Amyloid Precursor Protein Revisited

NEURON-SPECIFIC EXPRESSION AND HIGHLY STABLE NATURE OF SOLUBLE DERIVATIVES

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Background: Endogenous APP expression in CNS has not been rigorously examined.
Results: We characterized the expression, localization, and stability of endogenous APP and APPsβ using a highly specific antibody.
Conclusion: APP is a neuron-specific protein under basal and neuroinflammatory conditions, and soluble APP is highly stable.
Significance: Our studies clarify important properties of APP, which have direct implications in APP biology and AD pathogenesis.

APP processing and amyloid-β production play a central role in Alzheimer disease pathogenesis. APP has been considered a ubiquitously expressed protein. In addition to amyloid-β, α- or β-secretase-dependent cleavage of APP also generates soluble secreted APP (APPSα or APPsβ, respectively). Interestingly, APPsβ has been shown to be subject to further cleavage to create an N-APP fragment that binds to the DR6 death receptor and mediates axon pruning and degeneration under trophic factor withdrawal conditions. By performing APP immunocytochemical staining, we found that, unexpectedly, many antibodies yielded nonspecific staining in APP-null samples. Screening of a series of antibodies allowed us to identify a rabbit monoclonal antibody Y188 that is highly specific for APP and prompted us to re-examine the expression, localization, and stability of endogenous APP and APPsβ in wild-type and in APPsβ knock-in mice, respectively. In contrast to earlier studies, we found that APP is specifically expressed in neurons and that its expression cannot be detected in major types of glial cells under basal or neuroinflammatory conditions. Both APPsα and APPsβ are highly stable in the central nervous system (CNS) and do not undergo further cleavage with or without trophic factor support. Our results clarify several key questions with regard to the fundamental properties of APP and offer critical cellular insights into the pathophysiology of APP.

APP is a type-I transmembrane protein with high abundance in the CNS (1). APP undergoes alternative splicing to generate APP mRNAs encoding proteins of 695, 751, and 770 amino acids (referred to as APP695, APP751, and APP770). It is generally understood that APP695 is predominantly expressed in neurons, whereas APP751 and APP770 isoforms are expressed in most tissues examined, and their expressions are increased in astrocytes and microglia following brain injury (2–4).

APP is subject to cleavage by a number of secretases. The α-secretase or β-secretase cleaves the APP extracellular domain, generating soluble derivatives termed APPsα or APPsβ, respectively. APPsα has been reported to exhibit neurotrophic and synaptogenic activities (reviewed in Ref. 1). Our recent work indicated a potential signaling activity of APPsβ (5). Intriguingly, Nikolaev et al. (6) reported that APPsβ is further cleaved to create a 38-kDa N-terminal fragment (N-APP) found in the conditioned medium of trophic factor deprived axons in dorsal root ganglion cultures. This APPsβ fragment was shown to be a ligand of the death receptor 6 (DR6) and triggers axon pruning and neurodegeneration (6). These findings raise the question of whether N-APP is also produced in the CNS and to what degree it contributes to APPsβ function.

In this study, we first carefully examined a number of APP antibodies using APP-null samples as controls. We found that although most antibodies show excellent specificity for APP on Western blots, only one of them, APP-Y188, a rabbit monoclonal antibody recognizing the YENPTY motif of APP, is able to clearly distinguish the APP knock-out neurons from wild-type neurons on fluorescence immunocytochemistry. We subsequently characterized APP expression in mouse neurons and glial cells in vitro and in vivo, under resting or traumatic brain injury (TBI) conditions, or in the presence of Aβ plaques in AD mouse models. The results demonstrate that APP is only expressed in neurons and is undetectable in most glial cells examined. Furthermore, taking advantage of our APPsβ knock-in mouse model (5) and using a variety of culture conditions, we report that in contrast to Nikolaev et al. (6), APPsβ is highly stable and remains as an intact protein under normal or trophic factor deprivation (TFD) conditions.
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EXPERIMENTAL PROCEDURES

Animals Used in This Study—Mice were housed 2–5 per cage with ad libitum access to food and water in a room with a 12-h light/dark cycle in a specific pathogen-free mouse facility. All procedures were performed in accordance with National Institutes of Health guidelines and with the approval of the Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC). All animals used in this study were either C57BL6/J wild-type mice or mice on C57BL6/J background. 

