheimer’s pathogenesis in endocytosis, recycling and degradation pathways of APP and Aβ42. In addition, we presented Rab proteins with limited association with neurodegeneration and how these could provide future insights in this process. Future directions could be finding mechanisms mediated by Rab proteins that offer protective effects towards neurotoxicity. In addition, therapeutic approaches that could rescue processes such as endocytosis and recycling pathways in early stages of neurodegeneration could prove to be effective in controlling cascade of events that lead to AD pathology.
Figure 1 – Schematic of Rab GTPases involved in APP trafficking in the cell. This figure represents a likely model for the role of specific Rab proteins in APP and AB transport in the cell. Retrieved from (Udayar, Buggia-Prevot et al. 2013).
References:


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Chapter 2: Expression Effects of Rab10 and Sar1A GTPases in APP and Aβ Levels in N2A-695 Cells

Abstract

Rab proteins are members of a large family of monomeric GTP binding proteins that are key players in numerous steps of cellular transport ranging from intracellular transport to endocytosis and autophagy. Several studies show increased levels of particular Rab proteins in Alzheimer’s pathogenesis. Previous experiments indicate an association with Rab10 with amyloid generation and Alzheimer’s disease risk. Here we show an experimental approach used to characterize the effects of Rab10 and Sar1A GTPases in APP and amyloid processing. N2A cells were transient transfected with overexpression and shRNA plasmids and their effects in APP expression and Aβ40 and Aβ42 were measured using sandwich ELISA. We found that Rab10 expression does not affect APP production but significantly changes AB generation, particularly the toxic Aβ42. On the other hand, we found no significant effect of Sar1A expression on either APP or amyloid beta generation. These findings partially confirm the work done by Kauwe et al (2015) and provide preliminary evidence for two potential targets for protective effects in neurodegeneration.

Introduction

Alzheimer’s disease is the 6th most prevalent cause of death in US with an estimated 500,000 fatalities each year due to the disease (Alz.org retrieved 2014). A major neurological hallmark of the disease is the accumulation of senile plaques composed of amyloid beta molecules. The most accepted theory behind the pathogenesis of Alzheimer’s is known as the amyloid cascade hypothesis (Hardy and Higgins 1992, Ridge, Ebbert et al. 2013). Briefly, amyloid beta molecules are produced by cleavage of APP by beta and gamma secretases.
producing fragments ranging from 39-43 amino acids. Aβ42 is the peptide most susceptible to aggregation and it is the main component of the senile plaques (Thinakaran and Koo 2008, Tam, Seah et al. 2014). Accumulation of senile plaques in the brain disrupts neuronal connections and synapses, causing oxidative stress on neurons that form neurofibrillary tangles and apoptosis that lead to widespread neurodegeneration (Masters, Multhaup et al. 1985, Hung, Koo et al. 1992, Selkoe 2001, Ring, Weyer et al. 2007, Oh, Savonenko et al. 2009, Hashimoto and Matsuoka 2014). Interestingly, Aβ40 is by far the most common product from APP cleavage but does not aggregate into senile plaques. The change in Aβ40:42 ratio is generally considered to contribute to Aβ42 deposition and neurotoxicity.

Transmembrane APP undergoes the constitutive secretory pathway in the cell where it is post-translationally modified in the ER and TGN to be transported via anterograde transport to the cell membrane. APP is then internalized via endocytosis to the cell where it is processed by the recycling and autophagy machineries (Kins, Lauther et al. 2006, Nixon 2007, Thinakaran and Koo 2008, Thomas, Lelos et al. 2011, Haass, Kaether et al. 2012). Aβ production occurs mainly on the endosomal – lysosomal systems and has also been detected in compartments of the TGN (LeBlanc and Goodyer 1999, Haass, Kaether et al. 2012, Lu and Hong 2014). The majority of AB fragments are then secreted via exocytosis where Aβ42 aggregates in the extracellular space (Yu, Cuervo et al. 2005, Nixon 2007). However, small quantities of Aβ42 stay in the cell where they contribute in disrupting cellular pathways. Polymorphisms in proteins involved in the transporting and processing of APP have been associated with differential Aβ-42 deposition and a change in the Aβ40:42 ratio (Citron, Westaway et al. 1997, Cataldo, Peterhoff et al. 2000, Jankowsky, Fadale et al. 2004, Lee, Yu et al. 2010, Ridge, Ebbert et al. 2013). In this context, GTPases are small proteins involved in processes such as vesicle formation, transport, signal

