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Alterations of Annexin Expression in Pathological Neuronal and Glial Reactions

Immunohistochemical Localization of Annexins I, II (p36 and p11 Subunits), IV, and VI in the Human Hippocampus

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Annexins are Ca^{2+}-dependent membrane-binding proteins that are potentially important in Ca^{2+}-induced neurotoxicity or neuroprotection. To address the possible involvement of annexins in cellular reactions to brain injury and neurodegenerative disease, we studied the immunohistochemical localization of annexins I, II (p36 and p11), IV, and VI in the adult human hippocampus. Formalin-fixed, paraffin-embedded tissue from autopsy cases representing hypoxic-ischemic injury, seizure disorders, Alzheimer's disease, and age-related controls were examined. Neurons showed cytoplasmic immunoreactivity for annexin I, whereas annexin VI was distributed in patterns suggesting plasma membrane and perisynaptic locations. The cytoarchitectural distribution of annexin VI within neurons was altered in pathological states and annexin VI was strongly associated with neuronal granulovacuolar bodies in Alzheimer's disease. Reactive astrocytes expressed annexins I, II (p36 and p11), and IV, whereas quiescent astrocytes were minimally immunoreactive. Significant annexin immunoreactivity was also detected in oligodendrocytes (annexin IV), ependymocytes (I, II, and IV), choroid plexus (I, IV, and VI), meningothelium (I, II, IV, and VI), and vascular endothelium (II and IV) and smooth muscle (I, IV, and VI). This is the first comparative study of immunoreactivities for multiple annexins in human brain. Neurons and glia display selective and different profiles of annexin protein expression and show immunohistochemical changes in pathological conditions, which suggest involvement of annexins in neuronal and glial reactions to injury. (Am J Pathol 1994, 145:640–649)

The annexins are a family of proteins that are defined by a conserved COOH-terminal domain that confers Ca^{2+}-dependent binding to membranes containing acidic phospholipids. The NH_2-terminal sequence of each annexin is unique and presumably confers functional specificity to the protein. A variety of roles for annexins in cellular physiology have been proposed, such as mediation of membrane trafficking events and membrane-cytoskeleton interactions, regulation of phospholipase activity and eicosanoid release, receptor signal transduction, modulation, or formation of Ca^{2+} channels, and control of cellular proliferation and differentiation.[1,2] However, at present there is little understanding of the specific functions of particular annexins *in vivo*.

Annexins are widely distributed among species and tissues. In the mammalian nervous system, different annexins are expressed in various cell types.[3–6] The patterns of annexin expression in the brain may change during development[3,7] and in pathological states.[8,9] To better define the relationship of annexins to cellular reactions to injury and de-

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generation in the human central nervous system, we have compared the distributions of annexins I, II (p11 and p36 subunits), IV, and VI in the hippocampus after a variety of conditions associated with glial reactions and neuronal damage. In this study, hypoxic ischemic damage, chronic seizure-related injury, and Alzheimer's disease were accompanied by specific alterations in both the cellular distributions and cytoarchitectural patterns of the annexins. The most prominent changes occurred with the cytoarchitectural distribution of annexin VI in neurons and increases of annexins I, II, and IV in reactive astroglia. These data suggest that the annexins may be involved in neuronal and glial responses to acute and chronic injury.

Materials and Methods

Case Material

Hippocampal sections from a total of 20 postmortem cases (13 male, 7 female) were examined. Four patients (ages 35 to 66 years) with no history of either acute or chronic neurological disorders were regarded as controls. Six patients had histories of seizure disorders; two had asymmetric hippocampal sclerosis and four showed mild to moderate hilar astrogliosis without significant neuronal loss. Four patients with histories of chronic dementia had pathological findings diagnostic of Alzheimer's disease.[10] Four patients had significant hypoxic/ischemic events that occurred 4 days to 2 weeks before death; three showed neuronal loss in hippocampal CA1 with gliosis and one had a subacute infarct in CA1. Two patients were terminally unresponsive after shock and showed acute neuronal injury without gliosis. Postmortem intervals for all cases ranged from 2 to 24 hours (median, 12 hours). Neurosurgical specimens were used as controls to assess the effects of postmortem fixation delay on annexin immunohistochemistry. These consisted of gray and white matter from cerebral neocortex and hippocampus and represented a variety of diseases, including inflammatory processes, infarcts, seizure disorders, and Alzheimer's disease.

