The EMBO Journal vol.14 no.20 pp.4932–4938, 1995

Production of intracellular amyloid-containing fragments in hippocampal neurons expressing human amyloid precursor protein and protection against amyloidogenesis by subtle amino acid substitutions in the rodent sequence

Bart De Strooper[1,2], Mikael Simons[2,3], Gerd Multhaup[3], Fred Van Leuven[1], Konrad Beyreuther[3] and Carlos G.Dotti[2,4]

[1]Center for Human Genetics, Herestraat 46, 3000 Leuven, Belgium, [2]Cell Biology Program, European Molecular Biology Laboratory, Meyerhofstrasse 1, Postfach 10.2209, D-69012 Heidelberg and [3]Center for Molecular Biology Heidelberg (ZMBH), University of Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany

[4]Corresponding author

A distinguishing feature of Alzheimer's disease (AD) is the deposition of amyloid plaques in brain parenchyma. These plaques arise by the abnormal accumulation of βA4, a proteolytic fragment of amyloid precursor protein (APP). Despite the fact that neurons are dramatically affected in the course of the disease, little is known about the neuronal processing of APP. To address this question we have expressed in fully mature, synaptically active rat hippocampal neurons, the neuronal form of human APP (APP695), two mutant forms of human APP associated with AD, and the mouse form of APP (a species known not to develop amyloid plaques). Protein expression was achieved via the Semliki Forest Virus system. Expression of wild type human APP695 resulted in the secretion of βA4-amyloid peptide and the intracellular accumulation of potential amyloidogenic and non-amyloidogenic fragments. The relative amount of amyloid-containing fragments increased dramatically during expression of the clinical mutants, while it decreased strongly when the mouse form of APP was expressed. 'Humanizing' the rodent APP sequence by introducing three mutations in the βA4-region also led to increased production of amyloid peptide to levels similar to those obtained with human APP. The single Gly601 to Arg substitution alone was sufficient to triple the ratio of βA4-peptide to non-amyloidogenic p3-peptide. Due to the capacity of these cells to secrete and accumulate intracellular amyloid fragments, we hypothesize that in the pathogenesis of AD there is a positive feed-back loop where neurons are both producers and victims of amyloid, leading to neuronal degeneration and dementia. Moreover, our observations may explain why aging rodents (in contrast to other species such as human, polar bear, dog and monkey), never develop amyloid deposits.

Keywords: amyloid precursor protein/Alzheimer's disease/hippocampal neurons

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by the presence of extracellular amyloid plaques and intracellular fibrillary tangles. The amyloid plaques contain βA4 amyloid peptide, a cleavage product of amyloid precursor protein (APP). APP is an integral membrane protein with ubiquitous expression and unknown function (Selkoe, 1994a). Several alternatively spliced forms have been detected. One of these, APP695, is the dominant species in neurons (Golde *et al.*, 1992; Sandbrink *et al.*, 1994). The βA4-peptide sequence is located at the junction between the integral membrane domain and the extracellular domain of APP and is 39–43 amino acids long (Kang *et al.*, 1987). Genetic and cell biological evidence indicates that point mutations in the APP gene cause familial AD, although other proteins are also involved in the pathogenesis of familial and sporadic AD (Roses, 1994; Selkoe, 1994b). The cellular metabolism of APP is complicated (Haass and Selkoe, 1993). In non-neuronal and neuroblastoma cell lines, APP is proteolytically processed by several (not yet identified) secretases, which are located in different subcellular compartments (Weidemann *et al.*, 1989; Haass and Selkoe, 1993). α-secretase exerts its activity in a post-Golgi compartment on N- and O-glycosylated, phosphorylated and sulphated APP, and cleaves APP in the amyloid sequence itself, preventing further amyloid formation (Esh *et al.*, 1990; Sambamurti *et al.*, 1992; Sisodia, 1992; De Strooper *et al.*, 1993; Kuentzel *et al.*, 1993). β-secretase (probably active in an early endosome) cleaves APP at the N-terminus of the βA4-peptide sequence, yielding a potential amyloidogenic C-terminal fragment (Koo and Squazzo, 1994; Citron *et al.*, 1995). γ-secretase, located in an unknown subcellular compartment, releases βA4-peptide at its C-terminus. βA4-peptide and a non-amyloidogenic p3-fragment, released by combined α- and γ-secretase cleavage (Buxbaum *et al.*, 1993; Hung *et al.*, 1993; Näslund *et al.*, 1994), are secreted by all cells investigated until now (Haass *et al.*, 1992; Shoji *et al.*, 1992). The cleavages in APP are thus all clustered around the βA4 amyloid sequence and yield APP_s (90–120 kDa), cellular C-terminal fragments (10–14 kDa) and small secreted peptides (3–4 kDa). These proteolytic products are secreted in a strictly polarized fashion in epithelial MDCK cells (Haass *et al.*, 1994b, 1995; Lo *et al.*, 1994; De Strooper *et al.*, 1995). The clinical APP mutations known to cause familial AD affect one or more of the secretase cleavages, and lead to increased amyloid peptide secretion or to the production of a longer, more amyloidogenic form of the βA4-peptide (Citron *et al.*, 1992; Cai *et al.*, 1993; Haass *et al.*, 1994a; Suzuki *et al.*, 1994). In addition, the Swedish APP mutation causes partial missorting of APP_s to the apical compartment in MDCK cells (Lo *et al.*, 1994; De Strooper *et al.*, 1995).

