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Macromolecules Involved in Production and Metabolism of Beta-Amyloid at the Brain Barriers

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Abstract

One of the notable features of Alzheimer's disease (AD) is the overabundance of beta-amyloid peptides in brain fluids, leading to the formation and deposition of insoluble amyloid plaques. Previous work in this lab demonstrates that the normal choroid plexus, a primary component of the blood-cerebrospinal fluid barrier, has the capacity to remove beta-amyloid from the cerebrospinal fluid, potentially preventing the formation of beta-amyloid plaques. The purpose of this work was to determine whether the choroid plexus and/or the brain capillaries, a primary component of the blood-brain barrier, possessed the capacity to produce or degrade beta-amyloid peptides. Using quantitative real-time RT-PCR, immunodetection and enzyme activity assays, we demonstrated the presence in brain barriers of several key enzymes involved in beta-amyloid production, namely, amyloid precursor protein and beta-secretase, and in beta-amyloid metabolism and alternate processing, such as insulin degrading enzyme, endothelin-converting enzyme-1, neprilysin and alpha-secretase. Furthermore, beta-amyloid presence, in the absence of its application in culture media, was detected in an immortalized choroidal epithelial cell line, known as Z310 cells. The ability of the choroid plexus to produce and degrade beta-amyloid, in addition to its transport function, suggests a vital role of this tissue in maintaining beta-amyloid homeostasis. Disruption of this homeostasis due to aging, injury or toxicant exposure may contribute to accumulation of beta-amyloid peptides in the brain fluids, leading to AD.

Keywords

Alzheimer's disease; brain barriers; choroid plexus; beta-amyloid; beta-secretase; neprilysin

1. Introduction

The accumulation and aggregation of beta-amyloid peptides into insoluble plaques are thought to be crucial steps in the etiology of Alzheimer's disease (AD). The amyloid cascade hypothesis suggests that stopping or slowing formation of the A β plaques would delay the onset of the disease symptoms. Beta-amyloid (A β) is found in the extracellular fluids of the brain, including the cerebrospinal fluid (CSF) supporting the brain and circulating in brain ventricles, and the interstitial fluid surrounding the neurons and glial cells in brain lobes (Price et al., 1998; Selkoe, 2001; Seubert et al., 1992; Vigo-Pelfrey et al., 1993). It is unclear, however, whether the

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accumulated A β is produced inside the brain or imported across brain barriers from other tissues.

Brain barriers contribute to the etiology of AD mainly in three aspects: (i) the aging of cerebral vascular structure in the overall aging process of the brain, (ii) as the site of transport of extracerebral A β into the brain, and (iii) the ability to prevent A β aggregation by removing it (Zheng, 2001). The blood-brain barrier (BBB) separates blood from the interstitial fluid surrounding the neurons and neuroglia, and the blood-CSF barrier (BCB) separates the blood and CSF in and around the brain. Several studies have shown the presence of A β and its transport across the BBB (Deane et al., 2003;Mackic et al., 2002;Pluta et al., 2000;Poduslo et al., 1999;Shibata et al., 2000;Zlokovic, 2004) and BCB (Crossgrove et al., 2005;Monro et al., 2002;Sasaki et al., 1997;Serot et al., 2003;Serot et al., 2001). The choroid plexus, a major component of the BCB, has been demonstrated to be immunoreactive to antibodies against A β and its precursor protein, APP (Sasaki et al., 1997), but the origin of the A β peptide has not been definitively established. Our recent finding suggests that the choroid plexus may remove A β from the CSF (Crossgrove et al., 2005). It is also possible that the choroid plexus may metabolize A β sequestered from the CSF, as it exhibits high levels of metabolic enzymes effective in the detoxification of endogenous compounds, drugs and other xenobiotics (Gherssi-Egea et al., 1994;Lee et al., 2001;Morse et al., 1998;Philbert et al., 1995).

The objective of this work was to determine whether the choroid plexus produces and/or degrades A β peptides. We investigated the presence of several key enzymes involved in A β production and metabolism, including amyloid precursor protein (APP), α - and β -secretase, insulin degrading enzyme (IDE), endothelin-converting enzyme-1 (ECE), and neprilysin. We also examined the presence of A β in an immortalized cell line of choroid plexus epithelia, known as Z310 cells, established in this lab as a model for the BCB (Zheng and Zhao, 2002). Taken together with the body of knowledge concerning β -amyloid homeostasis, we sought to identify the relative roles of the BCB and the BBB in A β production and metabolism.

2. Results

By real time RT-PCR analysis, the choroid plexus, the brain capillaries and surrounding parenchyma, and Z310 cells expressed genes capable of producing and metabolizing A β peptides (Fig. 1 and 2). APP and β -secretase contribute to the production of A β peptides, while the remaining genes function to degrade the peptides (ECE-1, IDE and neprilysin) or form an alternate APP product (α -secretase). The brain capillaries in the striatum tended to have relatively higher expression of A β producing and metabolizing genes than the choroid plexus (Fig. 2). This was not due to a difference in GAPDH expression in the choroid plexus compared to the striatal brain capillaries (28.3 ± 1.4 ng vs. 27.3 ± 1.8 ng, respectively; mean \pm SEM; $p = 0.67$). In fact, there were no statistically significant differences in GAPDH expression among any of the regions tested by one-way ANOVA with post hoc comparisons using Bonferroni's test for multiple comparisons. Furthermore, the brain capillaries from the striatum had significantly greater expression of these genes than the surrounding brain parenchyma and greater expression than the capillary endothelia of the other tested regions. Compared to the immortalized choroidal epithelial cell line, the freshly isolated choroid plexus usually had a greater expression of the selected genes.