Tissue Processing and Immunohistochemistry

In most cases the entire brain was fixed by immersion in 10 to 20% phosphate-buffered formalin at room temperature for 2 weeks before dissection and routine embedding in paraffin. In three cases, the brains were freshly dissected and hippocampal slices were fixed for 3 to 4 days at 4 C in phosphate-buffered 10% formalin before paraffin embedding. The control neurosurgical specimens were fixed and processed under a variety of conditions, ranging from rapid immersion and fixation of fresh tissue in 10% buffered zinc formalin for 48 hours at 4 C to routine fixation for 8 to 24 hours at room temperature. Fixed tissue was embedded in either regular temperature or low melting point paraffin (42 C). Variations in tissue processing did not affect the cellular and cytoarchitectural localizations of specific annexin immunoreactivities related to either normal or disease states. The overall intensity of immunostaining was somewhat less robust in deeper regions of the postmortem brains fixed by whole immersion compared with fresh tissue sections that were more rapidly fixed.

Immunohistochemistry was performed for annexins I, II (p11 and p36), IV, and VI and for glial fibrillary acidic protein (GFAP) in all cases; for S100β and βA4-amyloid in Alzheimer's disease cases; and for synaptophysin, HAM 56, and factor VIII in selected other cases. Primary antibodies were obtained from the following sources and used at the indicated concentrations in phosphate-buffered saline (PBS): 1) monoclonal mouse IgG antibodies directed to annexin I, annexin II (p36 monomer), annexin II (p11 subunit), annexin IV, and annexin VI (each at 1:500; Zymed Laboratories, Inc., South San Francisco, CA); 2) rabbit polyclonal antibodies directed to GFAP (1:1400; Dako Corp., Carpinteria, CA); 3) monoclonal mouse IgG directed to synaptophysin (SY38, 1:10; Boehringer Mannheim Corp., Indianapolis, IN); 4) rabbit polyclonal antibodies directed to β4-amyloid (1:10; Boehringer Mannheim Corp.); 5) rabbit polyclonal antibodies directed to S100β (1:2000; Chemicon International, Inc., Temencula, CA); 6) monoclonal mouse IgG antibodies directed to human macrophage antigen (HAM 56, 1:100; Dako Corp.); and 7) monoclonal mouse IgG antibodies directed to factor VIII (1:10; Dako Corp.). The specificities of the antiannexin antibodies were confirmed by Western blotting of normal and neoplastic human brain tissues and of cultured U251 human glioma cells.

Paraffin sections, 5 μ thick, were prepared for immunohistochemistry by deparaffinization in xylene, preincubation for 30 minutes at 22 C with methanolic H_2O_2 (1.75%), and hydration in graded alcohols. For annexin II (p11 subunit or p36) immunohistochemistry sections were then treated either with pepsin (Sigma Chemical Co., St. Louis, MO) 4 mg/ml in 0.01 N HCl for 15 to 30 minutes at 37 C or by microwaving at 750 W in 10 mmol/L sodium citrate buffer (pH 6) for three to four consecutive 5-minute intervals, replacing evaporated buffer volume with H_2O after each inter-

val. The patterns of p11 immunoreactivity produced after each of these treatments were identical. For p36, microwave pretreatment allowed detection of immunoreactivity but pepsin pretreatment did not. For detection of βA4 epitopes, sections were incubated with 88% formic acid for 5 minutes at 22 C. All sections were then rinsed in PBS, blocked with 1.5% nonimmune serum in PBS (horse serum for monoclonal antibodies or goat serum for polyclonal antibodies), and incubated with the primary antibodies for 18 to 24 hours at 4 C. Primary antibody was labeled by the avidin-biotin complex method[11] using the Vectastain Elite kit (Vector Laboratories Inc., Burlingame, CA) and visualized with peroxidase-coupled anti-mouse or anti-rabbit antibodies using diaminobenzidine (DAB) as the chromogen. The sections were counterstained with hematoxylin.

Dual-label immunohistochemistry for p11 and GFAP was performed by first visualizing p11 with microwave pretreatment as described above using aminoethylcarbazole (AEC) as chromogen. The sections were then incubated with pepsin (4 mg/ml in 0.01 N HCl, 15 minutes at 37 C), rinsed in PBS, and blocked with nonimmune goat serum. GFAP was then visualized as described above, using DAB-Ni complex as chromogen. Dual labeling for βA4 and p11 was performed by first visualizing βA4 with formic acid pretreatment as described above using AEC as chromogen; p11 was then visualized with pepsin or microwave pretreatment as described above using DAB-Ni complex as chromogen.

Annexin Nomenclature

Studies cited herein have used a variety of designations for the annexin proteins. The system used here is that of Crumpton and Dedman:[12] p37, annexin I; p36, annexin II monomer; p32, annexin IV; p67/68, annexin VI.

