Large Quantities of Aβ Peptide Are Constitutively Released during Amyloid Precursor Protein Metabolism in Vivo and in Vitro*

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‡The abbreviations used are: AD, Alzheimer disease; APP, amyloid precursor protein; Aβ, amyloid-β-peptide; sAPP, soluble amyloid precursor protein ectodomain; PSD-95, postsynaptic density protein, 95 kDa; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

The metabolism of the amyloid precursor protein (APP) has been extensively investigated because its processing generates the amyloid-β-peptide (Aβ), which is a likely cause of Alzheimer disease. Much prior research has focused on APP processing using transgenic constructs and heterologous cell lines. Work to date in native neuronal cultures suggests that Aβ is produced in very large amounts. We sought to investigate APP metabolism and Aβ production simultaneously under more physiological conditions in vivo and in vitro using cultured rat cortical neurons and live pigs. We found in cultured neurons that both APP and Aβ are secreted rapidly and at extremely high rates into the extracellular space (2–4 molecules/neuron/s for Aβ). Little APP is degraded outside of the pathway that leads to extracellular release. Two metabolic pools of APP are identified, one that is metabolized extremely rapidly ($t_{1/2} = 2.2$ h), and another, surface pool, composed of both synaptic and extrasynaptic elements, that turns over very slowly. Aβ release and accumulation in the extracellular medium can be accounted for stoichiometrically by the extracellular release of β-cleaved forms of the APP ectodomain. Two α-cleavages of APP occur for every β-cleavage. Consistent with the results seen in cultured neurons, an extremely high rate of Aβ production and secretion from the brain was seen in juvenile pigs. In summary, our experiments show an enormous and rapid production and extracellular release of Aβ and the soluble APP ectodomain. A small, slowly metabolized, surface pool of full-length APP is also identified.

Alzheimer disease (AD) is a progressive, neurodegenerative process characterized pathologically by accumulation of the β-amyloid peptide (Aβ) in the form of extracellular plaques. On the basis of genetic, cellular, and animal studies the amyloid hypothesis postulates that increased deposition of Aβ in the brain is the primary influence in the pathogenesis of AD. Aβ is part of the amyloid precursor protein (APP) that is expressed ubiquitously by neuronal and non-neuronal cells (3–5). APP is a type 1 transmembrane glycoprotein that undergoes sequential site-specific proteolytic cleavages by either α- or β-secretase followed by γ-secretase, which cleaves APP in the transmembrane domain to yield several secreted derivatives. Cleavage of APP by the β-secretase BACE1 releases a large fragment called sAPPβ leaving behind a fragment that is acted upon by γ-secretase at different sites to release several short peptides of which Aβ40 and Aβ42 are the major components (6). Alternatively, cleavage of APP by α-secretase occurs within the Aβ domain, precluding the formation of Aβ and resulting in the secretion of sAPPα, a short peptide called p3, and a C-terminal domain, none of which are amyloidogenic.

Both in vitro and in vivo evidence suggests that Aβ is secreted from cells under normal physiological conditions (3, 4, 7–9) but the absolute amount, an important consideration given its proposed roles and pharmacologic interest, has not been precisely calculated. In addition, the relative amount of intracellular versus extracellular Aβ production and its ultimate disposition in neurons is unclear (10). Most models of Aβ metabolism depend on the analysis of cells transfected with human APP constructs or cells from transgenic mice. Establishing a model to obtain detectable, consistent, and reliable levels of soluble Aβ and APP ectodomain directly from non-transgenic and non-transfected neurons is important to understand the normal metabolism of APP and Aβ and to explore novel therapeutic approaches aimed at manipulating soluble Aβ levels. Toward this end we have developed a series of assays for endogenous rodent Aβ and APP and confirmed the key result in vivo using juvenile pigs. The picture that emerges is one of an enormous quantity and rapid turnover of APP, all leading to the extracellular secretion of sAPP and Aβ. A second, slowly metabolized, largely extrasynaptic, surface pool of APP is also identified, confirming and extending prior work (11).

**EXPERIMENTAL PROCEDURES**

**Antibodies**—An antibody (2B) to the first 16 amino acids of rat amyloid peptide was raised in rabbits by immunizing them with the peptide DAEGHDSGFVHRHKPC coupled to keyhole limpet hemocyanin. The resultant serum was affinity purified. Three different APP antibodies were used in this study. Two were commercially obtained (rabbit anti-APP CT and mouse monoclonal 22C11, both from Chemicon). One was made in our laboratory, using a GST fusion protein coupled to amino acids 30–300 of rat APP, injected into rabbits. This anti-
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body, termed N-terminal APP, was used after affinity purification. A polyclonal antibody that specifically recognizes the C terminus of sAPPβ was purchased from IBL.