The choroid plexus clearly produces APP protein as demonstrated by Western blot analysis and immunohistochemical staining (Fig. 3). The band in both choroid plexus tissue and choroidal Z310 cells (Fig. 3A) corresponds to the band in the positive control, the whole brain homogenate. The upper band (about 150 kDa) in Fig. 3A is a non-specific band that was uniquely eliminated when the primary antibody was pre-exposed to APP (data not shown).

Immunocytochemical staining further confirmed the presence of APP in Z310 cells (Fig. 3C). Negative controls, including a lack of primary antibody (Fig. 3B) and the addition of antibody that was pre-exposed to APP (data not shown), revealed no specific binding to the cells, suggesting that the APP staining was not an artifact. Subsequently, the presence of A β peptides was detected in Z310 cells (Fig. 4A&B) as well as in the choroid plexus tissue (Fig. 4C&D). Within the choroid plexus tissue, A β appears to be present both intracellularly and along the plasma membrane. It should be noted that the culture medium of Z310 cells did not contain exogenous A β peptides. Furthermore, any bovine A β that may be present from the serum would not be expected to react with the 6E10 antibody against A β , since the predicted bovine sequence of A β has at least eight amino acid substitutions within the 17-amino acid immunogenic fragment.

The level of neprilysin mRNA in our experiment (Fig. 2) appeared to be lower than that reported in the literature (Matsas et al., 1986). This may be due to an inefficient primer. Nonetheless, our immunohistochemical data confirmed the presence of neprilysin in the choroid plexus tissue (Fig. 5). As a positive control, the kidney also produced a significant signal in the glomeruli (data not shown). Z310 cells did not exhibit any significant immunoreactivity to the neprilysin antibody (data not shown), suggesting that the epithelial cell line lost neprilysin expression following immortalization.

The enzymatic assay of β -secretase activity revealed a detectable enzyme activity in all tested samples with an activity order of choroid plexus > BBB > Z310 cells (Fig. 6). The activity is consistent with the design of the assay, with 5–20% of the substrate being cleaved. The β -secretase activity in the choroid plexus, the structural basis of BCB, was 38% higher than that in brain capillary endothelia, which comprise the BBB.

3. Discussion

The brain barrier system, i.e., BBB and BCB, protects the central nervous system against the imbalanced buildup of neuroactive materials such as toxic chemicals, endogenous metabolites, and macromolecules (Zheng et al., 2003). The dynamic homeostasis of A β in brain extracellular fluid compartments, namely interstitial fluid and CSF, is presumed to be similarly maintained by both the BCB and BBB. Our previous study has established that the choroid plexus is capable of capturing A β molecules in the CSF with a relatively large capacity (Crossgrove et al., 2005). This study was designed to address whether the choroid plexus possesses the ability to produce A β , whether there is an enzymatic system in the tissue to degrade the captured A β , and what the relative role of the BCB is in comparison to BBB on these aspects.

Our data clearly demonstrate that both the BBB and the BCB are capable of producing A β peptides. Consistent with previous reports of APP expression in the choroid plexus and BBB (Kalaria et al., 1996; Sasaki et al., 1997), the mRNA expression of APP and β -secretase were confirmed by these protein and activity assays. While we did not directly examine γ -secretase activity or the presence of any of its four subunits (presenilin-1 or -2, nicastrin, Aph-1, and presenilin enhancer-2) in the choroid plexus, the presence of a correctly-cleaved APP product (A β) in the cultured choroidal cell line, in the absence of its addition in the cell culture medium, implies that both β - and γ -secretase are present and active in the choroid plexus. Other studies have confirmed the existence of γ -secretase mRNA and protein complex (determined by immunoprecipitation with presenilin) in whole brain (Hebert et al., 2004), but not specifically at either barrier.

This study also provides evidence that the BBB and BCB are capable of metabolizing A β by IDE, ECE and/or neprilysin, the enzymes known to degrade A β (Eckman and Eckman, 2005). This is consistent with previous reports in the literature of IDE in cultures of human

brain endothelial cells (Lynch et al., 2006), ECE in human brain cerebral vessels (Davenport and Kuc, 2000; Vatter et al., 2002) and in rat choroid plexus (Sluck et al., 1999), and neprilysin protein expression in choroid plexus (Matsas et al., 1986) and its activity in bovine brain microvasculature (Brownson et al., 1994). Although we were unable to determine the protein level of IDE due to the difficulty in obtaining rat-specific antibody, our real-time RT-PCR data indicated that the gene was being transcribed in the choroid plexus at a rate marginally greater than in the BBB ($p=0.06$). The presence of α -secretase in the BBB and BCB, which processes APP into a non-amyloid product, was also confirmed by PCR in this study.

The presence of these enzymes in both the BBB and the BCB raises several interesting questions for further investigation. First, in which direction(s) do the brain barrier systems secrete the produced $A\beta$ and to what extent does this contribute to the overall $A\beta$ homeostasis? The finding that brain capillary endothelia possess a significantly higher level of APP and β -secretase than brain parenchyma is remarkable. $A\beta$ production has been commonly viewed as a process uniquely related to the functions or malfunction of neurons and neuroglia. Our data provide evidence of an additional source of brain $A\beta$ that is derived from brain barrier cells. As the function of brain barriers deteriorates with aging and is affected by exposure to other damaging substances in blood circulation (Serot et al., 2001; Shah and Mooradian, 1997; Zheng et al., 1996), there is sound reasoning to postulate that the damaged brain barriers may become the primary source of $A\beta$ in the brain, which in turn facilitates the course and progression of AD. Clearly, an in-depth study is warranted to explore the contribution of brain barriers in the production of brain $A\beta$.