Results

Normal Hippocampus

The cellular localizations of annexins I, II (p36 and p11), IV, and VI are summarized in Table 1.

Table 1. *Cellular Localization of Annexins in Human Hippocampus and Associated Structures*

	Annexin			
	I	II	IV	VI
Neurons	++	–	–	+++
Subependymal/subpial astrocytes	++	+++	++	–
Parenchymal astrocytes	–	–	+	–
Oligodendrocytes	–	–	++	–
Ependyma	+++	+++	+++	+
Choroid plexus	+++	+	+++	+++
Meningothelium	+++	+++	+++	+++
Endothelium	+	+++	+++	–
Smooth muscle	++	–	++	++

Annexin I

Moderate to strong immunoreactivity for annexin I was present in neurons, subependymal and subpial astrocytes, choroid plexus epithelium, ependyma, and vascular smooth muscle (Figure 1A). In neurons, annexin I was primarily confined to the soma in a diffuse to granular cytoplasmic pattern (Figure 3F), sometimes with a perinuclear distribution. The intensity of the annexin I immunoreactivity of the hippocampal neurons ranged from most intense in bipolar neurons of the stratum oriens, to moderate in the pyramidal neurons of the cornu ammonis, and to only mild in the granular cells of the dentate gyrus. Immunoreactivity in other cell types generally showed a diffuse cytoplasmic pattern. Subependymal and subpial astrocytes exhibited moderate immunoreactivity in cell bodies and larger processes. In contrast, annexin I was not detected in nonreactive parenchymal astrocytes and oligodendroglia. The neuropil showed a faint to mild diffuse staining, somewhat less in white matter compared with gray matter. Immunoreactivity in vascular endothelium was mild and variable.

Annexin II (p36 and p11)

Immunoreactivities for annexin II (p36 and p11) showed similar cellular and cytoarchitectural distributions (Figure 1B). In ependymocytes and subependymal and subpial astrocytes, immunoreactivity was usually most intense at the cell periphery but was sometimes cytoplasmic. Immunostaining in deep white matter was variable and when present was associated with perivascular astroglia. Annexin II p36 and p11 were absent in neurons, nonreactive parenchymal astrocytes, oligodendrocytes, and neuropil. In other cell types, p11 and p36 immunoreactivities were strong in arachnoid meningothelium and vascular endothelium, whereas staining of the choroid plexus epithelium and vascular smooth muscle was variable and mild.

Annexin IV

Moderate to strong cytoplasmic immunoreactivity for annexin IV was present in subependymal and subpial