**Rat Primary Cortical Cultures**—Embryonic day 19 rat cortical cultures were grown at a density of 2,000,000 cells/60-mm dish for 14–21 days as described (12) in the Neurobasal plus 2% B-27 supplement (Invitrogen). Serial counts of these cultures using randomly selected live \( \times 40 \) fields \( (n = 4) \) or \( \times 60 \) fields of fixed neurons stained with DAPI (\( n = 5 \)) demonstrate a 31 \( \pm \) 5% loss of cells over the 2–3 week period of growth, a number calculated into the Aβ release kinetics. When Aβ and APP secretion assays were performed, a complete change of the neuronal growth medium to 1.5 ml of new Neurobasal B27 medium was performed at the start of the assay. This fresh medium was preincubated with glia for 8 h prior to use.

**Aβ ELISA**—96-Well ELISA plates (Kirkegaard & Perry Laboratories) were coated with affinity purified 2B antibody at 1 ng/well as the capture antibody with biotinylated 4G8 antibody as the detection antibody. The intra-assay and inter-assay coefficients of variation were both less than 10%; the lower limit of detection of rat Aβ was 8 pg/ml. Verification experiments were performed using two commercially available ELISA kits, the rat/human Aβ40 assay purchased from Wako and the rodent Aβ1–40 kit purchased from Invitrogen. Both assays use antibodies distinct from those used in our assay including a monoclonal antibody specific for the C-terminal portion of Aβ40 common to both. The Wako kit recognizes full-length Aβ1–40 as well as Aβ truncated prior to amino acid 11, a result we have confirmed with rat and human Aβ1–40 standards (purchased from AnaSpec). The Invitrogen kit does not recognize Aβ11–40, a result we have also confirmed. For all 3 rodent ELISA we used the same synthetic rodent Aβ1–40 standard (Bachem) from 1 ng/ml to 8 pg/ml. In the Wako Aβ40 ELISA, rodent and human Aβ1–40 (Bachem) standards gave O.D. results within 18.3 \( \pm \) 6.1% of each other (rodent higher; \( n = 4 \)) at a standard concentration of 400 pg/ml, a concentration relevant for comparison between Fig. 1 (rodent) and Table 1 (pig).

**Quantitation of APP Secretion**—Rat cortical neurons were switched to fresh medium. Samples of the supernatant were collected at various times after the medium switch. Samples of the supernatant were collected at various times after the medium switch. Absolute levels of APP secretion were determined with quantitative immunoblotting using either a purified APP-GST fusion protein (amino acids 30 to 300 of rat APP) or a His\(_6\) APP fusion protein (purchased from R&D Systems) as standards. Both standards were highly purified by several criteria. During experiments, multiple concentrations of the synthetic APP were run on a SDS-polyacrylamide gel along with the culture supernatants and probed with our APP N-terminal antibody or with the monoclonal 22C11 antibody, which recognizes an unglycosylated epitope on human and rodent APP between amino acids 66 and 81 (13). Both antibodies and fusion proteins gave quantitatively similar results. Protein band intensity for secreted APP and the protein standards were quantitated using a densitometer. A standard curve was generated for each experiment, such that band intensity could be converted to a protein concentration. As an independent method to quantitate the secreted APP ectodomain, we used an ELISA from Meso Scale Discovery that detects the β-cleaved form of APP using a sAPPβ C-terminal specific antibody (which recognizes the identical C terminus of rodent and human sAPPβ) and the 22C11 antibody that also detects rat and human APP. This kit was standardized using a supplied, highly purified sAPPβ fusion protein.

**Metabolic Labeling of APP in Primary Cortical Cultures**—Cultured neurons were labeled with 0.5 mCi/ml of \([35S]\)Met-Cys (PerkinElmer Life Sciences) for 10 or 30 min and then rinsed thoroughly and switched to growth medium supplemented with unlabeled methionine and cysteine. After baseline labeling, two 60-mm dishes of neurons were harvested in 1 ml of RIPA lysis buffer (total cellular APP) and supernatants and cells from other dishes were collected at various time points. Labeled APP in the supernatant was immunoprecipitated with 10 μl of our N-terminal rat APP serum (a saturating dose). Total cellular APP signal at \( t = 0 \) and at other time points was immunoprecipitated with saturating amounts of both the APP CT antibody and our N-terminal APP antibody. By saturating, we mean that no further material could be immunoprecipitated with a second round of immunoprecipitation. The immunoprecipitates were washed and fractionated on 4–20% Tris-HCl gels. The gels were dried, exposed, and radioactive bands were quantified using densitometry with appropriate subtraction of background. A series of dilutions of the \( t = 0 \) sample were used to construct a standard curve for quantitating the densitometry. The radioactive signal in the immunoprecipitates of supernatants and cells was also counted using liquid scintillation, subtracting the nonspecific signal due to immunoprecipitation with a similar amount of nonspecific IgG.