Neurons are the most dramatically affected cells in the brain of AD patients and clues about the pathogenesis of

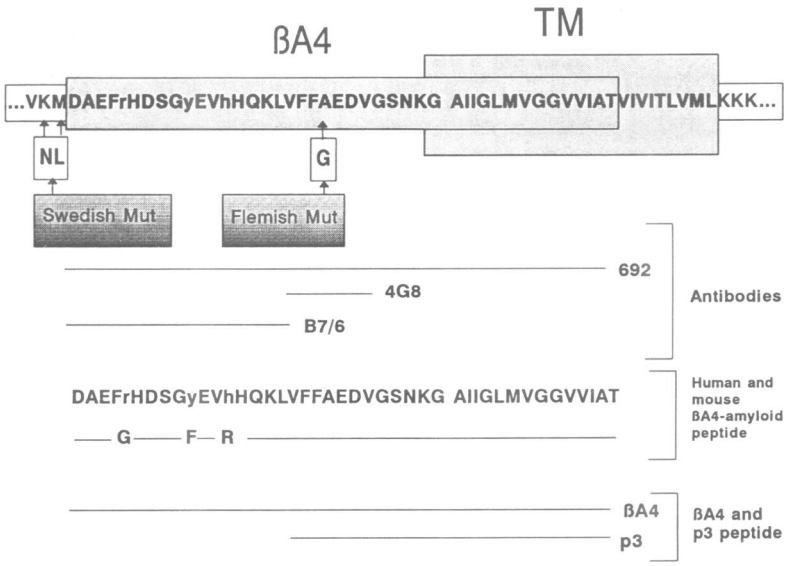

Fig. 1. Schematic representation of the human amyloid sequence (βA4) (Kang *et al.*, 1987) at the border of the transmembrane (TM) and extracellular domain of APP. The Swedish and Flemish point mutations (Hendriks *et al.*, 1992; Mullan *et al.*, 1992) causing FAD are indicated. The epitopes of the different antibodies used in this study are indicated. The mouse APP695 sequence (De Strooper *et al.*, 1991) contains 17 amino acid residue differences compared with human APP695. Three substitutions are located in the amyloid peptide sequence. They were mutated by site directed mutagenesis (Deng and Nickoloff, 1992) to yield APP/Mo/GRFYRH. Analogously, APP/Mo/GR is mouse APP containing only the Gly601/Arg mutation, close to the β-secretase site. Below, the sequence of the p3 peptide is indicated.

the disease will likely come from studies on neuronal cells. Previous studies using primary cultures of rat neurons already gave insight into the intracellular trafficking of rodent APP (Yamazaki *et al.*, 1995). Since rodents, in contrast to species such as human, monkey, polar bear and dog (Selkoe *et al.*, 1987; Johnstone *et al.*, 1991), do not spontaneously develop amyloid plaques when aging, it is not unreasonable to speculate that rodent APP is metabolized differently from human APP. Moreover, the effects of the clinical mutations responsible for familial Alzheimer's disease (FAD) on the metabolism of APP in neurons are, finally, unknown. To answer these questions, we expressed human wild type APP, two clinical mutants of APP and mouse APP in primary cultures of rat hippocampal neurons using the Semliki Forest Virus (SFV) protein expression system (Liljeström and Garoff, 1991). Recombinant SFV has previously been used to investigate the polarized trafficking of proteins in neurons (Ikonen *et al.*, 1993; de Hoop *et al.*, 1994, 1995) including APP (Simons *et al.*, 1995). Here we report the repercussions of the clinical mutations on the metabolism of APP in cells which are a primary target of the disease; i.e. fully polarized, hippocampal neurons. We demonstrate that the Swedish (APP/Sw) and Flemish (APP/Fl) mutations (Hendriks *et al.*, 1992; Mullan *et al.*, 1992) cause not only an increase in amyloid peptide secretion (Citron *et al.*, 1992; Cai *et al.*, 1993; Haass *et al.*, 1994a), but also an increase in the relative amount of intracellular, amyloid-containing fragments generated by β-secretase cleavage. In contrast, mouse APP is processed with little β-secretase cleavage and three times less βA4-peptide secretion. 'Humanization' of the mouse sequence by site directed mutagenesis demonstrates that amino acid differences within the βA4 amyloid sequence itself are responsible for the decreased amyloid production from mouse APP relative to human APP.

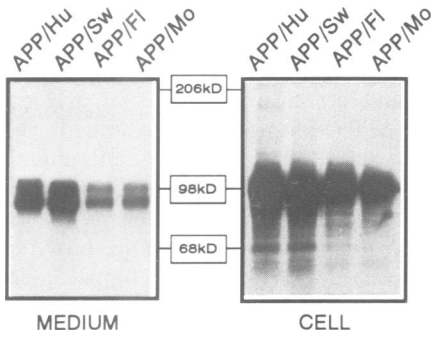

Fig. 2. Biosynthesis and secretory processing of human APP (APP/Hu), human APP containing the Swedish (APP/Sw) or Flemish (APP/Fl) mutations associated with FAD and mouse APP (APP/Mo), in rat hippocampal neurons. The first four lanes show secreted APP_s, immune precipitated from the culture medium with APP antiserum 203, the next four lanes show cellular APP immune precipitated from the cell extracts with APP C-terminal domain specific B12/4.

Results

Expression of human and rodent APP in hippocampal neurons

Wild type human APP, mutated human APP containing either the Swedish or the Flemish mutations associated with FAD and mouse APP (Figure 1) were expressed in rat hippocampal neurons using recombinant SFV. Secreted and intracellular fragments were recovered from the medium or cell lysate by immunoprecipitation with N- and C-terminal specific antibodies and analyzed by SDS–PAGE (Figure 2). In neurons expressing wild type human APP a broad band, corresponding to the precursor and N- and O-glycosylated forms with apparent M_r of 90–120 kDa was observed in total cell extracts. A similar band is recognized in immunoprecipitates of uninfected rat neurons (data not shown; M.Simons, B.De Strooper,

