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Monitoring "De Novo" APP Synthesis by Taking Advantage of the Reversible Effect of Cycloheximide

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By blocking "de novo" protein synthesis using cycloheximide, we previously described a dynamic model system to monitor turnover of a specific population of the Alzheimer's amyloid precursor protein. Here we show that cycloheximide is nontoxic and its effect is reversible, allowing protein synthesis to reinitiate. Upon cycloheximide removal protein synthesis restarted and by 1 hour the amyloid precursor protein–green fluorescent protein could be clearly detected, permitting the monitoring of amyloid precursor protein anterograde transport, particularly the secretory pathway. The consensus NPTY motif in amyloid precursor protein, typically associated with endocytosis, was mutated to NPTF or NPTE to mimic a constitutively dephosphorylated or phosphorylated residue, respectively. Our data reveal that disruption of this motif affects amyloid precursor protein endocytosis, as shown previously, but also its incorporation into trans-Golgi network budding vesicles. Thus, cycloheximide can be a useful tool to study both anterograde and retrograde "in vivo" protein transport.

Keywords: APP-GFP; phosphorylation; "de novo" pro-tein synthesis; monitoring; anterograde transport

Introduction

Monitoring intracellular targeting and processing of the Alzheimer's amyloid precursor protein (APP) is a challenging task, given the many pathways via which it is processed and the various stages at which protein cleavage can occur. Among its many functions, APP has been proposed to be a signal transduction molecule. This places it among a class of proteins that are cleaved to produce fragments that can mediate a process of regulated intramembrane proteolysis (RIP), with an important role in signaling cascades.[1] Amyloid precursor protein routing in the cell entails export from the trans-Golgi network (TGN), via the secretory pathway, followed by insertion into the cell membrane. Subsequently, APP is endocytosed and can suffer several fates typical of this pathway. Once endocytosed, the toxic fragment Abeta (amyloid beta) can also be produced, an event of specific pathological interest. Both the anterograde and retrograde transport of APP are highly regulated events, with protein phosphorylation likely to play a significant modulatory role. For example, data from our laboratory (da Cruz e Silva et al, in preparation) and previous reports indicate that TGN budding is dependent on protein kinase C, as is the production of Abeta.[2,3]

Although APP is itself a phosphoprotein, few events regarding APP processing have been shown to be governed by specific phosphorylations of APP itself. Thr^{668}, within the ^{667}VTPEER^{672} functional domain, has been shown to be important in determining binding of FE65 to APP in a phosphorylation state–dependent manner.[4] Another important domain in the C-terminus of APP is ^{682} YENPTY^{687}, which comprises a typical internalization signal (NPTY) for membrane-associated receptor proteins.[5-7] Both Tyr^{682} and Tyr^{687} are phosphorylated in vivo. Amyloid precursor protein is tyrosine phosphorylated in cells

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expressing a constitutively active form of the Abl protooncogene,[8] a nonreceptor tyrosine kinase similar to c-Src, which can form a stable complex with APP and FE65. Abl-dependent Tyr^{682} phosphorylation appears to involve the nerve growth factor receptor TrkA, to mediate APP membrane internalization and to result in reduced γ-secretase APP processing.[6,7,9] This phosphorylation may also mediate protein–protein interactions.[8,9] Consequently, attention was drawn away from Tyr^{687}, although this residue was reported to be phosphorylated in brain samples from patients with Alzheimer's disease (AD).[10] Furthermore, this residue is important in mediating endocytosis and we have previously shown that the Y687E mutant, mimicking constitutive phosphorylation, produces significantly less Abeta.[11,12]

To determine the role of Tyr^{687} phosphorylation in APP processing, a model system was developed taking advantage of cycloheximide (CHX) that blocks "de novo" protein synthesis.[13] Amyloid precursor protein was tagged with green fluorescent protein (GFP) and low levels of transfection permitted monitoring its turnover as it disappeared from the cell. Amyloid precursor protein turnover is virtually complete 5 hours after CHX addition. Hence, we were able to address retrograde processing of APP. To analyze anterograde transport, we expanded the model by removing CHX from the cells in culture, followed by "de novo" APP synthesis. We were able to determine that CHX, as used in this context, is not detrimental to the cell and that its effects are reversible. In essence, removal of CHX permits "de novo" protein synthesis to resume and subsequently one is able to monitor intracellular protein targeting. The reversibility of this model is discussed, as is its application to unraveling APP trafficking, particularly its anterograde transport.

