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Rapid Communication

Alzheimer Amyloid β-Protein Precursor in Sperm Development

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We prepared antisera to both the N and C termini of amyloid β-protein precursor (APP). Both antisera labeled 110 to 140 kd proteins from rat testis by immunoblotting. Northern blot analysis using oligonucleotide probes complementary to respective APPs showed that APPs expressed in rat testis contained Kunitz-type protease inhibitor domains. Immunocytochemically brain APP was localized in neurons and their processes. During sperm formation, APPs labeled by both antisera were localized only in acrosome and the growing tail of spermatids in the seminiferous tubules. This shows that APPs appear only in particular phases of spermatogenesis, that is, in the morphologic change phase from spermatid to mature sperm. Amyloid β-protein precursors in testis may play a role in cellular differentiation or morphologic change. (Am J Pathol 1990, 137:1027–1032)

Alzheimer-type dementia is a type of dementia that occurs frequently in the elderly. Abundant neurofibrillary tangles and senile plaques are the characteristic changes in Alzheimer-affected brains and are closely related to the dementia.[1] The component of senile plaque amyloid, β protein,[2,3] has been shown to be derived from a larger amyloid β-protein precursor (APP).[4] Amyloid β-protein precursors with Kunitz-type protease inhibitor domain (KPI) also have been reported.[5] Amyloid β-protein precursor has been conserved in mammalian evolution, and highly homologous APPs are expressed in many species.[6] Furthermore APP is known to be expressed in many systemic organs in addition to the brain.[7,8] However their precise role is still unclear. Therefore we studied extraneural APP expression in several rat systemic organs and found evidence for its expression in rat testis. Furthermore we found that testis APP might be related to sperm development. We also examined rat brains for APP expression as a control.

Materials and Methods

Antisera

Two peptides corresponding to the N terminus (residues 18 to 38) and C terminus (residues 666 to 695) of APP_{695} were synthesized and HPLC purified. These peptides were coupled to keyhole limpet hemocyanin and administered to rabbits. Two kinds of antisera against both the N terminus (W63N) and C terminus (W61C) of APP were obtained. The characteristics and specificity of these antisera have been reported elsewhere.[9,10]

Immunoblotting

Brain and testis tissues from 9-week-old male Sprague-Dawley rats were homogenized in 9 volumes of TS buffer (50 mmol/l [millimolar] TRIS, 150 mmol/l NaCl, 5 mmol/l ethylenediamine tetraacetic acid disodium salt (EDTA), 2 mmol/l phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 μg/ml pepstatin, and 1 μg/ml p-toluenesulfonyl-L-lysin chloromethyl ketone hydrochloride (TLCK), pH 7.6) and centrifuged at 100,000*g*.

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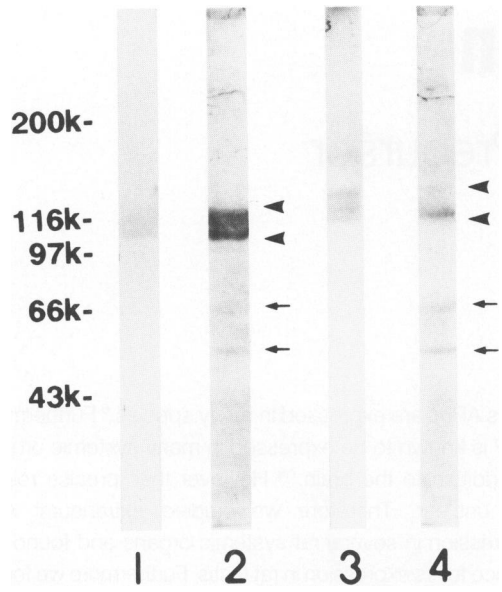


Figure 1. *Immunoblots of rat brain and testis stained with antisera to APP. W61C labeled 100 to 120 kd proteins from rat brain extract (lane 1), and 110 to 140 kd proteins from rat testis extract (lane 3). W63N labeled the same 100 to 120 kd proteins from rat brain (lane 2), and the same 110 to 140 kd proteins from rat testis (lane 4). Lower-molecular-weight bands (70 kd and 55 kd) are found with W63N antibody and are considered to be fragments of APP.*