Secondly, which barrier plays a more important role in $A\beta$ production and processing? The BBB expression of these genes was remarkably region-specific. For example, only the striatal BBB expressed significantly more APP, β -secretase and neprilysin than the choroid plexus. Interestingly, these same genes were also expressed significantly less in the neurons and neuroglia comprising the striatal parenchyma than in the striatal BBB. The striatal BBB also expressed more APP, β -secretase and neprilysin than the capillary endothelia of the other tested regions. There were no differences between or among the capillary endothelia and brain parenchyma of any other tested region, nor did they differ from the choroid plexus. Because the striatum had more expression than the BCB for both production (APP and β -secretase) and metabolism (neprilysin) enzymes, it is difficult to determine whether the striatal BBB increases or decreases the net $A\beta$ amount in the normal brain and how that contribution may compare to the $A\beta$ production by the BCB. That the choroid plexus has marginally more IDE than the combined BBB is important due to a recent finding that IDE inhibitors had a greater effect on $A\beta$ degradation than either ECE or neprilysin inhibitors in a BBB cell model (Lynch et al., 2006). Since $A\beta$ permeability at the choroid plexus is about 10-fold greater than that of the BBB in either flux direction (Crossgrove et al., 2005; Strazielle et al., 2000), and the choroid plexus surface area has been estimated to be about half that of the BBB (Keep and Jones, 1990), it is possible that the choroid plexus sequesters five times more $A\beta$ than the BBB. Thus, even a marginal increase in IDE expression at the BCB versus the BBB may contribute to considerably more $A\beta$ degradation by this barrier. It is therefore tempting to presume that the BBB of the striatum may play a larger role in the volume of $A\beta$ produced than other tested regions, whereas the BCB may have a larger role in $A\beta$ degradation.

Finally, what is the role of transthyretin (TTR), an $A\beta$ -binding protein produced in the brain exclusively by the choroid plexus, to the etiology of AD? First identified as a thyroxine-binding protein in blood and CSF, TTR has been implicated in $A\beta$ transport in the CSF and also functions to bind $A\beta$ and prevent amyloid aggregation and/or deposition in the CSF (Lignelid et al., 1997; Schreiber et al., 1990; Schreiber et al., 2001; Schwarzman et al., 1994). The level of TTR in the CSF decreases with aging and by exposure to the toxic metal lead (Zheng et al., 2001; Zheng et al., 1996). In AD patients, TTR levels in the CSF are reportedly significantly

reduced compared to age-matched controls (Merched et al., 1998). In a genetically engineered animal model, over-expression of TTR molecules appears to protect against the progression of AD pathology (Stein et al., 2004). It would be interesting to determine how the production and degradation of A β in the choroid plexus may relate to its binding to TTR and/or TTR production.

In summary, the BCB and the BBB possess the definite capacity to produce and degrade A β peptides, with the BBB having higher expression of proteins associated with A β production. Taken together with our earlier studies on A β uptake and transport, this study suggests that the BCB plays a major and complex role in removing A β from the CSF by sequestration and metabolism in the choroid plexus under normal, healthy conditions. It is possible that any damage to the BCB or BBB due to illness, injury or toxicant exposure could alter the A β homeostasis and may contribute to the etiology of AD.

4. Experimental Procedure

4.1 Materials

Materials were purchased from the following sources: primary antibodies against APP from Sigma (St. Louis, MO), against A β (clone 6E10) from Signet (Dedham, MA), against neprilysin from Vision BioSystems (Norwell, MA); secondary antibodies labeled with horseradish peroxidase from GE Healthcare (Piscataway, NJ) or labeled with Alexa Fluor 488™ from Invitrogen (Carlsbad, CA); and custom-produced real-time RT-PCR primers from Sigma-Genosys (Woodlands, TX). Unless otherwise noted, all other chemicals were obtained from Sigma.

The immortalized choroidal epithelial cell line, known as Z310 cells, was maintained in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 40 μ g/mL gentamycin at 37°C as described previously (Zheng and Zhao, 2002). Fresh choroid plexus and kidney tissue were isolated from male Sprague-Dawley rats that were anesthetized by ketamine and xylazine mixture (75 and 10 mg/kg) prior to tissue collection and euthanasia. All animal experiments were conducted with the approval of the Purdue University Animal Care and Use Committee.

4.2 Real-time, reverse-transcriptase PCR

mRNA levels of A β processing genes were determined by real-time RT-PCR as described previously (Li et al., 2005). Choroid plexus and selected brain regions were extracted from health adult rat brains. Brain tissue was homogenized on ice and separated by centrifugation (5400 \times g, 15 min, 4°C) in 18% dextran into the capillary fraction (pellet) and the brain parenchyma fraction (supernatant). Total RNA was extracted from each sample by TRIzol (Invitrogen, Carlsbad, CA) and purified on RNeasy columns (Qiagen, Palo Alto, CA). One microgram of purified RNA was converted to cDNA by MuLV reverse transcriptase with oligo dT primers and stored at -80°C until use. Forward and reverse primers for each gene of interest and a housekeeping gene, GAPDH, were designed with Primer Express 2.0 software. The primer sequences (forward; reverse, both in the 5' to 3' direction) are: APP (ccttaccggtgcctagtgtgt; gtccatccgctcctggtgta), α -secretase (ggagagttggccccacagtt; ccctgggattggagttaaga), β -secretase (gatccccctggaccacatct; gctctggccacaggtaccat), IDE (ggttgagagttccccttca; aggccgcgcttgaattc), ECE-1 (caccacagcatcacctaaca; ctacacacaggtcacaagagctt) neprilysin (aggcctttacgggactacat; gcctccccacagcattctc), GAPDH (cctggagaacacctgccaagtat; agcccagatgcccttagt). Real-time PCR was completed with the ABsolute QPCR SYBR green mix kit (ABgene, Rochester, NY) in the Mx3000P real-time RT-PCR system (Stratagene, LaJolla, CA). The amplification process was 15 min at 95°C followed by 40 cycles of 30 sec at 95°C, 1 min at 55°C and 30 sec at 72°C. Values were

expressed in cycle threshold time (C_t) and were normalized to the C_t times for housekeeping gene, GAPDH. Primer specificity was confirmed by a single peak in a dissociation curve or by a single lane following gel electrophoresis of the primer products (Fig. 1).