Materials and Methods

Production of APP-GFP Fusion Proteins

The Tyr^{687} residue of APP_{695} was mutated by site-directed mutagenesis to either glutamate or phenylalanine to mimic the phosphorylated and dephosphorylated state of the protein, respectively.[13,14] Both phosphorylation-mimicking mutants and wild-type (Wt) APP were fused with GFP by inserting into the pEGFP-N1 mammalian expression vector (Clontech), as previously described.[11,12]

Maintenance and Transfection of Cell Cultures

Monkey kidney COS-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 3.7 g/L NaHCO3 at 37°C and 5% CO_2 until 90% confluent.[15] Transient transfections were performed at 80% to 90% cell confluency using LipofectAMINE 2000, a cationic lipid transporter (Invitrogen Life Technologies Alfagene, Portugal). After 8 hours, cells were divided into 6-well plates with coverslips pretreated with 100 μg/mL polyornithine (Sigma, Portugal), and left to recover for 4 hours.

Tracking "de novo" Synthesis of APP Fusion Constructs

Transfected cells were then incubated in serum-free DMEM supplemented with 50 μg/mL CHX (Sigma) for 5 hours. Cycloheximide-containing medium was removed, cells were washed 2 times with phosphate buffered saline (PBS), and then incubated at 37°C in DMEM supplemented with 10% fetal bovine serum for 1, 2, 3, 4, and 5 hours. At each time point, the cells were washed 3 times with PBS and fixed in 4% paraformaldehyde. Following cell fixation and subsequent coverslip preparation, the subcellular localization of APP was directly observed by fluorescence microscopy using an Olympus IX-81 inverted epifluorescence microscope. Cells were analyzed for the presence or absence of fluorescence in specific subcellular organelles. Subcellular structures were confirmed using specific markers. Calnexin was used as an endoplasmic reticulum (ER) marker (the 2 hours 30 minutes time point is shown as an example).

Results

Our previously described model allowed for the monitoring of phosphorylation-dependent APP trafficking and processing.[13] Briefly, first we mimic the phosphorylation and dephosphorylation of APP target residues, allowing the evaluation of the functional impact of phosphorylation of those residues. Second, the constructs were fused in-frame with a reporter gene (*GFP*) in a mammalian expression vector and used to achieve low-level transient transfections. Finally, the addition of CHX was particularly useful because it dramatically reduces the background of APP-GFP molecules being continuously synthesized. In essence, "de novo" synthesis of APP-GFP was blocked and previously synthesized

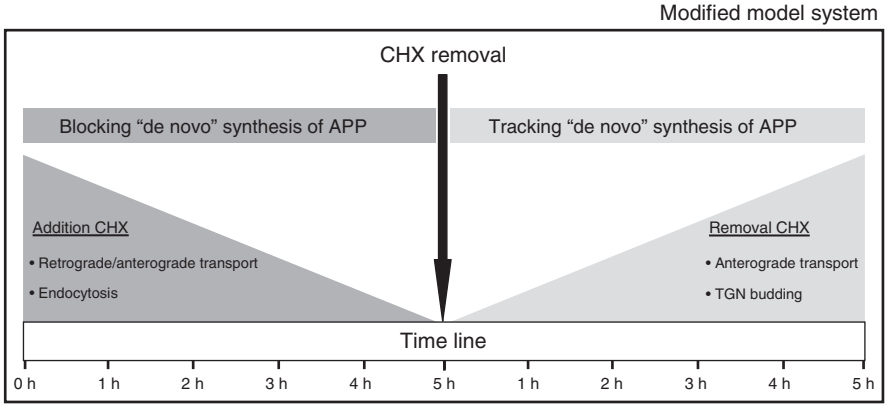

Figure 1. Modified model system. Schematic representation of the modified model system. The vertical arrow indicates the time point when cycloheximide (CHX) was removed. APP indicates amyloid precursor protein; TGN, trans-Golgi network.

molecules were detected and followed over a period of time (Figure 1). After 5 hours of incubation with CHX, APP-GFP turnover was complete and inhibited from further processing and fluorescence was absent in cells (not visible/black by epifluorescence), because of the absence of "de novo" protein synthesis. Thus, by using this approach, we previously showed the importance of Tyr^{687} in APP-GFP turnover rate, intracellular trafficking, processing, and particularly endocytosis and Abeta production.[11,12] Hence, the retrograde transport of APP was monitored. Removal of CHX, on the other hand, permits the study of the anterograde transport of APP (Figure 1).