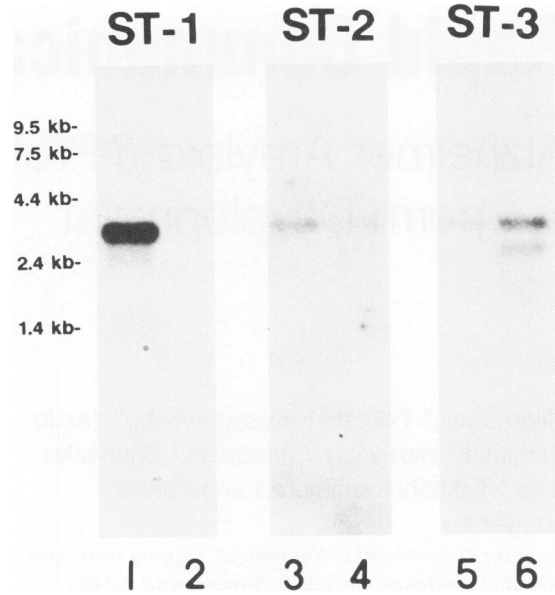


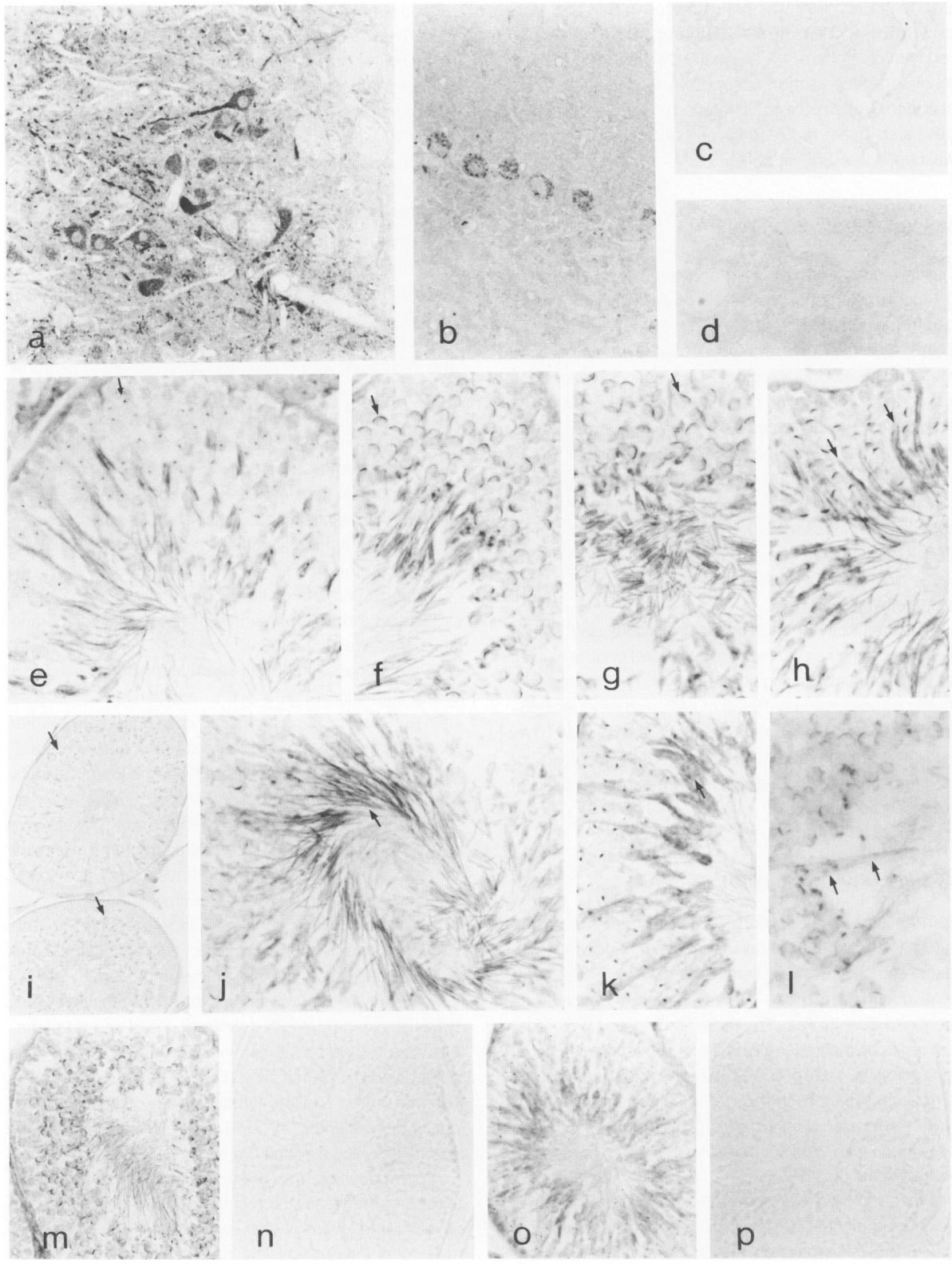
Figure 2. *Northern blot analysis of rat brain and testis poly (A)$^+$ RNAs. Lanes 1, 3, 5: poly (A)$^+$ RNA from rat brain; lanes 2, 4, 6: poly (A)$^+$ RNA from rat testis. An RNA ladder (0.2 to 9.5 kb; Bethesda Research Laboratories, Gaithersburg, MD) was used as the size marker. Probe ST-1 hybridized with a 3.5-kb mRNA of rat brain (lane 1); however there was no signal in rat testis (lane 2). Probe ST-2 and ST-3 hybridized with 3.6-kb mRNA of both rat brain (lanes 3 and 5) and testis (lanes 4 and 6). An additional 2.7-kb mRNA from rat testis hybridized with ST-3 (lane 6). The signal intensities were higher in lane 1 and lane 6.*