Mice were bred together to generate double heterozygous APP<sup>H9252</sup>/H11001 knock-in (ki) mice have been produced homozygous APP<sup>SL/A</sup>/H11001 and APP<sup>SL/A</sup>/H11002 animals, which were intercrossed to produce homozygous APP<sup>H9252</sup>/H11001 and APP<sup>H9252</sup>/H11002 double knock-in mice (APP<sup>hAβ/PS1</sup>).

Antibodies—The following APP-related antibodies were tested and used in this study: APP Y188 (rabbit monoclonal, 1:500, Epitomics), APP A8967 (mouse monoclonal, 1:100–1:1000, Sigma), APP ab15272 (rabbit polyclonal, 1:100–1:1000, Abcam), APPC (rat polyclonal, 1:100–1:1000, generated in the laboratory, (10)), 22C11 (mouse monoclonal, 1:100–1:1000, Millipore), 4G8 (mouse monoclonal, 1:100–1:1000, Covance), APP SIG-39184 (mouse monoclonal, 1:100–1:1000, Covance), APP SIG-39180 (mouse monoclonal, 1:100–1:1000, Covance), and 5A3/1G7 (mouse monoclonal, 1:100–1:1000, courtesy of Dr. Edward Koo (11)). The following antibodies were also used: anti-V5 tag antibody (mouse monoclonal, ab27671, 1:4000, Abcam), anti-DYKDDDDK tag antibody (L5, rat, 1:500, BioLegend), anti-GFAP (mouse monoclonal, MAB360, 1:1000, Millipore), anti-NeuN (mouse monoclonal, MAB377, 1:500, Millipore), anti-CD11b (rat monoclonal, ab8878, 1:100, Abcam), anti-myelin basic protein (MBP) (rat monoclonal, 1:1000, Millipore), anti-KDEL (rat, 1:500, Biosciences), anti-GM130 (mouse monoclonal, 1:500, 610822, BD Biosciences), anti-EEA1 (mouse monoclonal, 1:500, H00008411-M02, Abnova), anti-M6PR (mouse monoclonal, ab2733, 1:500, Abcam), anti-LAMP1 (rat, 553792, 1:1000, BD Biosciences), and anti-Grp78 (mouse monoclonal, 610979, BD Biosciences).

Mouse Primary Hippocampal/Cortical Culture—Cerebral hemispheres from postnatal day 0 mice were isolated, and meninges were carefully removed under the stereoscope in dissection medium (Hanks’ balanced salt solution 1× with 0.1 M HEPES, 0.6% glucose, 100 units/ml penicillin, and 100 μg/ml streptomycin, Invitrogen). Brain tissue was cut into small pieces with a sharp scissor, and tissue pieces were transferred to a 15-ml tube with 10 ml of dissection medium. After adding 500 μl of 2.5% trypsin (Invitrogen), the brain tissue was then incubated in a 37 °C water bath for 15 min with frequent swirling. 400 μl of soybean trypsin inhibitor (1 mg/ml, Invitrogen) was then added to stop the trypsin activity. The dissection medium was replaced with 2 ml of culture medium (Neurobasal™ medium with 2% B-27 supplements and 0.5 mM l-glutamine, Invitrogen). The tissue was dissociated by passing it through a P1000 tip 10–20 times in the presence of DNase (10 μg/ml, Sigma). Dissociated cells were then transferred to a new 15-ml tube and centrifuged at 1,000 rpm for 5 min, washed with 5 ml of culture medium twice, resuspended with 5 ml of culture medium, and plated onto poly-d-lysine (Sigma) precoated 12-mm glass coverslips/plastic 6-well plate at a density of 10,000 cells/cm². One day after seeding, the culture medium was changed.