4.3 Western blot analysis

Total protein extracted from choroid plexus, whole brain homogenate and Z310 cells was separated by gel electrophoresis on a 4–20% Tris-HCl linear gradient gel (Bio-Rad, Hercules, CA) and transferred to a PVDF membrane. Membranes were blocked with 1% milk powder and immunoblotted with a 1:200 dilution of rabbit anti-APP antibody, followed by incubation with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase. The protein bands were visualized by an ECL system (GE Healthcare, Piscataway, NJ).

4.4 Immunohistochemistry

Confluent Z310 cells grown in Lab Tek II 8-well chamber slides, freshly isolated choroid plexus or kidney tissue was fixed in paraformaldehyde (0.3%) and glutaraldehyde (0.25%) in phosphate-buffered saline (PBS) for 10 minutes at room temperature. Tissues were permeabilized by incubation in 0.5% Triton-X100 in PBS for 30 min. Non-specific antibody binding was blocked by incubation with 1% bovine serum albumin in PBS. Primary antibody was added for overnight incubation at 4°C (anti-neprilysin, 1:80; anti- β -amyloid 6E10, 1:100, anti-APP, 1:200). After washing the cells and tissues 5X in PBS, secondary antibody conjugated to horseradish peroxidase was added for at least one hour incubation at 37°C. Samples were washed 5 times in PBS and protein visualized by 3,3'-diaminobenzidine staining.

4.5 Confocal microscopy

Freshly isolated choroid plexus tissue was fixed, permeabilized and blocked as above. Tissues were incubated in primary antibody (at above concentrations) for 1 hour at 37°C, washed and incubated with a conjugated secondary antibody conjugated to Alexa Fluor 488™ for at least one hour at 37°C. Tissues were mounted and dried before visualization with a BioRad MRC 1024 confocal microscope, Purdue University Cytology Lab.

4.6 Determination of β -secretase activity

β -Secretase activity was determined in freshly isolated protein fractions by an assay kit (Sigma, St. Louis, MO), according to the manufacturer's instruction and utilizing the fluorescence resonance energy transfer technique. Briefly, proteins from cells or tissues were incubated for 2 hours at 37°C with a substrate whose fluorescence signal is increased following substrate cleavage by β -secretase. Enzyme activity was then determined from a standard curve and was normalized to total protein concentration of the sample.

4.7 Data Analysis

All values are expressed as the mean \pm SEM. Gene expression was compared between the choroid plexus and Z310 cell line by student's *t*-test. For the regional tissue analysis of gene expression (Fig. 2), choroid plexus and brain regional data were analyzed by one-way ANOVA. Post hoc examination using Newman-Keuls multiple comparison test compared gene expression (i) in the choroid plexus versus each regional capillary fraction, (ii) in each regional capillary fraction versus its corresponding brain parenchyma fraction and (iii) in each capillary fraction versus the other capillary fractions. In the cases where no differences were noted between the choroid plexus and each capillary fraction, data from the capillary fractions were combined and tested against the choroid plexus fraction by student's *t*-test. For the β -secretase activity assay, the data were analyzed by ANOVA using Newman-Keuls comparison of the three groups as the post hoc test.

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Abbreviations

AD	Alzheimer's disease
APP	amyloid precursor protein
Aβ	beta-amyloid
BBB	blood-brain barrier
BCB	blood-cerebrospinal fluid barrier
CSF	cerebrospinal fluid
ECE	endothelin converting enzyme-1
IDE	insulin degrading enzyme
PBS	phosphate buffered saline
RT-PCR	reverse-transcriptase polymerase chain reaction
TTR	

transthyretin

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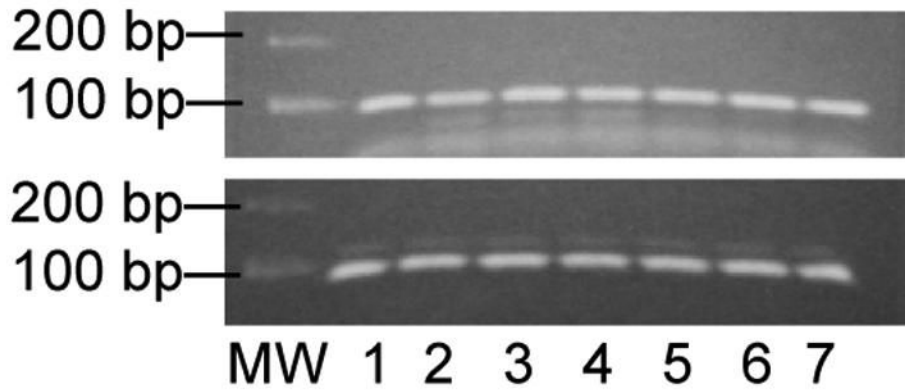


Figure 1. Expression of APP mRNA in choroid plexus tissues and Z310 cells by real-time RT-PCR. Following PCR, the double-stranded products were separated by gel electrophoresis and visualized with ethidium bromide. Upper: APP produced a double stranded product of 86 base pairs (bp). Lower: GAPDH produced a double stranded product of 82 bp. Lanes 1–5 used cDNA generated from Z310 cells. Lanes 6 and 7 are choroid plexus cDNAs.