Half-life Quantitation—The quantified APP data from metabolic labeling was fitted to a biexponential model by using Sigma Plot (SPSS Inc.). The model is described by the equation: \( y = A \times \exp(-\alpha t) + B \times \exp(-\beta t) \), where \( \alpha \) and \( \beta \) are the degradation rate constants in the initial and terminal phases of the degradation curve. The model fitting was achieved by the least squares criteria. For the biotinylation experiments, half-life determinations were based on previously published techniques (14). Protein band intensity was quantitated using a densitometer. A standard curve was generated for each film using APP fusion protein or an internal standard, such that band intensity of biotinylated material could be expressed as a percentage of \( t = 0 \) material. To calculate half-lives, we plotted the natural logarithm of the percentage of protein remaining as a function of time and found the slope of the resulting regression line. Assuming first-order decay kinetics, the half-life is equal to \( \ln 2/\text{slope} \).

**Immunoprecipitation and Immunoblotting of Aβ**—Ten μl of 4G8 antibody was incubated with 1 ml of the supernatant and with unconditioned growth medium containing known amounts of synthetic rodent Aβ1–40 (Bachem) overnight at 4 °C. The antibody was immunoprecipitated with 50 μl of Protein A. The amount of 4G8 antibody used for immunoprecipitation was sufficient to reduce the Aβ signal in the medium (determined with our ELISA assay) to levels detected in fresh, unconditioned medium. The immunoprecipitate was then boiled in sample buffer and run on a denaturing Tris-Tricine gel, transferred to a nitrocellulose membrane, and probed with purified rabbit anti-rat Aβ antibody and the signal visualized.
using chemiluminescence. We saw no oligomeric forms in our gels using either these antibodies or the oligomer-sensitive antibodies A11 (purchased from U.S. Biological) and 82E1 (purchased from IBL).

**APP Surface Biotinylation**—After 2–3 weeks in vitro, cortical cultures were cooled on ice for 15 min and surface biotinylated for 5 min using LC Biotin (Pierce) dissolved in PBS at 1 mg/ml for saturation biotinylation and at 0.1 mg/ml for subtotal biotinylation. The saturating conditions have previously been shown to result in complete biotinylation of all surface proteins (14). After washing, 1 ml of glial conditioned Neurobasal medium containing 1% B27 supplement was added to each dish, which was then placed back in the incubator. At different time intervals the cells were harvested in 0.5 ml of MPER (Pierce) with 1% protease inhibitor mixture. The cell lystate were then precipitated with streptavidin-coated beads as described earlier (14), and immunoblotted with the N-terminal or C-terminal APP antibodies as designated in the text. Quantitative estimates of surface APP were determined by comparing the total streptavidin-precipitated material to various dilutions of the total cell material as described (12).

**Immunostaining and Fab Fragments**—The APP N-terminal and 2B antibodies were primarily labeled with DyLight 633 and DyLight 488, respectively (Pierce), and then digested with papain using Pierce kit number 44985 to generate and purify Fab fragments. Fab fragments were used at a concentration of 7 μg/ml for 30 min of live staining of 2-week-old cultures in Neurobasal medium. Cultures were then fixed, permeabilized, and stained with mouse anti-synaptophysin or PSD-95 antibodies (Millipore). Consecutive, randomly selected neurons from 2 platings were photographed (cell centered) at ×100 and analyzed for colocalization off-line.

**Neuronal Transfections**—Human APP 695 was tagged mid-way through the ectodomain by inserting a HA tag into the unique XhoI restriction site starting at base pair 909 of 2087. Two-week-old cultures were transfected with this construct as described (15). Thirty-six hours after transfection, cells were stained live with rhodamine-labeled rat anti-HA and then fixed, permeabлизed, and stained with FITC-labeled mouse anti-HA. Axons and dendrites were identified by permeabлизed HA staining and differentiated as described previously (12).

**Measurement of Brain Aβ Production in Vivo**—Juvenile female Yorkshire pigs (90–102 pounds) were anesthetized with 2.5% isoflurane and maintained at 37 °C. The common carotid and internal jugular vessels were dissected on both sides. An angle-independent and calibrated dual-beam Doppler ultrasound device (Flowguard; CardioSonic Inc.) was used to measure right and left common carotid blood flow. Five ml of blood was collected from the right carotid artery and internal jugular vein in EDTA tubes twice, each separated by 15 min. Carotid blood flows were stable throughout the procedure. Each blood assay for Aβ40 was done in triplicate using the Wako rat/human Aβ40 kit at 1:6 and 1:8 dilutions using human standards from Bachem (which gave very similar results to the rodent standards) and at 1:6 using the Wako human Aβ1–40 ELISA using the same human standards from Bachem. By using the same standards across kits, the results can be more directly compared. In one pig (pig 3) we measured right and left jugular blood flows were stable throughout the procedure. Each blood assay for Aβ40 was done in triplicate using the Wako rat/human Aβ40 kit at 1:6 and 1:8 dilutions using human standards from Bachem (which gave very similar results to the rodent standards) and at 1:6 using the Wako human Aβ1–40 ELISA using the same human standards from Bachem. By using the same standards across kits, the results can be more directly compared. In one pig (pig 3) we measured right and left jugular