Immunocytochemistry—Cells on coverslips were fixed with 4% paraformaldehyde (PFA) in PBS at 4 °C overnight. Cells were washed with PBS three times and incubated in blocking solution (PBS with 3% BSA, 2% goat serum, and 0.1% Triton X-100) for at least 1 h at room temperature. Cells were then incubated with primary antibody solution (primary antibody diluted in blocking solution) for at least 2 h at room temperature or overnight at 4 °C. After washing with PBS three times, cells were incubated with secondary antibody solution for 1 h. Before being mounted onto glass slides, cells were washed with PBS three times.

Immunohistochemistry—Anesthetized animals were perfused with 4% PFA in PBS, and brains were dissected out for overnight post-fixation in 4% PFA. Brains were dehydrated, embedded in paraffin, and sectioned at 8 μm. For antibody staining, brain sections were then deparaffinized and rehydrated before being boiled in sodium citrate buffer (10 mM, pH 6.0) for 10 min for antigen retrieval. For plaque staining, sections were incubated in 80% formic acid for 5 min at room temperature. Brain sections were rinsed with PBS three times, incubated with blocking solution (PBS with 3% BSA, 2% goat serum, and 0.1% Triton X-100) for at least 1 h, incubated with primary antibody solution (primary antibody diluted in blocking solution) at 4 °C overnight, rinsed with PBS three times, incubated with secondary antibody solution for 1 h, and then rinsed with PBS three times. For fluorescence staining, brain sections were then mounted with DAPI-containing mounting medium (Vector Laboratories). In the case of diaminobenzidine chemical staining, the Elite ABC staining kit (Vector Laboratories) was used according to the user manual.

TBI—TBI was induced as described previously (12). Briefly, mice were anesthetized with isoflurane and intubated to control ventilation. After the mice were placed and secured in a stereotaxic frame, a 3-mm craniotomy was performed over the right parietal cortex. Injury was induced with controlled cortical impact using a voltage-driven impactor (3 m/sec, 1.5-mm deformation, 100 ms, Benchmark stereotaxic impactor, myNeuroLab, St. Louis, MO). The wounds were then sutured closed, and the mice were monitored until full recovery.

Immunoprecipitation—Immunoprecipitation of APPsβ was performed from brains of APPsβ mice using rat anti-DYKDDDDK (FLAG) tag antibody conjugated to magnetic beads (catalog number 143.11D, Invitrogen) according to the manufacturer’s protocol. Mice were sacrificed under anesthesia induced by isoflurane. Brains were retrieved and homogenized in PBS containing protease inhibitor mixture (catalog number 04693116001, Roche Applied Science), centrifuged at 20,000 × g for 15 min at 4 °C. Pellets were resuspended in PBS containing 1% Triton X-100 and protease inhibitor mixture, rotated for 1 h at 4 °C, and then centrifuged at 20,000 × g for 15 min at 4 °C.
Supernatants were used for immunoprecipitation by adding magnetic beads conjugated with anti-DYKDDDDK antibody. This mixture was incubated with rotation at 4 °C for 1 h, washed in ice-cold PBS containing 0.1% Triton X-100 five times, and eluted using 3× Triton X-100 (F4799, Sigma-Aldrich) at 4 °C with rotation for 30 min. Eluted protein was precipitated by adding 4× volume of acetone and incubating over-night at −20 °C. Protein precipitates were centrifuged, air-dried, and rehydrated in water and then mixed with 2× sample loading buffer for SDS-PAGE followed by Western blot analysis.

Immunoprecipitation of full-length APP was performed in similar manner as above from brains of wild-type mice using APPc rabbit polyclonal antibody and protein-G magnetic beads (catalog number 100.07D, Invitrogen). Proteins binding to the beads were eluted by boiling the beads in sample loading buffer and then subjected to SDS-PAGE and Western blot analysis. Normal rabbit IgG was used to control nonspecific bindings to protein-G beads. Brain lysate from APP−/− mice were used to control any nonspecific bindings of APPc antibody.