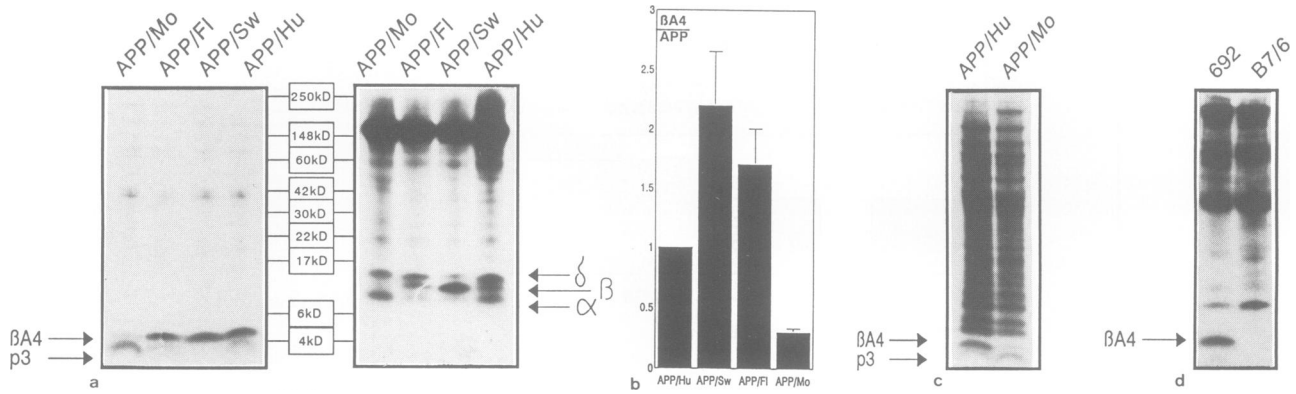

Fig. 3. Amyloidogenic processing of human APP, human APP containing the Swedish or Flemish mutations associated with FAD, and mouse APP, in rat hippocampal neurons. (**a**) The first four lanes show the relative amounts of βA4-peptide and p3-peptide (indicated by arrows) secreted into the culture medium. The next four lanes show the C-terminal fragments remaining in the cell extracts. The identity of all fragments has been confirmed by radiosequencing (M.Simons *et al.*, submitted). The delta-cleavage yields a fragment starting at Asn585 of APP695 and is further studied elsewhere (M.Simons *et al.*, submitted). (**b**) Densitometric quantitation of βA4-amyloid peptide production. βA4-amyloid production was quantitated by densitometric scanning and normalized to the levels of cellular APP. The ratio to wild type APP is displayed. The mean ratio ± SEM of three separate experiments is displayed. (**c**) Immune precipitation of βA4- and p3-peptide from hippocampal neurons infected with human or mouse APP using mAB 4G8. (**d**) Immune precipitation of mouse amyloid peptide from BHK cells with antibody 692. BHK cells were transiently transfected with the expression vector pSG5 coding for the mouse amyloid peptide fused to the signal peptide of APP. Immune precipitation with antibody B7/6 which is specific for human βA4 (Figure 1) revealed no signal, while immune precipitation with antibody 692 demonstrated its reactivity with mouse βA4.

G.Multhaup, P.J.Tienari, C.G.Dotti and K.Beyreuther, submitted). In the medium, two immunoreactive species with slightly lower mobilities than the cellular forms as expected for soluble, proteolytically processed APP were detected. These two secreted forms differ only in their levels of sialylation and are also produced by the endogenous neuronal rat APP (M.Simons *et al.*, sumitted). Overexpression of human APP in a pure glial cell culture results in a single secretory APP (sAPP) whereas sympathetic neurons do not produce sAPP (M.Simons *et al.*, submitted). These results show that the overall biosynthesis and secretory processing of APP by rat hippocampal neurons is very similar for the human and rodent forms of the protein.

Amyloidogenic processing of APP

The production of amyloid peptide and intracellular amyloidogenic fragments was investigated next (Figure 3). A peptide with the expected M_r of βA4 (4 kDa) was detected in the conditioned medium of neurons infected with either wild type human or the clinical mutant APP/Sw and APP/Fl when immunoprecipitated with polyclonal antibody 609, produced against synthetic human βA4-peptide (Figure 3a, left panel). In the immunoprecipitates from neurons infected with wild type but not mutant APP an additional much weaker fragment was visualized (~3 kDa). The identity of the precipitated peptides as true βA4- and the non-amyloidogenic p3-fragments was confirmed using antibodies B7/6 and 4G8 (Figure 1), which react either with the N- or the C-terminus of the βA4-peptide and by radiosequencing (M.Simons *et al.*, submitted). The amount of βA4 production with the different constructs was quantitated by densitometric scanning after normalizing the immunoprecipitated βA4-peptide to total APP (Figure 3b). Compared with wild type APP/Hu an increase in βA4-peptide production of 2.2 (± 0.5) and 1.7 (± 0.3) was observed with the APP/Sw and APP/Fl, respectively.

Hippocampal neurons produce three types of C-terminal fragments when expressing wild type APP/Hu, produced respectively by α-secretase cleavage, β-secretase cleavage and by a new secretase which we called δ-secretase (M.Simons *et al.*, 1995 submitted). This latter secretase yields a C-terminal fragment beginning at Asn585 of APP695, and therefore contains the entire amyloid peptide sequence. Upon expression of wild type and clinical mutant forms of APP we observed a strong relative increase in cell-associated C-terminal fragments generated by β-secretase cleavage (Figure 3a, right panel). The β-secretase cleaved form is dominant after transfection with APP/Sw, while the α-secretase product is almost completely absent from neurons expressing APP/Sw or APP/Fl.