The pellet was homogenized in 2 volumes of TS buffer containing 2% Triton X-100 and centrifuged again at 100,000*g*.[7] The membrane-rich supernatant (10 μl) was applied to a 4% to 12% gradient SDS-PAGE and transferred to Immobilon (Millipore, Bedford, MA), which was immunostained by avidin-biotin-complex (ABC) methods (Vector Labs. Burlingame, CA) using W61C (1:500) and W63N (1:200).[9,10]

Northern Blotting

Three kinds of 24-mer oligonucleotide probes were synthesized according to the reports of rat APP sequences.[11,12] ST-1 for APP_{695} (5′-CCG TCG TGG GAA CTC GGA CTA CCT-3′), ST-2 for APP_{751} (5′-CCG TCG TGG GAA ACA CGC TGC CAC-3′), and ST-3 for APP_{770} (5′-CCG TCG TGG GAA GTT TAA CCG GAT-3′) were complementary to the exon 6–9, 7–9, and 8–9 junction sequences,[13] respectively. Rat brain and testis poly (A)$^+$ RNAs were prepared from a 9-week-old male Sprague-Dawley rat by the guanidine thiocyanate method and by oligo (dT) cellulose column chromatography. Each 3 μg of formaldehyde-denatured poly (A)$^+$ RNAs from brain and testis were fractionated on a 1% agarose gel, run in MOPS buffer (pH 7.0; Wako, Oosaka, Japan), and subsequently transferred to Hibond-N (Amersham, Arlington Heights, IL) in 20 × SSC. These probes were labeled with ^{32}P-ATP at the 5′ end using a Megalabel kit (Takara, Kyoto, Japan) and purified by NICK-Column (Pharmacia, Uppsala