"De novo" protein synthesis was blocked by adding 50 μg/mL CHX for 5 hours. After that period, the CHX was removed and fresh medium without CHX was added, and the cells were allowed to resume "de novo" protein synthesis (Figures 1 and 2). Thus, transiently transfected COS-7 cells were observed at 1, 2, 3, 4, and 5 hours following CHX removal and analyzed for the presence or absence of fluorescence in specific subcellular organelles. At time 0 hour, no fluorescence was observed. A representative example at each time point both for Wt-APP and for the phosphorylation-mimicking mutants (Y687E and Y687F) is presented in Figure 2.

About 1 hour after CHX removal, we observed that the APP-GFP is already being abundantly "de novo" synthesized. Indeed, it is already detected in the ER and Golgi for Wt as well as for the 2 APP phosphomutants (Figure 2, 1 hour). At the 2-hour time point, the Wt-APP was already incorporated into the cytoplasmic vesicles, which was even more evident by 3 hours and reached the plasma membrane at that time point (Figure 2). The nucleus is notably fluorescent 1 hour later (Figure 2, 4 hours) and is clearer by the end of the experiment (Figure 2, 5 hours). The Y687E-APP mutant was also abundantly expressed after 1 hour of CHX removal. The protein, similar to Wt, could be detected in the ER and Golgi (Figure 2, 1 hour). However, some differences between the Wt-APP and the Y687E-APP mutant were already evident 1 hour later (2 hours), because few cytoplasmic vesicles are visible with this mutant. Additionally, the plasma membrane is already detected in some cells (Figure 2, 2 hours). At the 3-hour time point, a few cytoplasmic vesicles were visible with the Y687E-APP mutant when compared with Wt-APP, but the plasma membrane seems to be more intensely marked for the phosphorylation-mimicking mutant (Y687E-APP). During the last 2 time points, a few cytoplasmic vesicles as well as the nucleus were well visible (Figure 2, 4 and 5 hours). Contrasting effects were obtained when monitoring "de novo" protein synthesis of the dephosphorylation-mimicking mutant (Y687F-APP). As with Wt, 1 hour after CHX removal, localization in ER and Golgi was clearly evident. Moreover, the Y687F-APP protein trafficking was very similar to Wt-APP at all time points. There was a slight difference between them at the 2-hour time point, because the Y687F-APP mutant was already visible in the plasma membrane of some cells and generally appeared to possess more vesicles. Hence, the two phosphorylation state–mimicking mutants behaved differently and exhibited different distribution patterns.

Co-localization studies were validated with several markers, in particular calnexin that labels the ER. An example of co-localization with the