Non-amyloidogenic processing of rodent APP

Altogether the above results show the tendency of rat hippocampal neurons to produce large amounts of secreted βA4 petide and intracellular amyloidogenic fragments upon expression of wild type and clinical mutant forms of human APP. Since rodents, in contrast to other species (see Introduction), do not spontaneously develop amyloid deposits or other signs of AD-like pathology during aging, the possibility that rodent APP is metabolized along other pathways than human APP was investigated. Transfection of rat hippocampal neurons with wild type mouse APP695 (APP/Mo) yielded only 30% as much amyloid peptide as transfection with human wild type APP (Figure 3a and b). Differences in the ratio of amyloidogenic βA4-peptide versus non-amyloidogenic p3-peptide were observed as well (Figure 3a and quantitated in Figure 5b): APP/Hu expressing neurons generated two and a half times more amyloid than p3, whereas the reverse was seen with mouse APP. Consistent with the much lower βA4-peptide production, negligible to very low levels of β-secretase cleaved cellular fragments were produced from APP/Mo (Figure 3a, right panel).

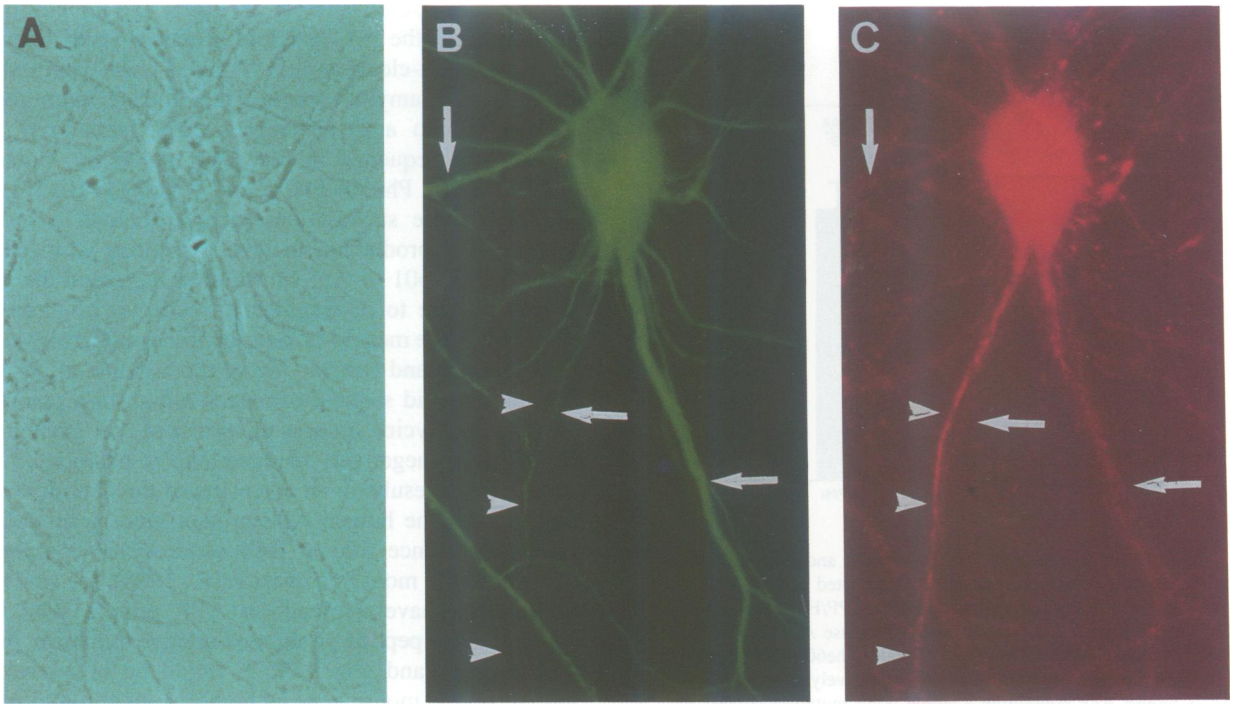

Fig. 4. Distribution of newly synthesized mouse APP in fully polarized hippocampal neurons in culture. (**A**) Phase contrast. (**B**) MAP-2 staining of the cell dendrites (arrows). (**C**) APP labeling. A single neurite, thin and of uniform diameter is clearly labeled with the anti APP antibody (arrowheads). This process is not stained with the anti-MAP2 antibody (arrowheads in B), demonstrating that this is the cell's axon.

It is well known that some polyclonal antisera directed against the human amyloid peptide react poorly with the mouse amyloid peptide. Therefore mAb 4G8 (Kim *et al.*, 1988) was used to corroborate the findings obtained with polyclonal serum 692 against human amyloid peptide. mAb 4G8 recognizes amino acid residues 17–24 of the amyloid sequence, which are identical in human and mouse (Figure 1). Double immune precipitation with this mAb confirmed the high amyloid production of human APP and the low production of mouse APP (Figure 3c). The excellent reactivity of antiserum 692 to mouse amyloid peptide in immune precipitation was further confirmed using the supernatant of BHK cells transfected with a construct yielding secreted mouse amyloid peptide (Figure 3d).

Polarized trafficking of mouse APP

Altogether the above results show that mouse APP is processed differently from human APP in rat hippocampal neurons. To determine whether the difference in processing is also reflected in a different intracellular routing, we analyzed sorting of mouse APP. Fully mature hippocampal neurons were infected with SFV expressing the mouse form of APP. The intracellular distribution of the newly synthesized protein was analyzed by immunofluorescence microscopy utilizing an antibody against APP at a dilution such that only the overexpressed protein could be detected and an antibody against MAP2, a cytoskeletal protein present exclusively in dendrites. At 3 h post infection, newly synthesized mouse APP was found in the cell body and axon of the infected cells (Figure 4). The dendrites were devoid of labeling at this post infection time. This result shows that despite differences in the processing, mouse APP is sorted in a manner similar to that of human APP (Simons *et al.*, 1995).