**RESULTS**

**The Aβ Peptide Accumulates Rapidly and Linearly in the Supernatant of Cultured Neurons**—To assess the normal production and metabolism of Aβ in rodent neurons we developed an ELISA sensitive to both of the common forms of the Aβ peptide (Aβ1–40 and Aβ1–42) released during β-cleavage of APP. Using this assay, Aβ levels in supernatants from cortical neuronal cultures were quantified at 6, 12, and 24 h after a complete change in medium using either our own standards or commercially available standards (which gave identical results). As seen in Fig. 1A the levels of Aβ in the supernatant increase linearly with time. Assuming there is no degradation or uptake of Aβ (see below), and that all neurons release similar amounts in a non time-varying pattern (see “Discussion”), the amount of Aβ released was quite substantial, calculated at roughly 4.7 ± 0.43 (S.D.) molecules/neuron/s (n = 4). Performing the assay in minimal essential medium (without protein) for periods up to 5 h gave the same result (4.3 ± 0.49 molecules/neuron/s; n = 3). Assays of supernatants from cultures derived from BACE1 or APP knock-out mice, or from cultures composed only of glia showed no Aβ in the supernatant. To further ensure the accuracy of the ELISA, we compared the amount of Aβ in the supernatant at 24 h as measured by ELISA to that measured by denaturing immunoblot. As seen in Fig. 1B, maximal immunoprecipitation of secreted Aβ from culture supernatants at 24 h

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![Figure 1](http://www.jbc.org/)

**FIGURE 1. Aβ and sAPP release by cultured neurons.** A, time course of Aβ secretion by primary rat neuronal cultures measured by ELISA. Data are representative of four independent experiments (each assayed in duplicate) and are shown with mean ± S.D. In B, an immunoblot of Aβ immunoprecipitated from culture medium at 24 h is compared with standards immunoprecipitated in a similar fashion. The blot was probed with 2B antibody. The arrows indicate the positions of the 3.8- and 8.4-kDa standards. In C, a portion of the supernatant from cultured cortical neurons (top) was collected (in duplicate) at the indicated times and probed with our N-terminal APP antibody along with specified amounts of an N-terminal APP fusion protein (bottom). In D, mean APP ectodomain accumulation per ml of medium (± S.D.) over time is presented from 4 experiments.

Aβ40 levels, which were identical (259 pg/ml (R) versus 265 pg/ml (L)).
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produced a signal that was visually similar to a 2 ng/ml Aβ1–40 standard, immunoprecipitated in a similar fashion. By constructing a standard curve of the densitometer signals of varying Aβ1–40 concentrations immunoprecipitated from culture medium under the same conditions as the secreted material, the concentration of secreted Aβ at 24 h, determined by denaturing immunoblot, was 1.8 ± 0.5 ng/ml (mean ± S.D. in 3 experiments), similar to the Aβ concentration determined by the ELISA. Finally, to ensure our assay was generalizable, we used two commercially available ELISA, a rodent/human Aβ40 kit (Wako) and a rodent Aβ1–40 kit (Invitrogen) for comparison with our own ELISA using our own rodent standards. Results at the 6-h time point for both commercial kits (520 ± 51 (S.D.) pg/ml of Aβ40 (n = 8) and 467 ± 43 pg/ml of Aβ1–40 (n = 4)) were in substantial agreement with our own assay (570 pg/ml; Fig. 1A).

APP Ectodomain Release Parallels the Rise of Aβ in Supernatant—To measure APP ectodomain secretion and accumulation we used two antibodies, the commercially available 22C11 antibody and our own N-terminal polyclonal APP antibody generated from an APP N-terminal fusion protein capable of distinguishing APP and its secreted ectodomain from other related molecules either by size or specificity (Fig. 2). Quantitative immunoblotting of supernatants from cultured neurons (n = 4, in duplicate) demonstrated that the levels of secreted APP ectodomain in the supernatant increased linearly with time, similar to the increase in Aβ. Using an APP ectodomain fusion protein of known concentration (and confirmed with a second commercially purchased fusion protein), we were able to quantitate the accumulation of the ectodomain. As shown in Fig. 1, C and D, the amount of APP ectodomain that accumulates in the supernatant by 24 h, on a nanogram basis, is 67 times greater than Aβ, and equates to 12.7 ± 2.9 (S.D.) molecules/neuron/s. Using the 22C11 antibody to quantify APP ectodomain accumulation at 24 h we obtained a very similar result (158 ± 23 (S.D.) ng/ml; n = 4). On a molar basis, the ratio of APP ectodomain accumulation to Aβ accumulation in the supernatant is 2.7 to 1 at 24 h.