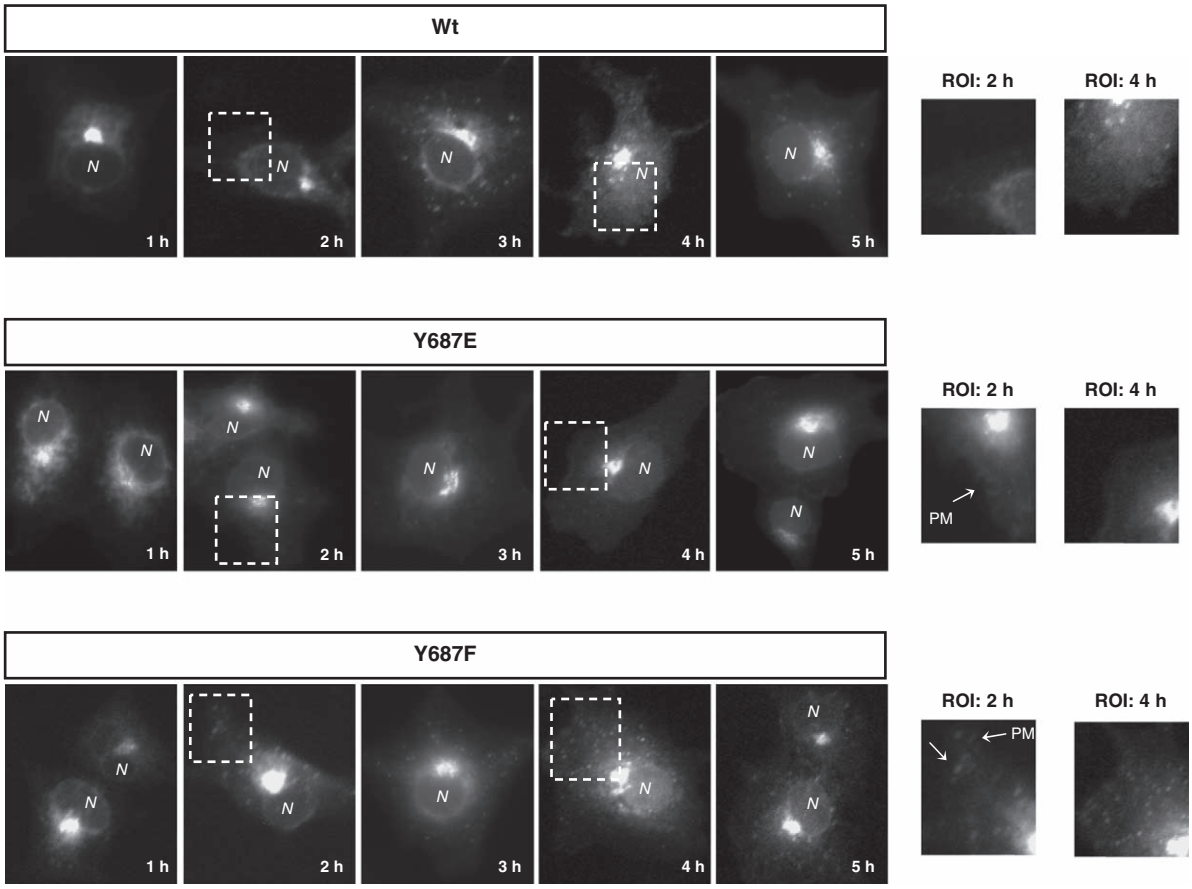

Figure 2. Wild-type and mutant (Y687E and Y687F) APP-GFP intracellular protein trafficking. Representative epifluorescence microphotographs of transiently transfected COS-7 cells at specific time points after CHX removal. APP-GFP indicates amyloid precursor protein–green fluorescent protein; CHX, cycloheximide; ROI, region of interest; N, nucleus; PM, plasma membrane; Wt, wild type.

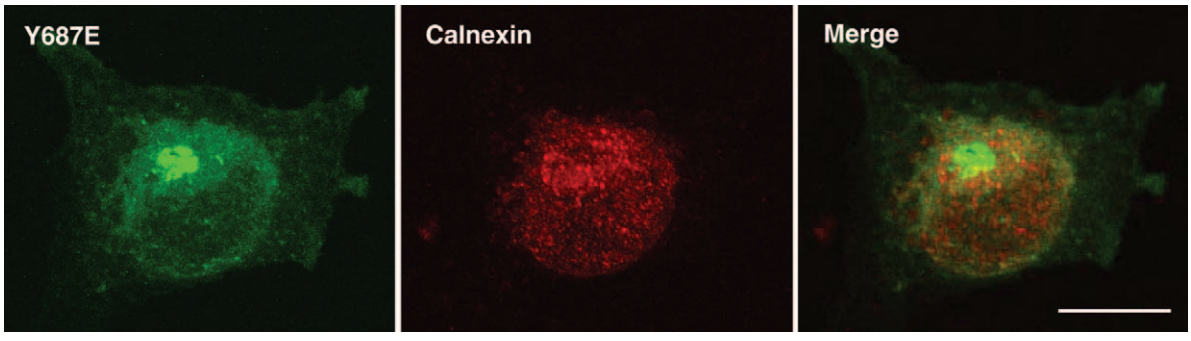

Figure 3. Y687E-APP-GFP co-localization with calnexin. COS-7 cells transfected with the phosphorylation-mimicking mutant (Y687E-APP) were fixed and processed for immunocytochemical analysis using a specific antibody to detect endogenous calnexin (an endoplasmic reticulum, ER, marker). Bar = 10 μm. APP-GFP indicates amyloid precursor protein–green fluorescent protein.