Humanizing mouse APP restores amyloidogenic processing

Mouse APP695 and human APP695 differ in only 17 amino acid residues (De Strooper *et al.*, 1991). In the amyloid peptide region itself, three amino acids are different, located between the β- and the α-cleavage sites (Figure 1). These three residues are identical in all species known to develop amyloid plaques when aging (Johnstone *et al.*, 1991; Selkoe *et al.*, 1987). We therefore 'humanized' the mouse APP sequence in the βA4 region by mutating Gly601 to Arg, Phe606 to Tyr and Arg609 to His (Figure 1). Expression of this humanized mouse APP (APP/Mo/GRFYRH) in neurons produced the same levels and the same ratio of βA4-peptide to p3-fragment as APP/Hu (Figure 5a and b). Mutating the single residue Gly601 to Arg in the mouse APP sequence was alone sufficient to increase the ratio of production of βA4-peptide to p3 by 3-fold (Figure 5b).

Discussion

While the metabolism of APP and its clinical mutants has been studied in considerable detail in non-neuronal and neuroblastoma cell lines, information on its processing in cells which are really affected by the disease, e.g. hippocampal neurons, is lacking. Only recently, using recombinant SFV, were we able to express wild type human APP in primary cultures of rat hippocampal neurons and determine its intracellular trafficking and processing (Simons *et al.*, 1995; M.Simons *et al.*, submitted). In the

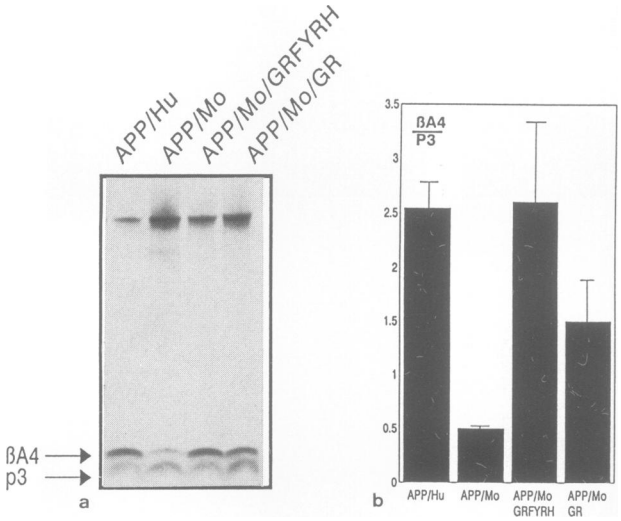

Fig. 5. (**a**) Immune precipitation of βA4-amyloid and p3-peptide from culture supernatant of hippocampal neurons infected with SFV containing cDNA coding for human APP695 (APP/HU), mouse APP695 (APP/Mo) and humanized forms of mouse APP695. APP/Mo/GRFYRH is mouse APP695 in which Gly601, Phe606 and Arg609 were mutated towards Arg, Tyr and His, respectively (Figure 1). APP/Mo/GR is mouse APP containing a single point mutation Gly601 to Arg. (**b**) Amyloidogenic potential of APP mutants indicated as the ratio of βA4-peptide to p3 peptide. The mean of four experiments ± SEM is displayed.

current study we show that hippocampal neurons have an intrinsic tendency to metabolize human wild type APP along amyloidogenic pathways, as exemplified by the secretion of relative high amounts of βA4-peptide relatively to p3-peptide, and by the accumulation of C-terminal fragments containing the amyloid sequence. We also report here that expressing the Swedish or the Flemish APP mutants in hippocampal neurons results in major alterations in the relative amounts of β- to α-secretase cleaved fragments. In the case of the Swedish mutation, only β-secretase cleaved fragments are observed, confirming that APP/Sw is a better substrate than APP/WT for this enzyme. It also suggests that APP/Sw is cleaved relatively early in the biosynthetic pathway before the α-secretase compartment is reached, because otherwise α-secretase cleaved fragments should also accumulate, similarly to APP/WT (De Strooper *et al.*, 1995). As with the APP/Sw, the Flemish mutation is preferentially cleaved at the β site. The results obtained after expression of the clinical forms of APP would suggest that not only increased secretion of amyloid peptide, but also intracellular accumulations of amyloidogenic fragments might be important for the pathogenesis of AD.

The fact that rat hippocampal neurons are able to produce βA4-amyloid when expressing human APP would indicate that they could also generate amyloid peptide from endogenous rodent APP. This does not seem to be the case. Expression of mouse APP yielded three times less amyloid peptide than its human counterpart and absence of β-secretase cleaved C-terminal fragments in the neuronal cell extracts. However, the intracellular distribution (this work) and secretory processing (M.Simons *et al.*, submitted) were similar. Since the human and rodent APP sequences differ at only 17 amino acid residues (De Strooper *et al.*, 1991) and three of these are located in the βA4-amyloid sequence itself between the β- and the α-cleavage sites (Figure 1), we hypothesized that the lack of amyloid production in rodents might reside in these amino acid differences. We 'humanized' the mouse APP sequence in the βA4 region by mutating Gly601 to Arg, Phe606 to Tyr and Arg609 to His (Figure 1). These three substitutions were sufficient to restore βA4-amyloid production to levels obtained with human APP. The Gly601 to Arg substitution increased the ratio of βA4-peptide to p3-fragment by a factor of three. As predicted by the method of Garnier-Robson (Garnier *et al.*, 1978; Kabsch and Sander, 1983), the arginine at position 5 in the amyloid sequence induces a high turn potential, whereas the glycine induces an extended coil structure at the following negatively charged Asp in position 7. The turn structure results in an extension of the peptide which might render the human APP protein more accessible to β-secretase. Since the rat βA4 sequence is completely identical to the mouse (Shivers *et al.*, 1988), we conclude that rodents have evolved an APP protein with low amyloidogenic peptide production relative to human, polar bear, monkey and dog APP. It would be of interest to 'humanize' the mouse gene by homologous recombination in transgenic mice *in vivo*, as was done here *in vitro*, and to investigate whether such mice spontaneously develop amyloid plaques, as we would predict. Obviously, such mice would be very useful to explore the contribution of environmental factors in the pathogenesis of AD.