Rab10

Rab10 is a member of the Ras superfamily of small GTPases. In general, Rab GTPases are involved in every step of the intracellular vesicle transport in the cell (Stenmark and Olkkonen 2001, Seabra, Mules et al. 2002). In addition to trafficking, some Rab proteins seem to play an important role in organelle shape and growth by associating with microtubules (Zerial and McBride 2001, Seabra, Mules et al. 2002, Grosshans, Ortiz et al. 2006). Rab proteins switch between an inactive (GDP-bound) cytosolic state and active (GTP-bound) membrane bound state. Among other cellular processes, Rab10 has two main roles: regulating ER dynamics and anterograde transport to the plasma membrane (Schuck, Gerl et al. 2007, English and Voeltz 2013, Liu, Xu et al. 2013, Deng, Lei et al. 2014). English et al (2013) showed that Rab10 localizes in the ER and associates with microtubules to direct ER growth. Mutant Rab10 show an altered ER morphology and reduced ability to fuse with adjacent ER tubules (English and Voeltz 2013). Independent studies show that Rab10 has an active role in transporting cell receptors to and from the TGN to the plasma membrane (Sano, Eguez et al. 2007, Schuck, Gerl et al. 2007, Lerner, McCoy et al. 2013, Deng, Lei et al. 2014). In neurons, Rab10 promotes axon development and neuronal polarization by transporting axonal receptors that interact with
extracellular growth factors (Wang, Liu et al. 2011, Liu, Xu et al. 2013, Xu, Deng et al. 2014). Additional evidence suggests a particular role of Rab10 in the endosome – recycling pathways (Babbey, Ahktar et al. 2006, Chen, Schweinsberg et al. 2006, Chen, Li et al. 2014). In a recent RNAi screen of all human Rab GTPases, Rab10 was found to decrease Aβ42 production and to affect sAPP-B levels in neural cells (Udayar, Buggia-Prevot et al. 2013).

**Sar1A**

Sar1A is another member of the Ras superfamily of small GTPases, involved in vesicle transport and membrane rigidity. In mammals, the two Sar1 isoforms (Sar1A and Sar1B) contain 89% homology. However, Sar1A is commonly referenced as Sar1 (Bi, Corpina et al. 2002, Loftus, Hsieh et al. 2012). Unlike Rab10, which localizes to vesicles throughout the secretory and recycling pathways, Sar1A is only localized to the ER, particularly in COPII vesicles. COPII transport is the first step of the secretory pathway for most secreted proteins starting with the ER (Wang and Wu 2012, Zanetti, Pahuja et al. 2012, Cutrona, Beznoussenko et al. 2013). Sar1A is a principal component of COPII vesicle formation and transport from the ER to the Golgi (Barlowe, d'Enfert et al. 1993, Kuge, Dascher et al. 1994, Long, Yamamoto et al. 2010). In its active GTP form, Sar1A triggers vesicle formation by binding with the ER membrane to recruit other COPII components. Depletion of Sar1A severely disrupts COPII assembly and transport from the ER to Golgi (Cutrona, Beznoussenko et al. 2013). Sar1A also decreases rigidity of the membrane that is bound, either by associating with lipid bilayers or with microtubules (Long, Yamamoto et al. 2010, Loftus, Hsieh et al. 2012, Riggs, Bergman et al. 2012, Fokin, Brodsky et al. 2014). This observation is supported by recent studies that link polymorphisms along Sar1A with the development of membrane structure deficiencies such as sickle cell and Anderson disease (Pepperkok, Lowe et al. 1998, Kumkhaek, Zhu et al. 2007, Kumkhaek, Taylor et al.
2008, Georges, Bonneau et al. 2011). Interestingly, both Rab10 and Sar1A have an important role in regulating anterograde transport and ER structure by interacting with microtubules. However, a specific link with neurodegenerative diseases has not been previously established.