Stoichiometry and Site of APP Cleavage—To determine the ratio of α- versus β-cleavage of APP, we immunoprecipitated the APP ectodomain component corresponding to cleavage of APP at the α-secretase site (sAPPα) from the supernatant of neuronal cultures after a 24-h incubation. To do this we used the 2B antibody that recognizes sAPPα 1–16 (cleaved before amino acid 17 of the Aβ fragment) and sAPP 1–10 (cleaved before amino acid 11 of the Aβ fragment) but not sAPPβ (cleaved prior to the Aβ fragment), so that any non-amyloidogenic cleavages of APP would be immunoprecipitated. When neuronal supernatants are immunoprecipitated using saturating concentrations of the 2B antibody, a substantial portion of the total APP ectodomain (30.5 ± 8.6%; n = 4) is left in the supernatant (Fig. 3A). This residual component presumably represents sAPPβ that portion of the APP ectodomain that should be produced in a 1:1 ratio with Aβ. Probing the immunodepleted supernatant with the 2B antibody (Fig. 3A) shows that no residual signal attributable to residual sAPPα remains. Repeat immunoprecipitation with the 2B antibody brought down no further sAPPα (not shown). Performing the complementary experiment (Fig. 3B), we found that an antibody that specifically immunoprecipitates sAPPβ left a residual sAPP signal of 61 ± 9.8% (n = 4) in the medium. Assuming 2 molecules of sAPPα are generated for every molecule of sAPPβ, and knowing the amount of total sAPP released by neuronal cultures (12.7 ± 2.9 molecules/neuron/s), the amount of Aβ expected to be released into the supernatant if the release of Aβ and sAPPβ is a 1:1 phenomenon would be 4.2 molecules/neuron/s, which is very close to that actually observed (4.7 molecules/neuron/s), suggesting that there is a close (possibly 1:1) relationship between β-cleavage of APP and the release of Aβ and sAPPβ. To confirm our results of a nearly 1:1 relationship between the β-cleavage of APP and the extracellular release of Aβ and sAPPβ we used a commercially available ELISA, which detects full-length rodent sAPPβ. Using this assay to test culture supernatants after a 24-h incubation, the sAPPβ content was 39 ± 7 ng/ml (n = 3) compared with a total Aβ content (done simultaneously) of 2.2 ± 0.3 ng/ml, a molar ratio of 1.1 molecules of Aβ for every molecule of sAPPβ detected in the supernatant. Of note, immunoprecipitation of the culture supernatant with the 2B antibody prior to the sAPPβ ELISA...
had no effect on the results (not shown), demonstrating specificity in the immunoprecipitation experiments of Fig. 3, A and B.

**Metabolic Labeling of APP Shows That the Majority of APP Is Metabolized through Extracellular Release**—Metabolic labeling with \(^{35}S\)methionine allowed us to examine the percentage of APP, which, once synthesized, is secreted extracellularly, and whether it is degraded. As shown in Fig. 4, APP is secreted at a rapid rate. The majority of the cellular APP signal at \(t = 0\) is present in the extracellular medium at 3 h, consistent with an extremely fast turnover as noted previously in cultured neurons (11). Approximately 90% of the total cellular APP present at time 0 appears in the extracellular medium by 9 h (Fig. 4B) when calculated by liquid scintillation following immunoprecipitation (91 ± 11%; \(n = 4\)) or by densitometry (92 ± 9%; \(n = 4\)). The percentage of metabolically labeled APP released into the medium was not dependent on the metabolic labeling time of the cultured neurons. A 10-min metabolic labeling time (as contrasted with the typical 30-min labeling time) resulted in the same release of total labeled cellular APP into the medium by 9 h. The fact that nearly all the cellular APP at \(t = 0\) is recoverable from the supernatant 9 h later at the correct molecular weight for the APP ectodomain indicates that nearly all APP catabolism leads to extracellular release and that little degradation of the secreted APP ectodomain is occurring in the supernatant over this time scale.

**Surface APP Represents a Small Proportion of Total Cellular APP and Turns Over at a Much Slower Rate**—Consistent with the rapid release of soluble APP from neurons, the metabolic half-life of radioactively labeled full-length endogenous APP is quite rapid (Fig. 5A) with the majority of the cellular signal gone by 12 h but with a small residual signal lasting past 24 h. Using liquid scintillation, we quantitated the cellular APP C-terminal

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**FIGURE 3. Stoichiometry of APP cleavage.** In A, neuronal supernatants were immunodepleted with the 2B antibody, which recognizes the 16 amino acids on the C terminus of sAPP\(_\beta\). The residual supernatant was then run on a denaturing gel and blotted with the 22C11 APP N-terminal antibody (top) or the affinity purified 2B antibody (bottom). A serial dilution of non-immunodepleted supernatant was run for quantitative purposes. As controls, the medium was immunodepleted with the rabbit N-terminal APP antisera (APP) and with control rabbit preimmune serum (IGG) at the same concentration as the 2B antibody. No residual 2B signal was seen in the supernatant after immunoprecipitation (IP) with the 2B antibody. In B, neuronal supernatants were immunodepleted with an sAPP\(_\beta\)-specific antibody (at 4 \(\mu\)g/ml) or with control antibodies at similar concentrations. The residual supernatant was then run on a denaturing gel and blotted with the 22C11 APP N-terminal antibody. A serial dilution of non-immunodepleted supernatant was run for quantitative purposes.