With the obvious limitation of the experimental paradigm utilized, rat hippocampal neurons in culture, our work supports the view that the early onset of AD observed in FAD patients might be due to abnormal APP metabolism in hippocampal neurons (Selkoe, 1994b). Moreover, the contribution of other cells, or transport of βA4-peptide through the blood–brain barrier, do not necessarily have to be invoked to explain the amyloid precipitates, since hippocampal neurons secrete high amounts of βA4-amyloid peptide and, importantly, retain intracellular C-terminal fragments containing amyloid peptide which are toxic for neurons (Yoshikawa *et al.*, 1992; Fukuchi *et al.*, 1993). Thus it appears that in the pathogenesis of AD there is a positive feed-back loop in which neurons produce, and are affected by, the toxic secreted and intracellular APP fragments. Unfortunately we cannot conclude from the results at hand whether the C-terminal fragments are involved in the neurodegeneration in AD. However, it is interesting to speculate that these cell-associated fragments, or derivatives thereof, could interact with cytoskeletal proteins or with their controlling kinases and/or phosphatases, thereby providing a possible link between disturbed APP metabolism and intracellular tangle formation.

In conclusion, the use of a well-characterized neuronal culture system, together with an efficient protein expression system, permitted for the first time the study of the metabolic pathway of human and rodent forms of APP in the cells which are really affected by the disease. In the future, the approach used here should permit the analysis of the mechanisms by which abnormal APP metabolism affects the normal function of neurons.

Materials and methods

Hippocampal cell culture, metabolic labeling and double immune precipitation

Hippocampal neurons were prepared from 18 day old fetal rats as described by Goslin and Banker (1991). After dissection of the hippocampi and dissociation of the cells by trypsinization, cells were plated on polylysine coated plastic dishes (4×10^5/6 cm dish) or on glass cover slips (1.5×10^5/6 coverslips). Cells were maintained in MEM with N2-supplement (N2-medium). Proliferation of non-neuronal cells was prevented by adding 5 μM cytosine arabinoside. Recombinant SFV diluted in conditioned N2-medium was placed on 7–14 day-old neurons. After viral absorption during 1 h, the virus solution was replaced by N2-medium and infection continued for 2 h. Medium was then replaced by methionine-free N2-medium containing 200 μCi/ml [^{35}S]methionine and labeling was continued for 3 h. Cell extracts and culture supernatants were processed as described previously (De Strooper *et al.*, 1993, 1995). Antibodies were added and immune complexes were recovered with either protein A– or protein G–Sepharose, as described. Immunoprecipitated material was analyzed on 6% Tris–glycine or 10–20% Tris–tricine SDS–polyacrylamide gels (Novex). Gels were enhanced by Intensify (Du Pont), dried and exposed for 1–3 days to Kodak XAR film. Quantitative analysis of the bands was done by densitometric scanning as previously described (De Strooper *et al.*, 1993, 1995).

Antibodies

Rabbit antiserum 692 was raised against synthetic human βA4-peptide (1-42) and immunoprecipitates both βA4-peptide and p3-fragment. Rabbit antiserum B7/6 was raised against synthetic human βA4-peptide (1-40) and recognizes only βA4- but not p3-fragment. It is also human sequence specific. mAb 4G8 reacts with amino acids 17–24 of the amyloid peptide, and was obtained from Drs K.Kim and H.M.Wisniewski (Kim *et al.*, 1988). The epitopes of the different βA4-peptide antibodies used in this study are indicated in Figure 1. Goat antiserum 203 reacts with the extracellular domain of APP (Lowery *et al.*, 1991) and was kindly provided by Dr B.Greenberg (Cephalon). Rabbit antiserum B12/4 was raised against the 20 C-terminal amino acids of the intracellular domain of APP (De Strooper *et al.*, 1993).

Preparation of recombinant SFV virus

cDNAs coding for human APP695, human APP695 containing the Swedish or Flemish mutations (De Strooper *et al.*, 1995) and mouse APP695 (De Strooper *et al.*, 1991, 1993) were cloned blunt end in the *Sma*I site of pSFV-1 (Liljeström and Garoff, 1991). The mouse APP mutants APP/Mo/GRFYRH and APP/Mo/GR (see Figure 1) were generated by site directed mutagenesis (Deng and Nickoloff, 1992). The SFV-1 plasmids were linearized with SPE I and run-off transcripts were generated using SP6 RNA polymerase. Each APP transcription mix was cotransfected with the Helper-transcription mix in BHK cells to yield infective recombinant SFV (Liljeström and Garoff, 1991).

Immunofluorescence

Hippocampal neurons maintained in culture for 14 days were incubated at 37°C for 1 h with recombinant SFV expressing mouse APP695. Excess virus was removed and the cells were further incubated at 37°C for 3 h. The cells were then fixed in 4% paraformaldehyde and processed for immunofluorescence microscopy essentially as described (Simons *et al.*, 1995). Polyclonal rabbit anti FdAPP (Weidemann *et al.*, 1989) and rhodamine conjugated donkey–anti-rabbit antibodies (Dianova) were used to detect APP.

Acknowledgements

We thank Liane Meyn for preparing the hippocampal cultures and Sigrid Reinsch for discussions and criticisms of the manuscript. B.D.S. is a post-doctoral fellow of the Nationaal Fonds voor Wetenschappelijk Onderzoek (Belgium) and was supported by the Human Frontiers of Science Program. This work was partially supported by SFB 317 to K.B. and C.G.D.

References

Buxbaum,J.D., Koo,E.H. and Greengard,P. (1993) Protein phosphorylation inhibits production of Alzheimer amyloid β/A4 peptide. *Proc. Natl Acad. Sci.*, **90**, 9195–9198.