Figure 3. *Immunocytochemistry of rat brain and testis using antisera to APP.* **a**: *Rat facial nerve nucleus. W61C, magnification ×140. Large motor cell bodies and their processes are labeled (arrow). Some glial cells and the periphery of blood vessels are also stained.* **b**: *Rat cerebellum. W63N, magnification ×280. Purkinje's cells were stained.* **c**: *Preabsorption study. W61C, rat facial nerve nucleus, ×280.* **d**: *Preabsorption study. W63N, cerebellum, ×280. Figures* **e** *to* **p** *were rat seminiferous tubles.* **e**: *W61C, magnification ×280. Immunoreactive dots first appeared near the nucleus of spermatids (arrow). Growing tails of spermatid labeled by W61C are also seen.* **f** *and* **g**: *This dotlike immunoreactivity gradually increased into a cap formation (arrow). Spermatogonia, spermatocyte, and interstitial tissue were not stained. W61C, magnification ×280.* **h**: *When nuclear condensation and the change in nuclear configuration were accomplished, APP1 in acrosome was no longer evident (arrow). W61C, magnification ×280.* **i**: *Periodic acid Shiff stain, magnification ×80. Acrosomes were stained (arrow).* **j**: *APP immunoreactivity also is evident in growing spermatid flagella (arrow). W61C, magnification ×280.* **k**: *W63N, magnification ×280. The APP1 detected by W61C antiserum was almost the same as that detected by W63N antiserum. However the vanishing cell bodies of spermatocytes were weakly stained by W63N antiserum.* **l**: *Head and tail of mature sperm in seminiferous tuble cavity show no APP1 (arrow). W61C, magnification ×700.* **m** *and* **n**: *preabsorption study by W63N, ×100. APP immunoreactivity with W63N* (**m**) *was not detected using preabsorbed antiserum* (**n**). **o** *and* **p**: *preabsorption study by W61C, ×100. APP immunoreactivities by W61C* (**o**) *were also abolished by preabsorption antiserum* (**p**).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Sweden). Each filter was prehybridized in 5 × SSC containing 50% formamide, 5 × Denhart's solution, 50 mmol/l sodium phosphate, 1% glycine, 0.1% SDS, and 200 μg/ml of sonicated salmon sperm DNA for 1 hour at 42°C, and then hybridized with ^{32}P-labeled probes in the above-described buffer at 55°C for 20 hours. The filters were washed in 6 × SSC at 55°C.[14]

Immunocytochemistry

Tissue blocks from rat brain and testis were fixed with 0.1 mol/l (molar) phosphate-buffered saline containing 4% paraformaldehyde and 0.3% picric acid (pH 7.6, 4°C). Eight-micrometer cryostat sections were immunostained with an Elite ABC kit (Vector Labs.). Intrinsic peroxidase activity and nonspecific binding were blocked by 0.5% periodic acid solution and 10% normal goat serum, respectively. The first antisera (W61C, 1:500; W63N, 1:200) were applied for 6 hours at room temperature. The peroxidase activity was developed for 3 minutes in diaminobenzidine-Ni solution (0.1 mol/l TRIS-HCl, 0.8 mg/ml DAB-tetrahydrochloride, 0.4 mg/ml $NiCl_2$, and 0.009% H_2O_2, pH 7.6). The sections then were counterstained with methylgreen.[9,10]

The specificity of each antiserum (W61C and W63N) was checked by a preabsorption test and control studies for each immunostaining step. Briefly, 5 mg of each synthetic peptide (residues 18–38 of APP, and residues 666–695 of APP) was incubated overnight at 4°C with 10 ml of diluted antiserum (W61C 1:500, W63N 1:200). After centrifugation at 16,000*g*, the supernatant was used in preabsorption studies.

Results and Discussion

Both antisera labeled 100 to 120 kd proteins from the Triton X-100 extracts of rat brains by immunoblotting. In testes samples, both antisera labeled 110 to 140 kd proteins (Figure 1). These bands were not observed in preabsorption studies. The proteins detected were considered to be the full-length native APP because they contained both terminal epitopes, and their molecular weights correlated with the findings of previous studies.[7,9,10,15] Lower molecular weight bands (70 kd and 55 kd) also were found with W63N antibody and were considered to be fragments of APP.[9]

We performed Northern blot analysis to determine which type of APP is expressed in the rat testis (Figure 2). Probe ST-1 hybridized with a 3.5-kb mRNA from rat brain; however there was no detectable signal in rat testis. Both ST-2 and ST-3 hybridized with a 3.6-kb mRNA from rat brain and testis. In the testis, ST-3 also hybridized with an additional 2.7-kb mRNA, which is presently characterized. These results show that the APP_{695} mRNA is the major mRNA species in rat brain, whereas the mRNAs of rat testis encode APP_{770} and APP_{751}. Therefore we confirmed that the APPs containing a KPI sequence are expressed in rat testis. These findings correspond to the previous reports that showed that primarily APP_{695} is present in the brain and that APPs containing KPI are expressed in systemic organs.[6,8] This might explain the differences in the molecular size of APPs between testis and brain as shown by immunoblotting.