**Purification of APPsβ-V5/His Recombinant Protein—Mouse** APPsβ was cloned into the pcDNA3.1-TOPO-V5/His (Invitrogen) mammalian expression vector with the V5/His tag fused to the C terminus of APPsβ. This pcDNA3.1-APPsβ-V5/His vector was transfected into CHO-S cells (Invitrogen) using Lipofectamine 2000 (Invitrogen). Stable clones were selected in serum-containing selection medium (1 mg/ml G418, 10% FBS, and 1× nonessential amino acids in DMEM medium, Invitrogen). After selecting a stable clone with the highest expression/secretion level of APPsβ-V5/His, this clone was further expanded in serum-containing selection medium. When the culture density reached 80% confluency, the medium was replaced with serum-free chemically defined medium (CDCHO medium containing 8 mM-glutamine and 10 ml/liter HT supplement, Invitrogen) for another 5 days. The conditioned serum-free medium was collected, centrifuged to remove large cell debris, passed through a 0.45-μm filter, and loaded onto a HisTrap® column (GE HealthCare). The APPsβ-V5/His was then purified by eluting the column with a gradient of imidazole in PBS. The fractions containing APPsβ-V5/His recombinant protein were dialyzed against PBS, filtered with a 0.20-μm filter, aliquoted, and stored in a −80 °C freezer.

**RESULTS**

**Identification of a Specific Antibody for APP Immunostaining—** A variety of APP antibodies are available either commercially or from academic research laboratories. Although these antibodies have been extensively used for biochemical and immunohistochemical assays, we found that when performing immunohistochemical staining and comparing wild-type with APP-null neuronal cultures, most of the antibody stainings were indistinguishable between the two cultures, suggesting nonspecific staining for endogenous APP. We therefore tested a selection of APP antibodies from different origins and against different epitopes on fixed mouse primary hippocampal cultures (Table 1 and Fig. 1A) and identified a rabbit monoclonal antibody (Y188), raised against the C-terminal sequences of APP, that displayed strong staining in wild-type neurons but almost no staining in APP KO neurons. Y188 antibody staining is also highly specific in paraffin-fixed brain sections (Fig. 1B). In addition, Western blot analysis showed that only the full-length APP, but not α- or β-secretase cleaved C-terminal fragments, is produced in cultured neurons at physiological levels (data not shown). As such, the Y188 antibody recognizes full-length APP translated from all mRNA isoforms (herein referred to as APP or FL-APP). Although different experimental conditions may give rise to different results, our data call for the importance of using APP-deficient samples as negative controls for APP expression studies.

**APP Is Specifically Expressed in Neurons but Not in Major Glial Cells—** We subsequently examined endogenous APP expression and localization using the Y188 antibody. Unexpectedly, we found that in primary hippocampal neurons cultured 14 days in vitro (DIV), we could only detect APP in NeuN-positive neurons, but not in GFAP-positive astrocytes (Fig. 2A). Another two major CNS glial cell types, microglia and oligodendrocytes, were not present in our in vitro culture system (data not shown). Therefore, we performed APP immunohistochemical staining on adult mouse hippocampal sections. Consistent with the neuronal culture result, the NeuN-positive CA1 neurons abundantly express APP, but nearby GFAP-positive astrocytes, CD11b-positive microglia, and MBP-positive oligodendrocytes were devoid of APP expression (Fig. 2B). Because APP has been shown to be induced in activated astrocytes under neuroinflammation or brain injury conditions (2–4), we further investigated possible astrocytic APP expression using several conditions known to lead to astrocyte activation. We first treated primary hippocampal cultures with 10 or 50 ng/ml human TNFα, concentrations known to trigger neuroinflammation in vitro (13) (Fig. 3A). When compared with vehicle controls, GFAP immunoreactivity increased dramatically in a dose-dependent manner in TNFα-treated groups, and astrocytes displayed hypertrophic morphology, suggesting activation. However, similar to vehicle-treated controls, APP expression was not detected in the reactive astrocytes (Fig. 3A). Next we tested APP expression in vivo in response to TBI using the controlled cortical impact protocol (Fig. 3B). When compared with uninjured controls where APP staining is observed within neuronal cell bodies (Fig. 3B, Control), 24 h after TBI, we observed the presence of punctate APP staining at the injury site, presumably in the swelling axons as reported previously (14, 15). Although there are limited GFAP-positive cells at this