Cai,X.D., Golde,T. and Younkin,S.G. (1993) Release of excess amyloid β protein from a mutant amyloid β protein precursor. *Science*, **259**, 514–516.

Citron,M., Oltersdorf,T., Haass,C., McConlogue,L., Hung,A.Y., Seubert,P., Vigo-Pelfrey,C., Lieberburg,I. and Selkoe,D.J. (1992) Mutation of the β-amyloid precursor protein in familial Alzheimer's Disease increases β-protein production. *Nature*, **360**, 672–674.

Citron,M., Teplow,D.B. and Selkoe,D.J. (1995) Generation of Amyloid β Protein from its precursor is sequence specific. *Neuron*, **14**, 661–670.

de Hoop,M.J., Huber,L.A., Stenmark,H., Williamson,E., Zerial,M., Parton,R.G. and Dotti,C.G. (1994) The involvement of the small GTP-binding protein rab5a in neuronal endocytosis. *Neuron*, **13**, 11–22.

de Hoop,M., von Poser,C., Lange,C., Hunziker,W. and Dotti,C.G. (1995) Intracellular routing of wild-type and mutated poly-immunoglobulin receptor in hippocampal neurons in culture. *J. Cell Biol.*, **130**, 1447–1459.

De Strooper,B., Van Leuven,F. and Van Den Berghe,H. (1991) The amyloid β protein precursor or proteinase nexin II from mouse is closer related to its human homolog than previously reported. *Biochim. Biophys. Acta*, **1129**, 141–143.

De Strooper,B., Umans,L., Van Leuven,F. and Van Den Berghe,H. (1993) Study of the synthesis and secretion of normal and artificial mutants of murine amyloid precursor protein (APP): cleavage of APP occurs in a late compartment of the default secretion pathway. *J. Cell Biol.*, **121**, 295–304.

De Strooper,B., Craessaerts,K., Dewachter,I., Moechars,D., Greenberg,B., Van Leuven,F. and Van Den Berghe,H. (1995) Basolateral secretion of Amyloid Precursor Protein in Madin- Darby Canine Kidney cells is disturbed by alterations of intracellular pH and by introducing a mutation associated with familial Alzheimer's Disease. *J. Biol. Chem.*, **270**, 4058–4065.

Deng,W.P. and Nickoloff,J.A. (1992) Site-directed mutagenesis of virtually any plasmid by eleminating a unique site. *Anal. Biochem.*, **200**, 81–88.

Esch,F.S., Keim,P.S., Beattle,E.C., Blacher,R.W., Culwell,A.R., Oltersdorf,T., McClure,D. and Ward,D.J. (1990) Cleavage of Amyloid β peptide during constitutive processing of its precursor. *Science*, **246**, 1122–1124.

Fukuchi,K., Sopher,B., Furlong,C.E., Smith,A.C., Dang,N.T. and Martin,G.M. (1993) Selective neurotoxicity of COOH-terminal fragments of the β-amyloid precursor protein. *Neurosci. Lett.*, **154**, 145–148.

Garnier,J., Osguthorpe,D.J. and Robson,B. (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.*, **120**, 97–120.

Golde,T., Estus,S., Younkin,L., Selkoe,D. and Younkin,S. (1992) Processing of the amyloid protein precursor to potentially amyloidogenic derivatives. *Science*, **255**, 728–730.

Goslin,K. and Banker,G. (1991) Rat hippocampal neurons in low-density culture. In Banker,G. and Goslin,K. (eds), *Culturing Nerve Cells.* MIT Press, Cambridge, MA, pp. 251–281.

Haass,C. *et al.* (1992) Amyloid β-peptide is produced by cultured cells during normal metabolism. *Nature*, **359**, 322–325.

Haass,C. and Selkoe,D. (1993) Cellular processing of β-amyloid precursor protein and the genesis of amyloid β-peptide. *Cell*, **75**, 1039–1042.

Haass,C., Hung,A.Y., Selkoe,D.J. and Teplow,D.B. (1994a) Mutations associated with a locus for familial Alzheimer's Disease result in alternative processing of Amyloid β-protein precursor. *J. Biol. Chem.*, **269**, 17741–17748.

Haass,C., Koo,E., Teplow,D. and Selkoe,D.J. (1994b) Polarized secretion of β-amyloid precursor protein and amyloid β-peptide in MDCK cells. *Proc. Natl Acad. Sci. USA*, **91**, 1564–1568.

Haass,C., Koo,E.H., Capell,A., Teplow,D.B. and Selkoe,D.J. (1995) Polarized sorting of β-amyloid precursor protein and its proteolytic products in MDCK cells is regulated by two independent signals. *J. Cell Biol.*, **128**, 537–547.

Hendriks,L. *et al.* (1992) Presenile dementia and cerebral haemorrhage linked to a mutation at codon 692 of the β-amyloid precursor protein gene. *Nature Genet.*, **1**, 218–222.

Hung,A.Y., Haass,C., Nitsch,R., Qiu,W.Q., Citron,M., Wurtman,R.J., Growdon,J.H. and Selkoe,D.J. (1993) Activation of protein kinase C inhibits cellular production of the amyloid β-protein. *J. Biol. Chem.*, **265**, 22959–22962.

Ikonen,E., Parton,R.G., Hunziker,W., Simons,K. and Dotti,C.G. (1993) Transcytosis of the polymeric immunoglobulin receptor in cultured hippocampal neurons. *Curr. Biol.* **3**, 635–644.

Johnstone,E.M., Chaney,M.O., Norris,F.H., Pascual,P. and Little,S.P. (1991) Conservation of the sequence of the Alzheimer's Disease amyloid peptide in dog, polar bear and five other mammals by cross-species polymerase chain reaction analysis. *Mol. Brain Res.*, **10**, 299–305.