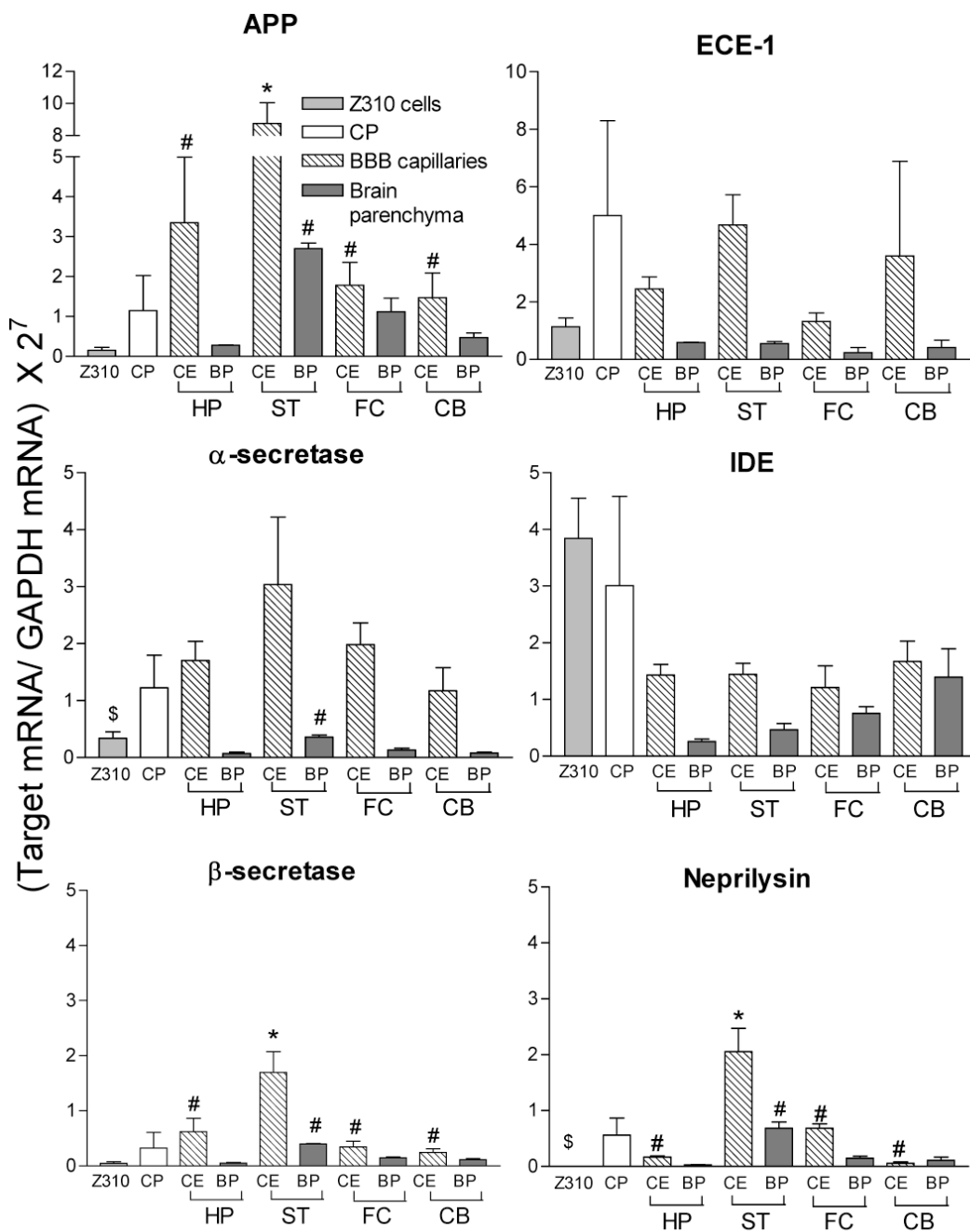


Figure 2. Gene expression of six Aβ-producing and -metabolizing enzymes in the choroid plexus, brain capillaries that comprise the BBB, and the surrounding brain parenchyma. Values are mean ± SEM (n=3). Significant differences are noted by: *different from choroid plexus by one-way ANOVA with Newman-Keuls post hoc test, \$different from choroid plexus by t-test, #different from capillary endothelium of the striatum. Abbreviations: APP: amyloid precursor protein; ECE-1 endothelin converting enzyme-1; IDE: insulin degrading enzyme; HP: hippocampus; ST: striatum; FC: frontal cortex; CB: cerebellum; CE: capillary endothelium; BP: brain parenchyma. Note the differences in the values of the y-axes.