Y687E-APP phosphorylation-mimicking mutant is presented in Figure 3. As mentioned above, the Y687E-APP mutant does not produce clearly visible vesicles. In fact, it exhibits strong localization to the ER throughout the experiment. Co-localizations were carried out at different time points, an example is shown at 2 hours 30 minutes. In conclusion, the use of CHX is reversible and permits monitoring anterograde APP transport to unravel the importance of residue Y687 in this process.

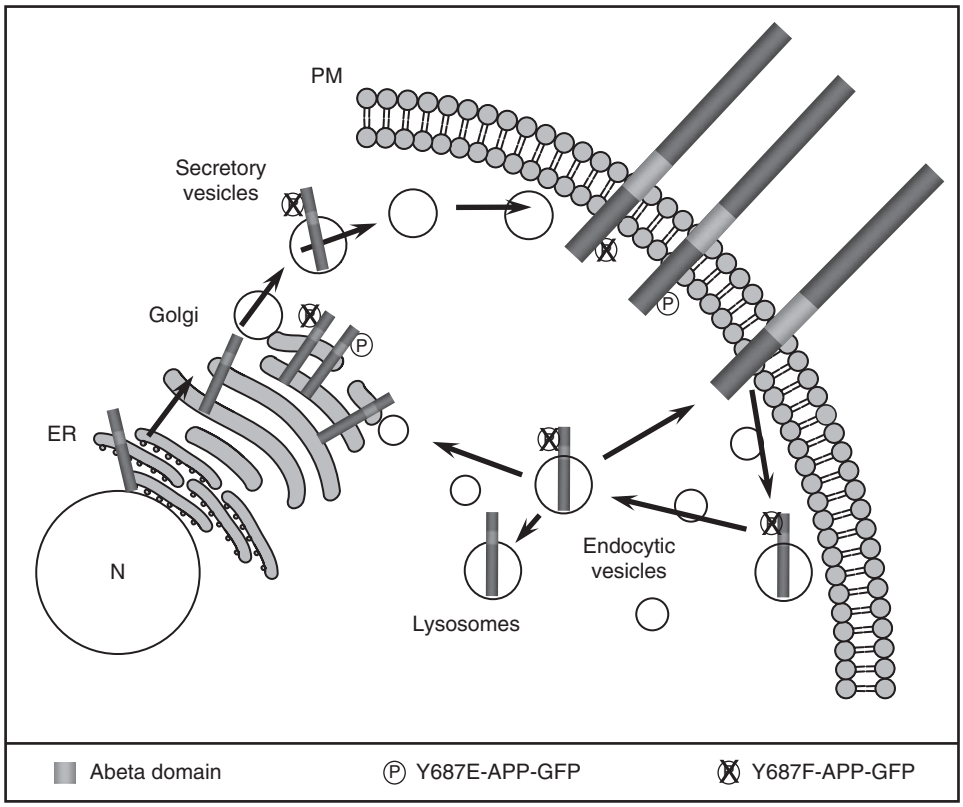

Figure 4. Tyrosine 687 phosphorylation−/dephosphorylation−dependent amyloid precursor protein subcellular targeting. APP-GFP indicates amyloid precursor protein–green fluorescent protein; N, nucleus; ER, endoplasmic reticulum; PM, plasma membrane.