**FIGURE 4. Metabolic labeling shows near complete release of cleaved APP ectodomain into the extracellular medium.** A, metabolic labeling of cellular APP followed by immunoprecipitation of APP from cells (\(t = 0\)) or supernatant (other time points). Cellular APP was immunoprecipitated with a combination of the APP CT and the N-terminal APP antibodies (APP) or with control IGG. The supernatants were immunodepleted with only the N-terminal APP antibodies (APP) or with control rabbit preimmune serum (IGG) at the same concentration as the 2B antibody. No residual 2B signal was seen in the supernatant after immunoprecipitation (IP) with the 2B antibody. In B, neuronal supernatants were immunodepleted with an sAPP\(_\beta\)-specific antibody (at 4 \(\mu\)g/ml) or with control antibodies at similar concentrations. The residual supernatant was then run on a denaturing gel and blotted with the 22C11 APP N-terminal antibody. A serial dilution of non-immunodepleted supernatant was run for quantitative purposes.

**FIGURE 5. Metabolic labeling and surface biotinylation demonstrate fast and slow APP turnover.** A, metabolic labeling of APP followed by immunoprecipitation (IP) from cells using the APP C-terminal antibody or control IGG. In B, the data (±S.E.) for the observed decay of cellular APP immunoprecipitable material is plotted and fitted to a theoretical curve for the biexponential decay of cellular APP as described in the text (\(n = 4\)). In C, cultured cortical neurons were surface biotinylated using a saturation protocol and streptavidin immunoprecipitated. 100% of the surface (IP) material was run along with a standard curve of varying percentages of the total cellular material and the blot was probed with the APP N-terminal antibody. D, 50% of the biotinylated surface material (Surf) from sister cultures at \(t = 0\) along with 20% of the total cell material (Tot) at each time point are displayed along with 7% of the total secreted supernatant material at the 3-h time point (Sup). E, 100% of the surface material from sister cultures following biotinylation at \(t = 0\) along with 10% of the total cell material at each time point are displayed along with an APP fusion protein standard curve run simultaneously. Both D and E were probed with the 22C11 APP antibody. F, the half-life curve calculated from the experiment shown in E.
antibody precipitable signal as a function of time, subtracting nonspecific signal immunoprecipitated by a similar amount of nonspecific IGG. As shown in Fig. 5B, the degradation of the radioactive signal can be fitted to a biphasic decay with an initial rapid degradation (t₁/₂) of 2.2 h (95% confidence interval 1.9–2.8; n = 4) and a subsequent relatively slow degradation (t₁/₂) of 29 h (95% confidence interval 15–68 h). The fraction that turned over at a slower rate amounted to 7% of the total signal. Using densitometry, rather than liquid scintillation, the half-life for the major portion of the decay was 2.5 h (95% confidence interval 2.0–3.2; n = 4) with a residual component of 5%, which had a t₁/₂ of 23 h (95% confidence interval 14–45 h). Similar results were obtained when the N-terminal APP antibody was used for immunoprecipitation.

The component of total cellular APP that turns over slowly was similar in amount and turnover rate to the fraction of APP we found present on the surface of neurons. Using saturating surface biotinylation (14), the amount of the total APP present on the surface (i.e. was subject to biotinylation using a non-permeable biotinylation reagent) was 5.3 ± 1.8% (n = 5) using the N-terminal APP antibody to quantitate APP (Fig. 5C) and 6.5 ± 2.2% using the C-terminal APP antibody. Degradation of the surface component was quite slow compared with release of the ectodomain. As shown in Fig. 5D, over a short time period (3 h) and using either a saturating or subtotal biotinylation strategy, there is almost no decrease in the surface APP component whereas, over the same period, the amount of ectodomain recoverable from the supernatant becomes a considerable fraction of the total cellular APP pool. Half-life experiments looking at the decay of the surface APP signal over longer time points (Fig. 5, E and F) show that the surface component decays with a half-life of 19 ± 4 h (mean ± S.D.; n = 4), a number well within the confidence intervals of the slow phase of APP degradation seen with metabolic labeling. Live surface immunolabeling of cultured neurons with tagged Fab fragments of the APP N-terminal antibody or the 2B antibody showed microclusters of immunostaining for each of these two APP epitopes, which overlapped completely (Fig. 6A). The surface clusters of APP were both synaptic and non-synaptic; 336 of 1142 surface APP clusters (30%) from 30 neurons showed colocalization with the synaptic marker synaptophysin. Approximately 28% of PSD-95 immunopositive excitatory synapses (184 of 663 PSD-95 positive synapses examined in 25 neurons) had colocalized surface APP immunostaining (Fig. 6B, arrows), with APP positive and negative excitatory synapses clustered nearby on the same neuron. Fourteen percent (38 of 254) of glutamic acid decarboxylase-positive inhibitory synapses also had colocalized surface APP clusters. Neurons derived from APP knock-out mice showed no surface staining (not shown), whereas permeabilized staining of neurons with both antibodies showed a diffuse pattern (not shown). Transfection studies using human APP695 HA-tagged in the extracellular ectodomain indicated that surface APP on neurons could be both axonal and dendritic in origin (Fig. 6, C and D), as all dendrites and axons of transfected neurons showed punctate, live, surface HA staining (red/yellow) in addition to a nonhomogeneous intracellular pattern (green).