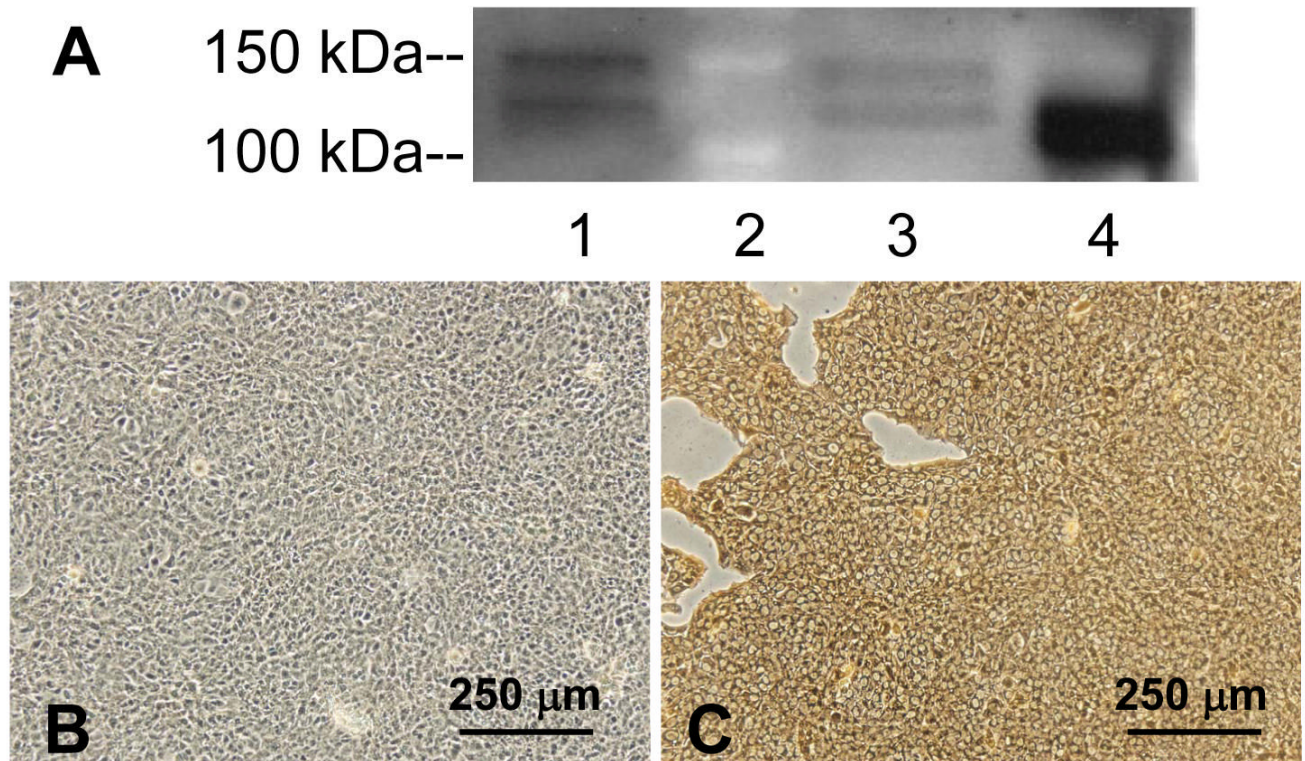


Figure 3.

APP protein is expressed in the choroid plexus tissue and the choroidal epithelial cell line. A) Representative Western blot for APP presence in Z310 cells (lane 1), choroid plexus (lane 3) and homogenized whole brain (lane 4; positive control). Lane 2 is the molecular weight markers for 100 and 150 kDa. Bands are present at 110 kDa. Results are not quantitative. B) Negative control in which the primary antibody against APP was excluded from the immunocytochemistry in Z310 cells. C) APP expression (brown stain) in Z310 cells. B and C are representative of three immunocytochemistry experiments.

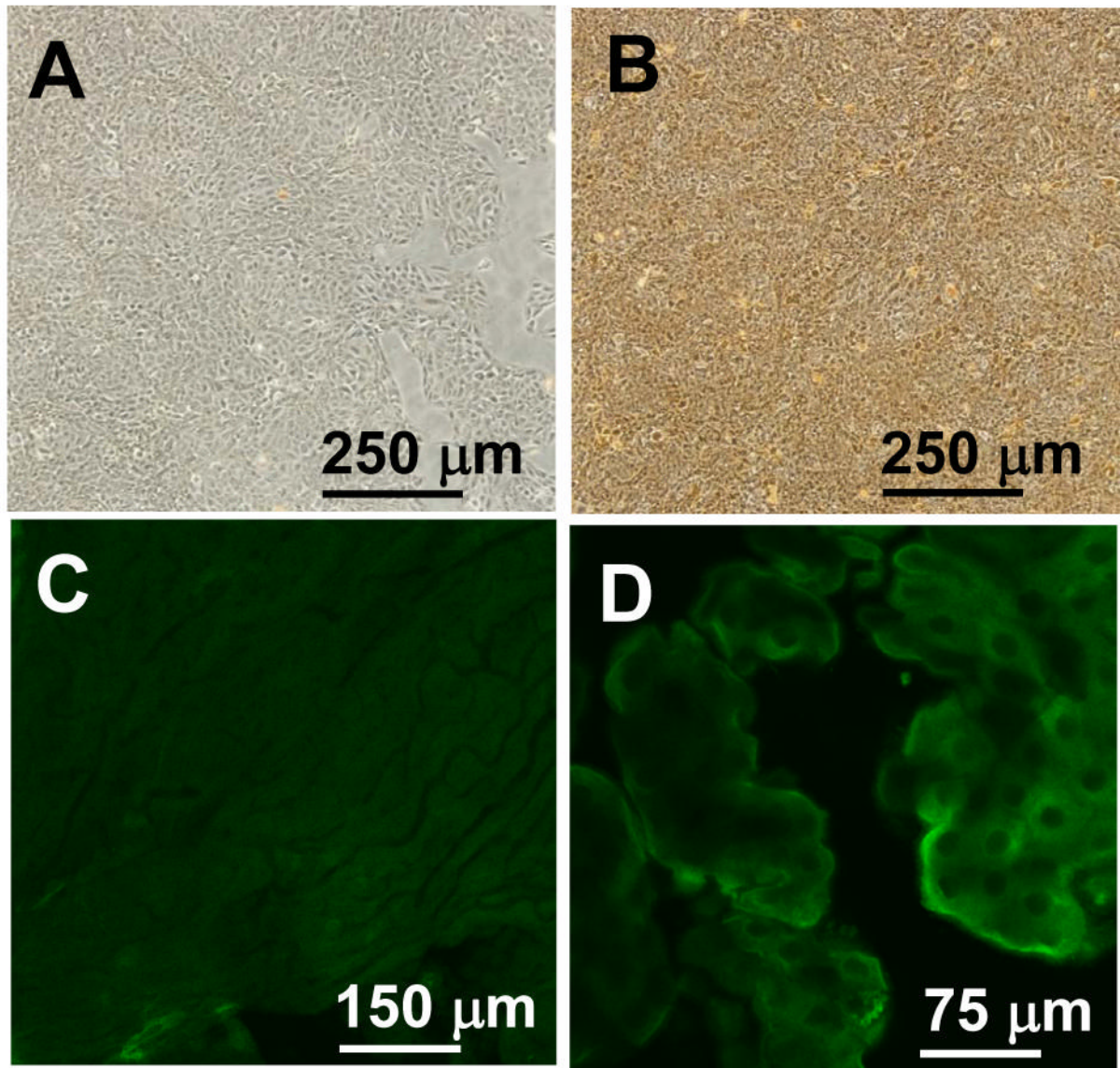


Figure 4.