Based on these studies, we examined the localization of testis APP by immunocytochemistry. First the localization of rat brain APP was studied as a control. Both W61C and W63N showed dotlike immunoreactivity in neuronal cell bodies and their processes (Figure 3a and b). This immunoreactivity was not detectable using preabsorbed sera (Figure 3c and d). Positive neurons were widely distributed and almost all neurons in the cerebral cortex and the subcortical nucleus were labeled. Some astrocytes also were immunostained. These findings correspond to the previous immunocytochemical studies of APP expression in the brain.[9,11,16]

During sperm formation, both antisera first labeled proteins in a dotlike pattern near the nuclear membrane of spermatids (Figure 3e). This APP immunoreactivity (APPI) changed with further spermatid differentiation into a cap formation (Figure 3f). In addition, APPI covered nuclei that were changing their shapes (Figure 3g). When nuclear condensation and the change in nuclear configuration were accomplished, APPI was no longer evident (Figure 3h). The dot and cap APPI configurations of spermatids corresponded exactly to periodic acid Shiff staining (Figure 3i), so these immunoreactive structures represent the acrosome (proacrosomal granules and acrosomal vesicles).[17] Amyloid β-protein precursor immunoreactivity also labeled growing spermatid flagella (Figure 3j). The APPI detected by W61C antiserum was almost the same as those detected by W63N antiserum (Figure 3k). However the vanishing cell bodies of spermatocytes were weakly stained by W63N antiserum. Head and tail of mature sperm localized in seminiferous tube cavities were not labeled by APP staining (Figure 3l). Spermatogonia, spermatocytes, and the interstitial tissue of the testis were not immunostained. All APPI was eliminated in preabsorption studies (Figures 3m to p).

Sperm develop from the epithelia of the seminiferous tubules in the testis. Each step in spermatogenesis is a recognizable event in the seminiferous tubules. Spermatogonia differentiate into spermatocytes and subsequently into spermatids, which become mature sperm after acrosome formation, a change in nuclear configuration, nuclear condensation, and flagella formation. The acro-

some can be stained using periodic acid Shiff reaction and is used to demonstrate cap formation of the spermatid. At the same time as cap formation, the spermatid nucleus condenses and the sperm head forms. The acrosome contains polysaccharides and several enzymes that are released during the acrosome reaction, when sperms make initial contact with the ovum. On this basis, the acrosome is suspected to play a role in the configurational change in the spermatid head and in sperm penetration into the ovum.[17]

The above immunocytochemical study of testis indicates that native APP is present in acrosomes and in growing flagella, suggesting that APP appears in particular phases of spermatogenesis, that is, the head and tail formation phase in spermatids. Although the precise role of APP is still unclear, cell-to-cell or cell-to-matrix interaction is suspected.[11] However, for the spermatid, these interactions are not necessary, unlike sperm, which must interact with the ovum and its matrix. Furthermore native APP is present in growing flagella. These interactions are not necessary for growing flagella. Therefore we speculate that APP of testis plays an important role in cellular differentiation or morphologic changes occurring between the spermatid stage and the mature sperm stage in spermatogenesis.

Recently the secreted form of APP[15] was shown to be protease nexin-II.[18,19] Nexin-II is a serine protease inhibitor produced by various cultured extravascular cells and exhibits nerve trophic activities. It also has been shown that the Drosophila genome encodes a protein resembling human APP and this gene corresponds to the Drosophila and locus, a gene required for nervous system development.[20] Furthermore APP is suspected to be regulated by a nerve trophic factor.[21] These reports suggest that APP is a protein necessary for neuronal development. The results of our study of spermatid differentiation indicate that APP also is associated with sperm development. Furthermore APP may play an important role in cellular differentiation or morphologic change of spermatids.

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