Brain Aβ Production and Secretion in Vivo Is Similar to That Measured in Cultured Neurons—To validate our experiments in cultured neurons, we measured the amount of Aβ that is produced and then transported intact from the brains of juvenile Yorkshire pigs. We simultaneously measured bilateral common carotid blood flows using a Doppler ultrasound probe while measuring blood Aβ40 (sensitive to both Aβ1–40 and Aβ1–40) and Aβ1–40 in the right carotid artery and jugular vein in 5 pigs. The common carotid blood flow was then corrected for the known fraction of common carotid blood flow, which supplies the cortex in pigs (58%) resulting in a calculated cortical blood flow (16). The cortical flow values we obtained are similar to those seen in humans (17–19) corrected for the differences in brain weight between human and pig. Carotid-jugular Aβ40 and Aβ1–40 differences were determined twice in each animal separated by 15 min and averaged. As shown in Table 1, brain Aβ production and secretion in the pig is also substantial, with a mean of 13.1 ± 3.4 (S.D.) billion Aβ40 and 11.4 ± 2.8 billion Aβ1–40 molecules released per second, assuming that release does not vary significantly over short time intervals (see “Discussion”). Given that the pig neocortex and basal ganglia (the part of the brain whose blood flow and venous drainage we are measuring) is composed of 7 × 10⁸ neurons, extrapolating from Fig. 2 of Braitenberg (20), and leaving out the cerebellum (because we do not sample vertebral blood flow or the vertebral venous system), we come to a rough
TABLE 1  
**Brain Aβ production in vivo**

Five juvenile Yorkshire pigs had simultaneous right carotid and right jugular Aβ40 and Aβ1–40 (parentheses) levels determined along with measures of bilateral common carotid blood flow (CCBF). The cortical blood flow is calculated. Carotid and jugular Aβ40 and Aβ1–40 levels were determined twice in each animal 15 min apart. Each assay was done in triplicate (the mean CV is 7.1%). The Aβ40 and Aβ1–40 levels are the means of the two measures (C.V. between means was less than 5.0%). Cortical blood flow was calculated from the measurement of bilateral common carotid blood flow as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Pig</th>
<th>Right carotid Aβ40 (Aβ1–40) levels</th>
<th>Right jugular Aβ40 (Aβ1–40) levels</th>
<th>Brain Aβ40 A-V (Aβ1–40) gradient</th>
<th>Bilateral CCBF</th>
<th>Cortical blood flow</th>
<th>Brain Aβ40 (Aβ1–40) production</th>
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<td>253 (227)</td>
<td>311 (264)</td>
<td>58</td>
<td>231 (47)</td>
<td>135 (109)</td>
<td>18.5 × 10⁹ (15.0 × 10⁹)</td>
</tr>
<tr>
<td>2</td>
<td>179 (246)</td>
<td>212 (286)</td>
<td>33</td>
<td>250 (40)</td>
<td>147 (109)</td>
<td>11.4 × 10⁹ (13.8 × 10⁹)</td>
</tr>
<tr>
<td>3</td>
<td>215 (230)</td>
<td>259 (261)</td>
<td>44</td>
<td>227 (31)</td>
<td>133 (9.1 × 10⁹)</td>
<td>12.8 × 10⁹ (9.1 × 10⁹)</td>
</tr>
<tr>
<td>4</td>
<td>415 (299)</td>
<td>439 (326)</td>
<td>24</td>
<td>283 (27)</td>
<td>166 (10.3 × 10⁹)</td>
<td>9.4 × 10⁹ (10.3 × 10⁹)</td>
</tr>
<tr>
<td>5</td>
<td>212 (241)</td>
<td>253 (268)</td>
<td>41</td>
<td>240 (27)</td>
<td>141 (8.9 × 10⁹)</td>
<td>13.5 × 10⁹ (8.9 × 10⁹)</td>
</tr>
</tbody>
</table>

DISCUSSION

The Quantity and Speed of APP Processing and Its Implications for Alzheimer Disease—To develop an effective strategy for reducing Aβ production in pathological conditions, it is important to precisely understand the quantity of APP metabolized under normal circumstances, as well as its pathways for degradation and Aβ release. In our cultures, APP is processed in excess of 10 molecules/neuron/s, and the vast majority of its metabolism leads to the extracellular release of its cleavage products. The only portion of APP metabolism that is slow is its surface component. Although we describe APP and Aβ release using the phrase “per neuron per second” as a means of conveying the amount released, the amount of Aβ that individual neurons release may differ substantially depending on the subclass of neuron, and there may be time relevant, activity dependent, fluctuations in APP and Aβ secretion on a second by second scale (21).