**TABLE 1**

| Antibody     | Species  | Epitope     | ICC specificity |
|--------------|----------|-------------|-----------------|
| Y188         | Rabbit mAb | YENPTY motif | High            |
| A8967        | Rabbit pAb | 46–60 aa    | No              |
| Ab15272      | Rabbit pAb | 44–62 aa    | No              |
| APPc         | Rabbit pAb | C terminus  | No              |
| 22C11        | Mouse mAb  | 66–81 aa    | No              |
| 4G8          | Mouse mAb  | AP17–24 aa  | No              |
| APP 39184    | Mouse mAb  | 61–77 aa    | No              |
| APP 39180    | Mouse mAb  | 573–596 aa  | No              |
| 5A3/1G7      | Mouse mAb  | N terminus  | Low             |

**Characteristics of Full-length and Soluble APP**

General characteristics of the APP antibodies and their specificity tested by immunohistochemical staining of 14 DIV wild type and APP null primary hippocampal cultures fixed with 4% PFA are shown. The origins of the antibodies can be found under “Experimental Procedures.” ICC, immunocytochemistry; mAb, monoclonal antibody; pAb, polyclonal antibody; aa, amino acids.
stage, profound reactive astrocytosis can be seen 72 h after TBI (Fig. 3B). Consistent with the \textit{in vitro} culture experiment, APP expression is absent in GFAP-positive reactive astrocytes in brain tissue after TBI. It is interesting to note that the induction of GFAP immunoreactivity at this stage is correlated with diminished APP punctates, suggesting that these reactive astrocytes play an active role in clearing APP deposits. Lastly, we examined APP expression in a homozygous knock-in mouse model of AD expressing APP with a humanized A\textsubscript{β} (4G8) and APP (Y188) co-immunostaining showed profound APP immunoreactivity surrounding the core of amyloid plaques (Fig. 3C), likely due to its accumulation in dystrophic neurites. However, although abundant GFAP-positive reactive astrocytes can be observed in the vicinity of amyloid plaques, these astrocytes are devoid of APP expression (Fig. 3C). Taken together, we conclude that endogenous APP expression is extremely low or absent in astrocytes under physiological or pathological conditions induced by TBI or AD pathology.

\textbf{Subcellular Localization of APP in Neurons—}APP has been reported to be localized to the plasma membrane (20), the ER and endosomes (21), and the Golgi apparatus (22, 23) and accumulated in the lysosomes (24). Because these studies were performed using overexpression systems or employing antibodies that may result in nonspecific staining, we thought to re-evaluate the subcellular localization of endogenous APP in primary wild-type neuronal cultures using the Y188 antibody and co-staining with a panel of organelle markers, including ER (KDEL), Golgi complex (GM130), early endosomes (EEA1), late endosomes (M6PR), and lysosomes (LAMP1) (Fig. 4, A–E). We found that APP is largely localized to the Golgi complex and late endosomes. Its localization is limited in the ER and early endosomes and almost completely absent in lysosomes.

We previously created a strain of FLAG-tagged APPs\textsubscript{6} ki mice by inserting the FLAG tag and stop codon immediately after the BACE1 cleavage site (5). The resulting knock-in allele expresses truncated APPs\textsubscript{6} fused with the FLAG tag and lacks the transmembrane and the intracellular domains. We reported that the APPs\textsubscript{6} is expressed and secreted similar to wild-type APP (5). Because APP is known to undergo intracellular processing, to understand the cellular dynamics of APP
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APP\textsubscript{β} is Highly Stable under Normal and TFD Conditions—Nikolaev et al. (6) showed that in dorsal root ganglion culture, APP\textsubscript{β} is further cleaved upon TFD to produce a truncated product named N-APP, which serves as a DR6 ligand to mediate axon pruning and degeneration (6). Generation of the APP\textsubscript{β} knock-in mice and the demonstration of the normal secretion of the APP\textsubscript{β} protein afford us with a unique opportunity to examine this cleavage event in the absence of APP\textsubscript{so}. We thus investigated the stability and metabolism of APP\textsubscript{β} in the APP\textsubscript{β} ki/\textit{ki} primary neuronal cultures while using wild-type cultures for total APP\textsubscript{so} and APP\textsubscript{β}. We collected 5% of the conditioned medium from 1, 5, 9, 13, 17, and 21 DIV cultures and replaced it with the same amount of fresh medium to maintain the total culture volume. Western blot analysis of the conditioned medium using the 22C11 antibody, which detects APP\textsubscript{so} and total APP\textsubscript{so} and APP\textsubscript{β}, showed that the sole band detected was ~100 kDa, representing intact APP\textsubscript{so}. No lower molecular weight product resembling N-APP was detectable.