The choroid plexus produces A β from APP. Left, negative controls excluding primary antibody. Right, expression of immunoreactive A β peptide in tissues and cells. A, B) A β expression (brown stain) in Z310 cells by immunocytochemistry. C, D) Green fluorescence corresponds to A β peptide in choroid plexus tissue. Note the differences in magnification.

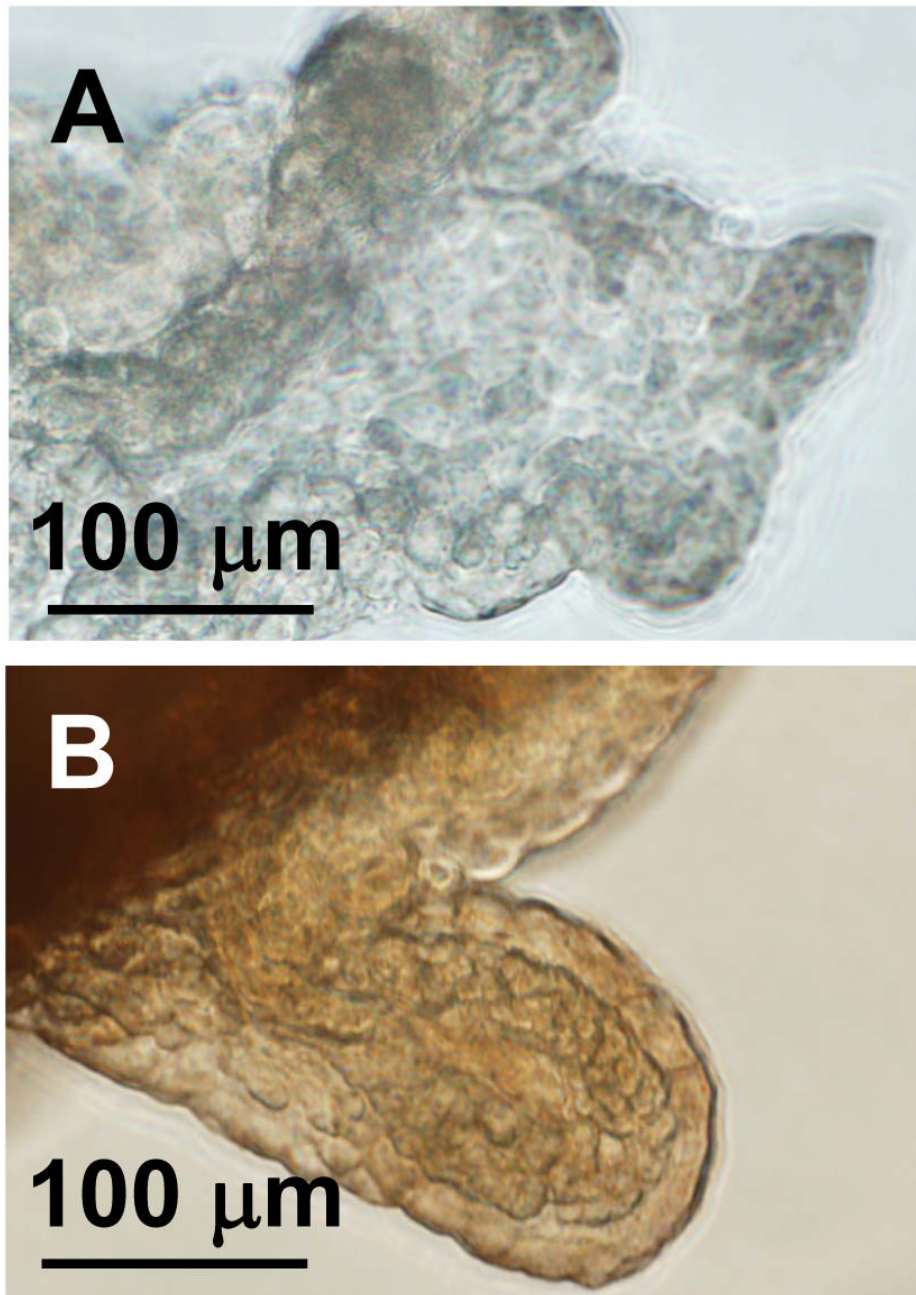


Figure 5. Immunohistochemistry of neprilysin in the choroid plexus. Neprilysin expression is evident in choroid plexus tissue (B) compared to a control tissue, in which the primary antibody was excluded (A). These images are representative of at least three trials.