Aβ is produced in 1 of 3 cleavages of APP in vitro and is produced and released from the brain at a similarly large rate in vivo. Although the rapid turnover of APP might be consistent with a role as a tightly regulated growth factor (22) or regulatory enzyme, the large amounts of APP and all its degradative metabolites that are produced and secreted by neurons would argue against these roles for the APP ectodomain and its peptide metabolites. The rapid turnover and large quantities of APP we have seen metabolized in vivo and in vitro is consistent with a proposed role for APP as a cargo protein in fast axoplasmic transport (23) with a compulsory degradation and release after one trip down the axon, although the significance of this role for APP is still in dispute (24). Our data are also consistent with work that suggests that large amounts of the APP ectodomain shed into the extracellular space may serve a nonspecific neuroprotective role (25). Our work is consistent with that of others (7, 9) who have shown large amounts of Aβ accumulate in the supernatant of cultured neurons. The work by Seubert et al. (7) is particularly relevant as they measured 72-h accumulation of Aβ in the supernatant of cultured human neurons at 4 ng/ml, a number not inconsistent with our results (by extrapolation). Our work more precisely quantitates Aβ release, rather than accumulation, and quantitatively places Aβ release in the overall context of APP metabolism. Moreover, our results in vivo are an important extension and validation of the tissue culture work.

The large quantities of soluble APP ectodomain we have seen released in these cultures and presumably in vivo needs to be reconciled with the recent work suggesting sAPPβ is a ligand for DR6-mediated axonal pruning (26). In this formulation, growth factor deprivation triggers BACE1 cleavage of APP releasing the ectodomain, which then binds to DR6 and activates caspase 6 and caspase 3, causing axonal and cell body apoptosis, respectively. How this could become manifest in an environment in which sAPPβ is in such plentiful supply is unclear, suggesting that quantities of sAPPβ may be more restricted in specific compartments. One further implication of the large quantities of APP and Aβ that are produced in vitro and in vivo is that clearance mechanisms (27, 28) and their regulation by aging should assume a more important target for translational research efforts.

Secretion of the APP Ectodomain and the Aβ Peptide—Our data suggests a tight relationship between the amount of β-cleavage of APP and the extracellular release of the sAPPβ ectodomain fragment and the Aβ peptide. In addition, most
Aβ Peptide Release

APP synthesized by these neurons appears in the extracellular space in short order. Reports of the amount of APP metabolism that leads to extracellular release versus intracellular accumulation have been variable depending on the cell lines studied and constructs used (10, 29, 30). In our neurons, processing endogenous APP, most Aβ is released into the extracellular medium. Intraneuronal Aβ has been implicated in the early pathogenesis of AD (31) and studies have documented the intracellular accumulation of Aβ and its effects on neurodegeneration (30–32). In this regard, it should be emphasized that even if only a small percentage of APP metabolism led to intracellular Aβ retention, this would still represent a large quantity of Aβ, given the overall quantities we have documented. Moreover, under pathological conditions the ratio of intracellular versus extracellular accumulation could certainly vary.

The Slow Turnover of Surface APP Suggests New Models for APP Metabolism—Our data shows a biexponential metabolism of APP and further suggests that the component of APP with the slower turnover rate is the surface component. The quantity of APP with a slow metabolic turnover rate matches the percent of the surface component of APP relative to the overall pool. Moreover, the slow rate of surface APP turnover is consistent with the slow phase of metabolism from metabolic studies. These observations are consistent with prior observations by Storey et al. (11) who described two pools of APP in cultured neurons with different metabolic half-lives. This group also presented evidence that the slowly metabolized pool was the surface component of APP. Our work extends these important results in a more quantitative fashion, accounting for nearly all APP metabolism and release. These results are important because the current model of APP metabolism postulates a cell surface phase for the full-length amyloid precursor protein prior to and after a model (6). If the surface component of APP has a much slower turnover rate than the total cellular pool of APP, it would exclude it from being the obligate precursor to metabolism of the total pool. However, other explanations are possible. First, there could be a second pool of surface APP that turns over so fast that it never amounts to a significant component of the total surface pool even though it might still be the main pathway for APP catabolism. Our results are also consistent with a model in which the majority of α- or β-cleavage occurs prior to the surface, except for the small pool of slowly metabolized, full-length, surface APP, and that reinternalization is only necessary for γ-cleavage. Whether the slowly metabolized surface pool serves a unique role or has simply escaped normal cellular processing mechanisms is unclear. Glutamate receptors are known to be stabilized on the cell surface by their synaptic localization (14), but we have not observed the same degree of synaptic localization for surface APP as is seen with glutamate receptors. Interestingly, however, the turnover rate for surface APP is quite similar to the turnover rate for a mixture of synaptic and nonsynaptic glutamate receptors (14), potentially offering a clue to the mechanism for degradation of these molecules. Our finding that much surface APP is non-synaptic and that the majority of synapses lack clustered APP is different from some previous reports (33, 34) but more consistent with other reports, which suggest surface func-

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Large Quantities of Aβ Peptide Are Constitutively Released during Amyloid Precursor Protein Metabolism in Vivo and in Vitro
Abhay Moghekar, Sneha Rao, Ming Li, Dawn Ruben, Andrew Mammen, Xiaopei Tang and Richard J. O'Brien


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