Recent unpublished work by Kauwe (2015) and collaborators discovered two rare variants in Rab10 (rs1427874) and Sar1A (rs7653) that influence Alzheimer’s disease risk. Rab10 and Sar1A have not previously been associated with APP transport or amyloid production. We sought to characterize the effects of differential expression of Rab10 and Sar1A on APP and Aβ production in murine N2A/APP695 cells. We found that differential expression of Rab10 change Aβ42/Aβ40 ratio and influences Aβ42 production.

Results
Rab10 & Sar1A expression does not affect full-length APP

We sought to first test the effects of cellular Rab10 and Sar1A overexpression on full length APP. We transfected pCMV6-Rab10 and pCMV6-Sar1A overexpression plasmids in N2A/APP695 mouse cells. At 48 h after transfection, cells were harvested and protein expression was measured using 6E10 and 9E10 antibodies as described in methods section. Fig 1 shows that full-length APP expression is the same in GFP control lanes with Rab10 and Sar1A transfected cells. We further tested whether this observation by immunoblotting with three different gradients of protein aliquots (25 ug/uL, 37.5 ug/uL and 50 ug/uL). The results were the same as before, indicating that APP, Rab10 and Sar1A exposure increase proportionally to the total protein immunoblotted (not shown). Similarly, we measured APP expression of transfected N2A/APP695 cells with shRNA plasmids that produced a 62% knockdown of Rab10 and a 26% knockdown of Sar1A. Fig 1 also shows no visible change in full length APP between the
scrambled plasmid and the knockdown plasmids. Together, these results show that full length APP is not affected by overexpression of Rab10 and Sar1A.

Rab10 expression alters Aβ whereas Sar1A does not change Aβ levels.

Next, we sought to quantify the effect of varying expression of Rab10 and Sar1A on Aβ levels. A sandwich ELISA measuring Aβ40 and Aβ42 levels was performed from cell media obtained from transient transfected cells with overexpression and shRNA plasmids for Rab10 and Sar1A. We observed increased levels of Aβ42 levels and an increased Aβ40:42 ratio (Fig 2A, p = 0.0017) in cells overexpressing Rab10. Conversely, we observed the opposite effect in knockdown Rab10 cells, resulting in decreased Aβ42 levels and a decreased Aβ40:42 ratio (Fig 2B), however, the p-value was verily significant p = 0.048. On the other hand, no change in AB42 levels and AB40:42 ratios were observed in both overexpressed and knockdown Sar1A cells (Fig 3, p-value not shown). Together, these results show that Rab10 plays a role in APP processing but not on full-length expression whereas the expression effects of Sar1A were did not significantly influence APP expression and processing.

Discussion

Here we show that Rab10 expression significantly affects amyloid generation while maintaining stable levels of APP in N2A-695 cells. This is the first study that presents primary evidence linking Rab10 with APP and Aβ production following preliminary results from Udayar suggesting this effect on Rab10 knockdown (Udayar, Buggia-Prevot et al. 2013). A possible hypothesis surrounding these observations might be that Rab10 does not directly interact in the secretory pathway of APP. Even though Rab10 heavily regulates ER dynamics and morphology (English and Voeltz 2013), it could be possible that Rab10 does not directly transport APP in anterograde fashion to the axons and synapses. APP is prevalent in axonal cones and evidence
shows that one of the trophic functions of APP is to promote axon development (Kins, Lauther et al. 2006, Sosa, Bergman et al. 2013). Interestingly, Rab10 also promotes axon development and elongation by facilitating the transport of membrane receptors such as GLUT4 (Liu, Xu et al. 2013, Sano and Eguez 2007). Nascent APP molecules could be a specific cargo to another Rab GTPases such as Rab3 and Rab6, which have been shown to transport APP to axons and neurites (McConlogue, Castellano et al. 1996, Szodorai, Kuan et al. 2009).