We next washed the APP\textsubscript{β} and wild-type neurons with fresh medium multiple times at 7 DIV, which depleted all the trophic factors secreted from the neurons, and then we replaced the medium with fresh medium for 24, 72, or 120 h (Fig. 5, A and B). Only a small amount of neurons survived from the severe TFD, which accumulated overtime along with increased APPs. However, no APP cleavage products could be detected at any
time following the TFD. To rule out the possibility that there was not enough APPs as substrate, we added purified V5/His-tagged APPsβ recombinant protein into the fresh medium in wild-type cultures immediately after TFD to test the existence of any enzymatic activity induced by TFD. Western blot analysis using the anti-V5 antibody showed that there was no difference between APPsβ exposed to TFD-treated neurons or fresh cell-free medium and that recombinant APPsβ protein is stable under both conditions (Fig. 5C). Taken together, we conclude that APPs is highly stable under normal or TFD conditions in the CNS.

DISCUSSION

In this study, we first tested a panel of APP antibodies to distinguish wild-type versus APP KO neurons by immunocytochemistry and identified a highly specific and potent APP antibody, Y188, which yields specific staining in paraffin-fixed brain sections. Taking advantage of this antibody, we show that only neurons, but not astrocytes, microglia, nor oligodendrocytes, express APP under basal or stressed conditions. Endogenous APP is mainly localized to the Golgi, whereas APPsβ expressed in APPsβ knock-in neurons is found to be enriched in the ER where it binds with the ER chaperone Grp78. Furthermore, biochemical analyses of total soluble APP or APPsβ expressed in wild-type or APPsβ knock-in neurons, respectively, allow the conclusion that secreted APP extracellular proteins are highly stable in regular or trophic factor deprivation conditions.

Neuron-specific Expression of APP and Its Implications in TBI and AD—In performing APP immunostaining using APP-null samples as controls, we found that surprisingly, many well established antibodies failed to distinguish between APP KO and wild-type neurons, indicating nonspecific staining under the conditions we employ. Although we cannot exclude the possibility that protocols used by other investigators may result in more specific signal, our result calls for the necessity of using APP knock-out mice
It has been accepted that neurons serve as the primary source of APP in the CNS. However, APP, in particular the 751/770 isoforms, have also been reported to be expressed in astrocytes (2, 3, 28–32). One study even stated that the total amount of astroglial APP is 90% higher than neurons (4). In addition, there are numerous studies reporting increased APP expression in reactive astrocytes induced by different insults, such as lesions or injury of the hippocampus (4, 33, 34), heterotopic transplantation (35), cerebral artery occlusion (36), needle stab injury (14), cuprizone intoxication (37), kainic acid-induced neurotoxic damage (38), and exposure to Aβ peptides (39). Increased APP expression in astrocytes was also suggested to contribute to Aβ plaque deposition and AD pathogenesis (4, 40). We show here that under basal conditions, we could not detect astroglial APP staining in primary cultures or in brain sections. APP expression remained undetectable in astrocytes when these cells were activated by TNFα treatment in vitro or upon traumatic brain injury in vivo. The punctate APP staining following TBI is likely due to its accumulation in the damaged neuronal axons (14, 15). Interestingly, axonal APP accumulation is transient, and most of the staining disappeared within 72 h of the TBI, when significant reactive astrocytosis takes place. Although correlative, this finding indicates that the activated astrocytes may play an active role in clearing APP deposits and possibly damaged axons. On the other hand, the transient and restricted nature of APP accumulation suggests that it is secondary to axonal damage rather than serving a primary role in the TBI response. In this regard, it is worth noting that TBI has been associated with increased risk of dementia (41). However, a consistent relationship between TBI and amyloid pathology has been difficult to establish in mouse models (41). Our APP expression analysis argues against a major effect of TBI in inducing Aβ pathology through astrocytic APP. Previous publications suggest that APP-independent processes, such as Tau modulation or neuroinflammation, are likely to play important roles in TBI-induced dementia (42, 43).