Discussion

Addressing a specific population of proteins is an important approach to study protein turnover. Methionine labeling is often used and certainly provides useful data. Here we present an alternative method that is nonradioactive, reversible and permits the study of anterograde and retrograde processing. In previous experiments,[11,12] we used CHX, thus inhibiting "de novo" protein synthesis and enabling protein turnover to be monitored, in particular endocytosis and subsequent targeting, such as to the TGN or the nucleus (Vieira et al., in preparation). By 5 hours, the fluorescent APP protein was absent and largely undetectable from the cell.[13] Thus, at this time point we removed the CHX, allowing "de novo" synthesis of APP to reinitiate. The CHX inhibition of protein synthesis was reversible and cells were able to resume "de novo" protein synthesis. The Y687E-APP mutant consistently failed to be incorporated into cytoplasmic vesicles under both conditions (in the presence and absence of CHX). Other aspects of transport of this mutant were also confirmed. Namely, the Y687E-APP mutant was still transported to the cell membrane, even in the absence of visible vesicles. Delivery of the Y687E-APP mutant protein to the cell membrane might be supported by the ER, consistent with previous findings by other authors.[16]

Considering the data thus far obtained with the Tyr^{687} mutants, it is possible to propose a model of phosphorylation-dependent APP targeting in the cell (Figure 4). Central to the development of this model was the observation that Y687E-APP and Y687F-APP exhibited contrasting behaviors, particularly with respect to TGN budding and incorporation into visible vesicles, as described above, and endocytosis.[11,12] In fact, Y687F-APP apparently incorporates more efficiently into vesicular structures than Wt-APP. The fact that the mutants behave so differently supports our hypothesis that phosphorylation of this key residue may in fact be a central regulatory event. In the model shown in Figure 4, we indicate subcellular structures abundant in both the dephosphorylation-mimicking and phosphorylation-mimicking mutants, and those where only the dephosphorylation-mimicking mutant was preferentially detected. Hence, it would appear that APP needs to be dephosphorylated at Tyr^{687} to be incorporated

into vesicular structures, both at the TGN and during endocytosis (Figure 4). These are two critical sites for Abeta production,[6,17-19] and this may explain why the dephosphorylation-mimicking mutant produces more Abeta, as we previously described.[12] In summary, and taking into account our previous findings, static APP populations in the plasma membrane and the TGN can de phosphorylated or dephosphorylated; whereas the mobile APP population associated with visible vesicles due to endocytosis or TGN budding seems to be more abundant in the dephosphorylation-mimicking APP at Tyr^{687}. APP export is likely to be mediated by multimeric complexes, the formation of which is probably being regulated by phosphorylation. Such an example has already been described for FE65 binding to APP, which is modulated by phosphorylation of APP at Thr^{668}.[4]

One can conclude that the CHX-based experimental model is useful to study both retrograde and anterograde processing, and that for APP the NPTY domain is important in both instances. The exposure to CHX appears to leave the cell machinery intact, such that the mutants behave in an identical manner both before and after the removal of the drug. That is, Y687E-APP failed to be incorporated into vesicles, whereas Y687F-APP was incorporated more efficiently than the Wt-APP. Another advantage of this method, particularly in comparison to pulse chase methods, is that it allows direct real-time in vivo monitoring of the GFP-tagged proteins. This is useful for AD-related studies addressing the use of signal transduction–based therapeutics, where phosphorylation of a single site on a given protein can result in a cascade of events and affect other proteins directly or indirectly. By visualizing APP directly, and given all other methodologies already evolved, such as measuring Abeta levels, the effects on APP metabolism can be easily evaluated.

We had previously shown that disrupting the NPTY domain dramatically impaired endocytosis of APP and affected Abeta production.[12] Amyloid precursor protein turnover was monitored in the absence of "de novo" protein synthesis by adding CHX to transfected cells in culture. The work presented here modified the previously described model system[13] by removing the CHX, thus permitting the "de novo" protein synthesis to resume. Consequently, all the cellular machinery should become available to permit "normal" protein targeting. Notably, under these conditions the Y687E-APP mutant still fails to be incorporated into visible vesicular structures (Figure 2). Overall, the method described for monitoring intracellular protein targeting has several advantages and can be applied to the study of proteins other than APP.