The significant observation of Rab10 in amyloid beta generation suggests a downstream effect in the APP processing pathway. A second hypothesis explaining the results of this study is that Rab10 participates in AB processing following APP endocytosis. Rab10 also localizes to the recycling and sorting endosomes and mediates vesicle transport from basolateral endosomes to early and late endosomes (Babbey, Ahktar et al. 2006, Chen, Schweinsberg et al. 2006). This transport is part of endosomal pathways where proteins are taken to and from the plasma membrane to be degraded by autophagy or recycled back to the plasma membrane. Expression changes of Rab10 might accelerate or suppress transport between endosomes as part of recycling and autophagy processes of APP. Future experiments specifically tracking endosomal transport will prove useful to determine the precise role of Rab10 in amyloid beta transport.

Conversely, we did not see a significant effect of Sar1A expression in both APP expression and amyloid processing. Since Sar1A only localizes to ER compartments and vesicles leading to the Golgi, it is possible that similar to Rab10, Sar1A is not directly involved in APP anterograde transport (Kuge, Dascher et al. 1994, Cutrona, Beznoussenko et al. 2013). Even though a direct role of Sar1A in amyloid beta production seems unlikely due to its localization in the cell, it should not be completely discarded. Our preliminary results support the former by not showing any interesting effects on Aβ. However, in N2A cells transfected with shRNA plasmids,
we only obtained a 26% total knockdown of Sar1A. We considered this knockdown percentage to be enough to obtain observable conclusions regarding Sar1A role in amyloid beta generation. Another factor that might influence in our results was the number of replicates used. We only used three replicates in our sandwich ELISA to evaluate total Aβ42 and Aβ40 production. It is possible that by increasing the number of replicates would and knockdown conditions by the use of other RNAi methods could influence these results. Further experiments including these recommendations and other cell imaging methods could be used to confirm these results.

In closing, these results support previous observations where silencing of Rab10 decreases only Aβ (Udayar, Buggia-Prevot et al. 2013) and show that the opposite also applies in higher Aβ generation. In addition, these results offer preliminary evidence supporting results by Kauwe et al (2015) where polymorphisms on the 3’UTR of Rab10 affect Alzheimer’s disease risk. The next step will be to repeat the methods used in this study in N2A-695 cells expressing rs14278789 (Rab10) to further confirm the functional effect of this genetic variation in Alzheimer’s.

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Methods

Plasmids

The plasmids used for this study were the following: pCMV6-Rab10 (Origene # RC201464), pCMV6-Sar1A (Origene # RC201450) for the overexpression experiments. pGFP-V-RS-Rab10 shRNA (Origene #TG501823) and pGFP-V-RS-Sar1A shRNA (Origene #TG501970). Four shRNA versions per gene were tested to obtain greater knockdown levels.

Cell culture

Mouse neuroblastoma cells (N2A/APP695) expressing human APP-695 isoform were kindly given by Celeste Karch, Ph.D. N2A/APP695 are a mouse neuroblastoma line that expresses human APP695 isoform and is commonly used in functional APP studies (Thinakaran, Teplow et al. 1996, Rajendran, Honsho et al. 2006, Wang, Wang et al. 2006). N2A/APP695 cells were plated and grown in Dulbecco’s modified eagle medium (DMEM) and Opti-MEM (1:1) supplemented with 1% L-glutamine, 5% FBS and 1% anti-mycotic solution. Cells were grown between 80% to 90% confluence for posterior analyses. Upon confluency, cells were transiently transfected using Lipofectamine 2000 (Invitrogen). Culture media was changed 24 h after transfection. Following 48 h after transfection, cell media was collected and centrifuged for 10 minutes at 4°C and protease inhibitor was added for peptide preservation. Cell pellets were collected, lysed and centrifuged with protease inhibitor to collect total protein. Protein concentration was measured using a BCA method in preparation for immunoblotting.