Examination of APP expression in an APP/hAβ/PS1 knock-in AD mouse model detected strong APP staining surrounding Aβ plaques, presumably originating from dystrophic neurites (44–46). However, and consistent with its neuron-specific expression, no APP can be detected in reactive astrocytes in the proximity of Aβ deposits. Because reactive astrocytosis and APP aggregates co-exist, the activated astrocytes do not appear to be effective in clearing APP deposits and dystrophic neurites. The reason for the apparently distinct actions of astroglia in the TBI and the AD model is not clear and may be due to differences in the nature of the insults (acute versus chronic). Nevertheless, these studies, combined, provide strong evidence that astrocytes express negligible amounts of APP under basal or stress/injury conditions and that neurons provide most, if not all, APP and, by extension, Aβ peptides.

**FIGURE 5. APPsβ is a highly stable protein.** A and B. Western blot analysis using the 22C11 antibody on conditioned medium collected from APPsβ ki/ki (A) or wild-type (B) cultures under normal neuronal culture conditions (day 1, 5, 9, 13, 17, and 21), at day 7 immediately before TFD, or 24, 72, or 120 h after TFD, showing the intact nature of APPs recovered from residual surviving neurons. C, anti-V5 Western blot of fresh medium containing APPsβ-V5/His recombinant protein incubated with TFD-treated neurons or cell-free culture for 24 h, 72 h or 120 h.
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greater responses to ER stress, was reported to decrease the risk of AD (49). We, for the first time, validated this interaction in an endogenous model in vivo and identified the APP N-terminal domain as its interaction site. It is tempting to speculate that this interaction indirectly contributes to Aβ production and AD pathogenesis through regulating APP maturation and trafficking and that molecules that can modulate the APPs-Grp78 interaction provide a potential therapeutic target.

An intriguing study reported that secreted APPsβ is cleaved upon TFD and that an N-terminal fragment generated from this cleavage (N-APP) serves as a DR6 ligand to trigger axonal degeneration (6). The creation of the APPsβ knock-in mice offers a unique opportunity to examine this cleavage event in the absence of APPsα and compare it with total soluble APP produced in wild-type neurons (5). Using regular neuronal cultures or trophic factor-depleted cultures, and by co-incubating the recombinant APPsβ with TFD-treated cells, we demonstrate that APPsβ and total APPs are both highly stable, and no further cleavage could be identified. Although intrinsic differences between primary cortical and dorsal root ganglion cultures or differences in the culture conditions could explain the different results found by us or by Nikolaev et al. (6), our results using CNS cultures nevertheless argue against a significant role of the N-APP/DR6 pathway in APP biological function or pathogenic processes.

In this study, we employed a highly specific APP antibody and an APPsβ knock-in mouse model and reinvestigated some of the fundamental biological properties of APP and APPs in vitro and in vivo. Our data demonstrate that endogenously produced APP is a neuron-specific protein not expressed in major types of glial cells. APP ectodomain binds to Grp78 in the ER, whereas the membrane and intracellular sequences of APP are required for its localization to the Golgi apparatus. Both neuron-secreted and recombinant APPsβ are highly stable and remain intact upon trophic factor deprivation. We conclude that neuronal APP is the origin of Aβ in both basal and brain injury conditions and that the APP ectodomain mediates downstream effects as a stable and intact protein.

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