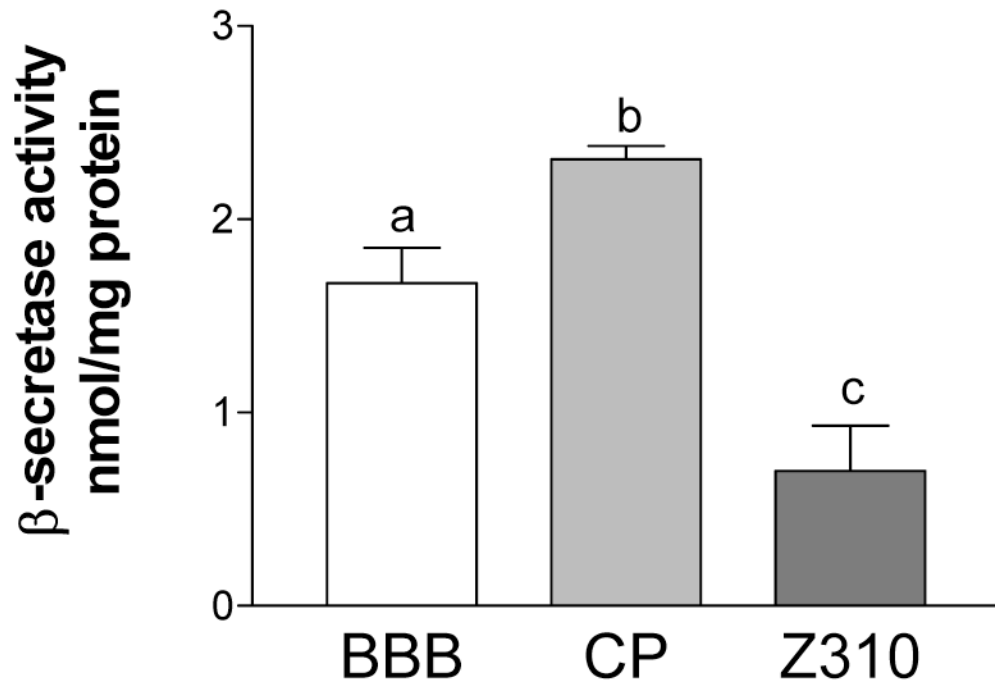
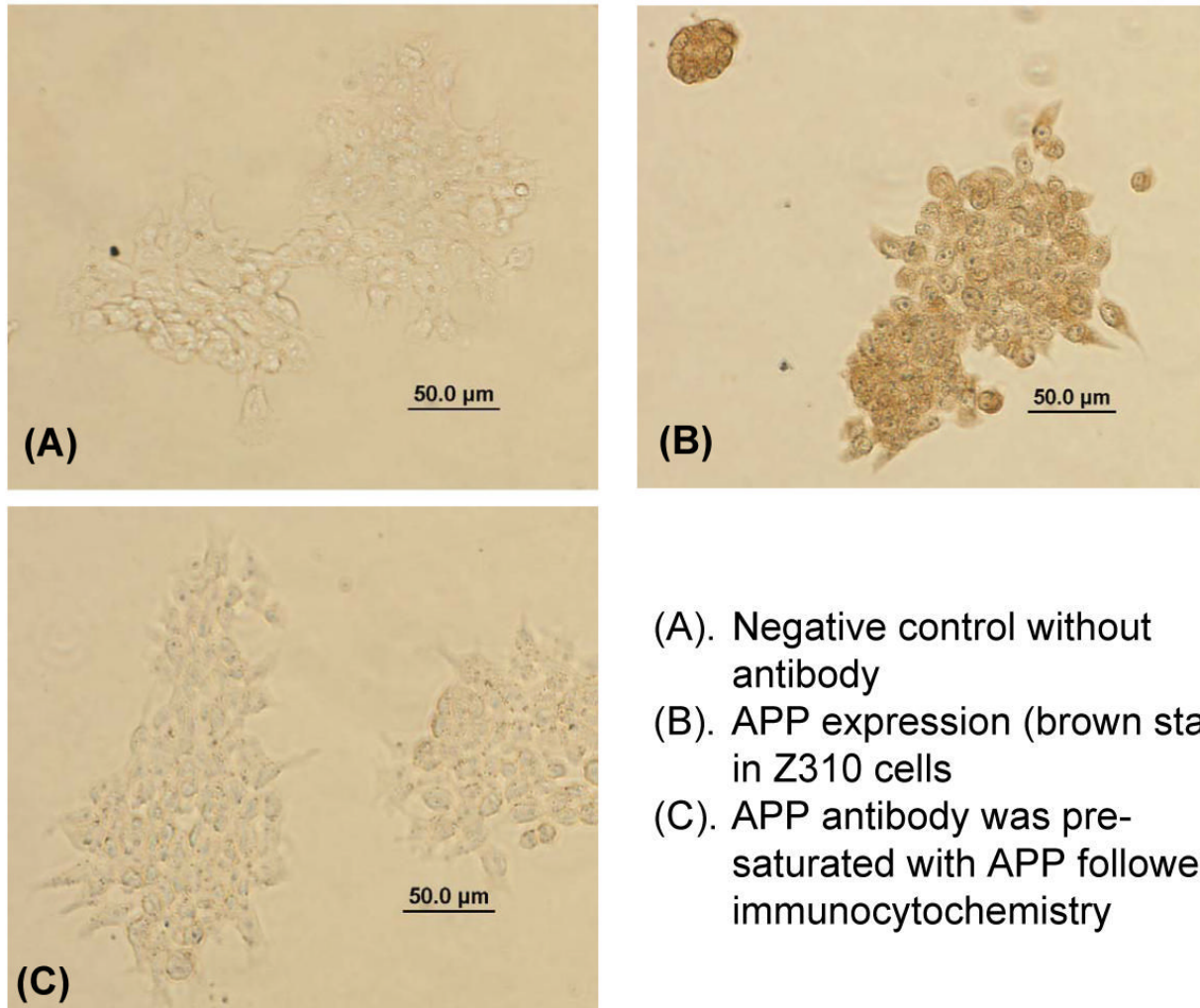


Figure 6. β -Secretase activity in the choroid plexus and the blood-brain barrier. The choroid plexus has more β -secretase activity (nmol product formed/ mg total protein) than either the brain capillaries (BBB; from whole brain) or Z310 cells. Values shown are mean \pm SEM (n=3). Each sample is significantly different from all others.



Appendix-1. Specificity Study of APP Antibody (supplement to Fig. 3)