Transfection and reporter gene assays

Functional assays of reporter gene constructs were performed by transient transfection of N2A/APP695 cells using Lipofectamine 2000 reagent (Life Technologies). Cells were allowed to confluence between 85 to 90% and transfected with and pCDNA control vector to bring total
DNA concentration 1.0 μg. Cells were growth for 48 h; following this time, media was collected for ELISA assays. Cells were washed and RNA was isolated from cells for RT-qPCR or lysed to assess protein concentration by Western blot analysis.

RNA isolation and RT-PCR analysis

In order to assess greatest knockdown, total RNA was isolated from N2A/APP695 cells after transfection with four plasmids containing specific shRNA for knockdown of either Rab10 or Sar1A genes. RNA was extracted from cells 48 h following transfection using RNeasy (Qiagen) following manufacturer protocol. RNA was converted to cDA using High-capacity cDNA reverse transcription (ABI). Following RT-PCR, Taqman real time PCR assays were used to observe the expression of Rab10 (Mm00489481_m1) and Sar1A (Mm01150424_m1) from ABI technologies. Total gene expression was quantified in triplicates using an ABI-7900 Real-Time PCR system. A housekeeping gene GAPDH was used for normalizing expression values using the CT method.

Protein analysis

N2A/APP695 cell lysates were used to assess Rab10, Sar1A and APP695 protein expression by SDS-PAGE and Western blot analysis with primary mouse 9E10 or 6E10 polyclonal antibody and goat-anti mouse polyclonal as secondary antibody. Briefly, equivalent amounts of total protein were evaluated by SDS-PAGE, blocked with 5% nonfat milk, and exposed to the primary antibody diluted at 1:5000 at 4°C overnight. Exposure to the secondary antibody was carried at 1:2000 for 2 h. Blots were exposed with enhanced chemiluminescence (Lumigen TMA-6).
Enzyme-linked immunosorbent assay

The levels of Aβ40 and Aβ42 were measured from collected cell culture media by sandwich ELISA as described by the manufacturer (Invitrogen). ELISA values were obtained (pg/mL) and corrected for total intracellular protein (ug/mL) based on BCA measurements.

Statistical analysis

Values are expressed as mean ± SD obtained from at least three separate experiments in each group. Data were assessed by one-way analysis of variance (ANOVA). When ANOVA indicated significant differences, the Student's t-test was used with Bonferroni correction for multiple comparisons. Results presented are representative and those with P values <0.05 were considered significant.
Figure 2: Expression of Rab10 and Sar1A does not affect full length APP. This figure shows an immunoblot using the following legends: lanes 1- GFP, lane 2 – GFP + Rab10, lane 3-GFP+Sar1A, lane 4 – Scrambled shRNA, lane 5 – scrambled + Rab10 shRNA, lane 6 – scrambled + Sar1A.
Figure 3: Rab10 expression influences Aβ42 and Aβ42/Aβ40 ratio. Figure 3A shows the results from a sandwich ELISA in N2A-695 cells transfected with overexpression and shRNA plasmids. Experiments and their respective controls (GFP for overexpression and Scrambled for shRNA) are depicted on Aβ42 (left), Aβ40 (middle) and Aβ42/Aβ40 ratio (right) panels. Each bar represents the results from six replicates. Figure 2B shows the effects of Rab10 for Aβ42/Aβ40 ratio only. Each bar represents the results from 10 replicates.
Figure 4: Sar1A expression does not influence Aβ42 and Aβ42/Aβ40 ratio. This figure shows the results from the sandwich ELISA in N2A-695 cells transfected with overexpression and shRNA plasmids. Experiments and their respective controls (GFP for overexpression and Scrambled for shRNA) are depicted on Aβ42 (left), Aβ40 (middle) and Aβ42/Aβ40 ratio (right) panels. Each bar represents the results from three replicates.
References:


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