Acknowledgments

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References

1. Weihofen A, Martoglio B. Intramembrane-cleaving proteases: controlled liberation of proteins and bioactive peptides. *Trends Cell Biol*. 2003;13:71-78.
2. Xu H, Greengard P, Gandy S. Regulated formation of Golgi secretory vesicles containing Alzheimer beta-amyloid precursor protein. *J Biol Chem*. 1995;270:23243-23245.
3. Buxbaum JD, Koo EH, Greengard P. Protein phosphorylation inhibits production of Alzheimer amyloid beta/A4 peptide. *Proc Natl Acad Sci U S A*. 1993;90:9195-9198.
4. Ando K, Iijima KI, Elliott JI, Kirino Y, Suzuki T. Phosphorylation-dependent regulation of the interaction of amyloid precursor protein with Fe65 affects the production of beta-amyloid. *J Biol Chem*. 2001;276:40353-40361.
5. Chen WJ, Goldstein JL, Brown MS. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J Biol Chem*. 1990;265:3116-3123.
6. Koo EH, Squazzo SL. Evidence that production and release of amyloid beta-protein involves the endocytic pathway. *J Biol Chem*. 1994;269:17386-17389.
7. Lai A, Sisodia SS, Trowbridge IS. Characterization of sorting signals in the beta-amyloid precursor protein cytoplasmic domain. *J Biol Chem*. 1995;270:3565-3573.
8. Zambrano N, Bruni P, Minopoli G, et al. The beta-amyloid precursor protein APP is tyrosine-phosphorylated in cells expressing a constitutively active form of the Abl protoncogene. *J Biol Chem*. 2001;276:19787-19792.
9. Tarr PE, Roncarati R, Pelicci G, Pelicci PG, D'Adamio L. Tyrosine phosphorylation of the beta-amyloid precursor protein cytoplasmic tail promotes interaction with Shc. *J Biol Chem*. 2002;277:16798-16804.
10. Lee MS, Kao SC, Lemere CA, et al. APP processing is regulated by cytoplasmic phosphorylation. *J Cell Biol*. 2003;163:83-95.
11. Rebelo S, Vieira SI, Esselmann H, Wiltfang J, da Cruz e Silva EF, da Cruz e Silva OA. Tyrosine 687

phosphorylated Alzheimer's amyloid precursor protein is retained intracellularly and exhibits a decreased turnover rate. *Neurodegener Dis*. 2007;4:78-87.
12. Rebelo S, Vieira SI, Esselmann H, Wiltfang J, da Cruz e Silva EF, da Cruz e Silva OAB. Try687 dependent APP endocytosis and Abeta production. *J Mol Neurosci*. 2007;32:1-8.
13. da Cruz e Silva O, Vieira SI, Rebelo S, da Cruz e Silva EF. A model system to study intracellular trafficking and processing of the Alzheimer's amyloid precursor protein. *Neurodegener Dis*. 2004;1:196-204.
14. da Cruz e Silva OA, Iverfeldt K, Oltersdorf T, et al. Regulated cleavage of Alzheimer beta-amyloid precursor protein in the absence of the cytoplasmic tail. *Neuroscience*. 1993;57:873-877.
15. Rebelo S, Henriques AG, da Cruz e Silva EF, da Cruz e Silva OA. Effect of cell density on intracellular levels of the Alzheimer's amyloid precursor protein. *J Neurosci Res*. 2004;76:406-414.
16. Shin RW, Saido TC, Maeda M, Kitamoto T. Novel alpha-secretase cleavage of Alzheimer's amyloid beta precursor protein in the endoplasmic reticulum of COS7 cells. *Neurosci Lett*. 2005;376:14-19.
17. Selkoe DJ, Yamazaki T, Citron M, et al. The role of APP processing and trafficking pathways in the formation of amyloid beta-protein. *Ann N Y Acad Sci*. 1996;777:57-64.
18. Greenfield JP, Tsai J, Gouras GK, et al. Endoplasmic reticulum and trans-Golgi network generate distinct populations of Alzheimer beta-amyloid peptides. *Proc Natl Acad Sci U S A*. 1999;96:742-747.
19. Perez RG, Soriano S, Hayes JD, et al. Mutagenesis identifies new signals for beta-amyloid precursor protein endocytosis, turnover, and the generation of secreted fragments, including Abeta42. *J Biol Chem*. 1999;274:18851-18856.