Kabsch,W. and Sander,C. (1983) How good are predictions of protein secondary structure? *FEBS Lett.*, **155**, 179–183.

Kang,J., Lemaire,H.G., Unterbeck,A., Salbaum,J.M., Masters,C.L., Grzeschik, K.G., Multhaup,G., Beyreuther,K. and Müller-Hill,B. (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature*, **325**, 733–736.

Kim,S.K., Miller,D., Sapienza,V., Chen,C.M., Bai,C., Grundke-Iqbal,I., Currie,J. and Wisniewski,H. (1988) Production and characterization of monoclonal antibodies reactive to synthetic cerebrovascular amyloid peptide. *Neurosci. Res. Commun.*, **2**, 121–130.

Koo,E.H. and Squazzo,S. (1994) Evidence that production and release of amyloid β-protein involves the endocytic pathway. *J. Biol. Chem.*, **269**, 17386–17389.

Kuentzel,S., Ali,S., Altman,R., Greenberg,B. and Raub,T. (1993) The Alzheimer β-amyloid protein precursor/protease nexin II is cleaved by secretase in a trans-Golgi secretory compartment in human neuroglioma cells. *Biochem. J.*, **295**, 367–378.

Liljeström,P. and Garoff,H. (1991) A new generation of animal cell expression vectors based on the Semliki Forest Virus Replicon. *Biotechnol.*, **9**, 1356–1361.

Lo,A.C.Y., Haass,C., Wagner,S.L., Teplow,D.B. and Sisodia,S.S. (1994) Metabolism of the 'Swedish' amyloid precursor protein variant in Madin-Darby Canine Kidney cells. *J. Biol. Chem.*, **269**, 30966–30973.

Lowery,D.E. *et al.* (1991) Alzheimer's amyloid precursor protein produced by recombinant baculovirus expression. *J. Biol. Chem.*, **266**, 19842–19850.

Mullan,M., Crawford,F., Axelman,K., Houlden,H., Lilius,L., Winblad,B. and Lannfelt,L. (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of β-amyloid. *Nature Genet.*, **1**, 345–347.

Näslund,J., Jensen,M., Tjernberg,L.O., Thyberg,J., Terenius,L. and Nordstedt,C. (1994) The metabolic pathway generating p3, an Aβ-peptide fragment, is probably non-amyloidogenic. *Biochem. Biophys. Res. Com.*, **204**, 780–787.

Roses,A.D. (1994) Apolipoprotein E affects the rate of Alzheimer Disease expression: β-amyloid burden is a secondary consequence dependent on APO E genotype and duration of the disease. *J. Neuropath. Exp. Neurol.*, **53**, 429–437.

Sambamurti,K., Shioi,J., Anderson,J.P., Pappolla,M.A. and Robakis,N. (1992) Evidence for intracellular cleavage of the Alzheimer's amyloid precursor in PC12 cells. *J. Neurosci. Res.*, **33**, 319–329.

Sandbrink,R., Masters,C.L. and Beyreuther,K. (1994) βA4-amyloid Protein Precursor mRNA isoforms without Exon 15 are ubiquitously expressed in rat tissues including brain, but not in neurons. *J. Biol. Chem.*, **269**, 1510–1517.

Selkoe,D.J. (1994a) Cell Biology of the amyloid β-protein precursor and the mechanism of Alzheimer's Disease. *Annu. Rev. Cell Biol.*, **10**, 373–403.

Selkoe,D.J. (1994b) Alzheimer's Disease: a central role for amyloid. *J. Neuropathol. Exp. Neurol.*, **53**, 438–447.

Selkoe,D.J., Bell,D.S., Podlisny,M.B., Price,D.L. and Cork,L.C. (1987) Conservation of brain amyloid proteins in aged mammals and human with Alzheimer's Disease. *Science*, **235**, 873–877.

Shivers,B.D., Hilbich,C., Multhaup,G., Salbaum,M., Beyreuther,K. and Seeburg,P. (1988) Alzheimer's Disease amyloidogenic glycoprotein: expression pattern in rat brain suggests a role in cell contact. *EMBO J.*, **7**, 1365–1370.

Shoji,M. *et al.* (1992) Production of the Alzheimer Amyloid β protein by normal proteolytic processing. *Science*, **258**, 126–129.

Simons,M., Ikonen,E., Tienari,P.J., Cid-Arregui,A., Monning,U., Beyreuther,K. and Dotti,C.G. (1995) Intracellular routing of human Amyloid Protein Precursor: axonal delivery followed by transport to the dendrites. *J. Neurosci. Res.*, **41**, 121–128.

Sisodia,S. (1992) β-Amyloid precursor protein cleavage by a membrane-bound protease. *Proc. Natl Acad. Sci. USA* **89**, 6075–6079.

Suzuki,N., Cheung,T.T., Cai,X.D., Odaka,A., Otvos,Jr,L., Eckman,C., Golde,T.E. and Younkin,S.G. (1994) An increased percentage of long amyloid β protein secreted by familial amyloid β protein precursor (βAPP717) mutants. *Science*, **264**, 1336–1340.

Weidemann,A., König,G., Bunke,D., Fischer,P., Salbaum,J.M., Masters, C.L. and Beyreuther,K. (1989) Identification, biogenesis, and localization of Precursors of Alzheimer's disease A4 amyloid protein. *Cell*, **57**, 115–126.

Yamazaki,T., Selkoe,D.J. and Koo,E.H (1995) Trafficking of cell surface β-amyloid precursor protein: retrograde and transcytotic transport in cultu red neurons. *J. Cell Biol.*, **129**, 431–442.

Yoshikawa,K., Aizawa,T. and Hayashi,Y. (1992) Degeneration *in vitro* of post-mitotic neurons overexpressing the Alzheimer amyloid protein precursor. *Nature*, **359**, 64–67.

Received on June 19, 1995; revised on July 20, 1995