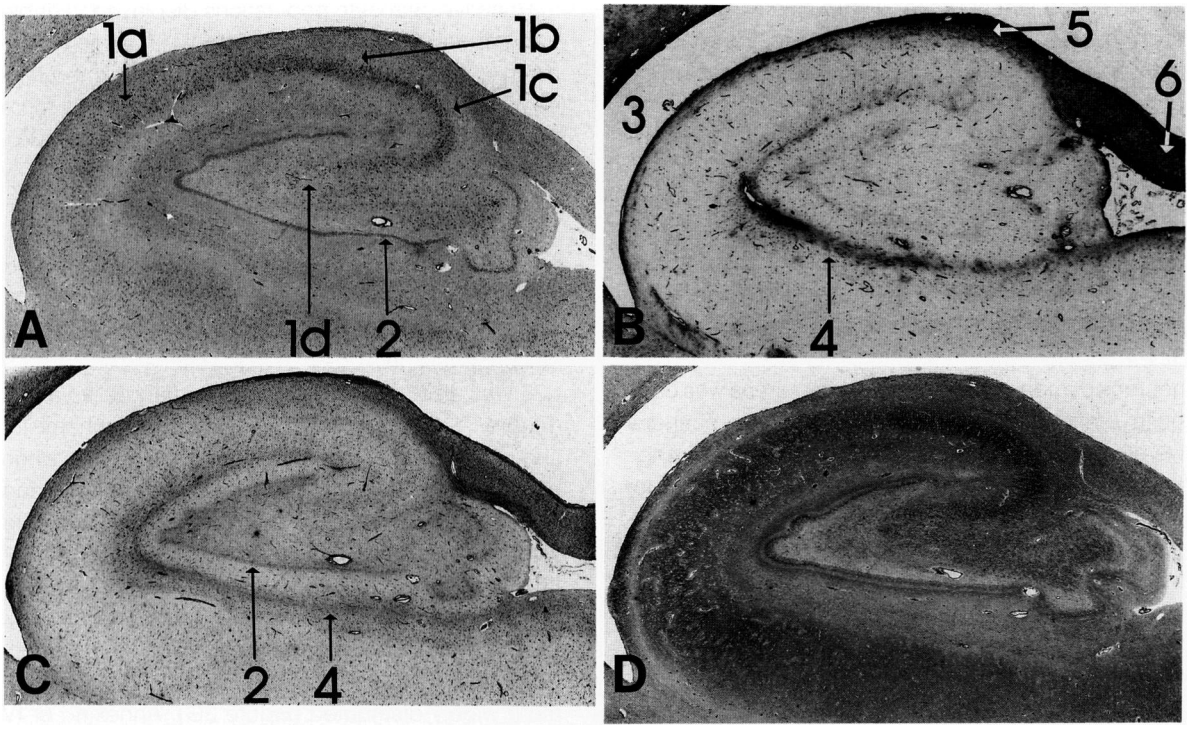


Figure 1. *Annexin immunohistochemistry of normal hippocampus. Adjacent tissue sections were processed for annexin I* (**A**), *p11* (**B**), *annexin IV* (**C**), *and annexin VI* (**D**) *using biotin-avidin immunohistochemistry with hematoxylin counterstain.* **A:** *Annexin I was present in neuronal cell bodies in the dentate gyrus and Ammon's horn, glia in hippocampal fissure, fimbria, and alveus, and ependyma and vessels.* **B:** *Dense p11 immunoreactivity was associated with glia in fimbria, alveus, and hippocampal fissure, and with vessels and ependyma.* **C:** *The distribution of annexin IV immunoreactivity resembled that of p11 but the staining was less dense and more diffuse. Immunoreactivity in dentate granule cell layer was associated with glia.* **D:** *Annexin VI was prominent in pyramidal and granule cell layers and dendritic fields. Staining in fimbria and alveus was primarily axonal. Original magnification, ×17.5. 1, Ammon's horn (a, CA1; b, CA2; c, CA3; d, CA4); 2, granule cell layer of dentate gyrus; 3, temporal horn of lateral ventricle; 4, hippocampal fissure; 5, alveus; 6, fimbria.*

astrocytes, oligodendroglia, choroid plexus, ependyma, arachnoid meningothelium, vascular smooth muscle, and endothelium (Figure 1C). The subependymal and subpial astrocytes contained diffuse or granular staining patterns in cytoplasm and larger processes. In choroid plexus epithelium, annexin IV was distributed in a relatively coarse granular cytoplasmic pattern, often with subapical accumulation, and in ependymocytes was usually localized to cell borders. Annexin IV in oligodendroglia was most apparent in populations exhibiting perineuronal and perivascular satellitosis where it was present as a thin perinuclear cytoplasmic rim or outlining the plasma membrane (Figure 3H). Annexin IV was never detected in neurons.

Annexin IV was also detected in the nuclei of some ependymocytes, astrocytes, oligodendrocytes, and endothelial cells. The presence of nuclear staining was not correlated with cytoplasmic staining or the type of lesion, clinical history, or patient age.

Annexin VI

Moderate to strong annexin VI immunoreactivity was present in neurons, choroid plexus epithelium, arachnoid meningothelium, and vascular smooth muscle (Figure 1D). In pyramidal neurons, annexin VI was primarily localized to the plasma membranes of dendritic processes and perikarya and in neuropil in a granular or punctate pattern similar to that of synaptophysin (Figure 3, A and B). Bipolar neurons in the stratum oriens displayed dense cytoplasmic staining of soma and proximal processes, whereas the immunoreactivity in granule cells was less prominent. The only immunoreactivity in the white matter was confined to axonal fibers. Annexin VI in the choroid plexus epithelium was concentrated in the apical and basal regions, whereas ependyma displayed negligible immunoreactivity. Annexin VI was not detected in nonreactive astrocytes, oligodendroglia, and endothelial cells.

Pathological States

All the types of acute central nervous system damage and chronic degeneration were associated with alterations of the cytoarchitectural distribution of annexin VI in affected neurons and increased expression of annexins I, II, and IV in reactive astrocytes.

However, the intraneuronal distribution of annexin VI and the relative degree of expression for the annexins showed some differences in the various pathological processes.

Neuronal Annexins

In subacute hypoxic ischemic injury, seizure damage, and Alzheimer's disease, the surviving CA2 pyramidal neurons displayed increased annexin VI immunoreactivity within the somal cytoplasm (Figure 3E). The differences in staining intensities of the CA2 neurons compared with those in other areas was often quite marked. Similar but more variable changes were present in the CA3 and CA4 neurons. In Alzheimer's disease, the granulovacuolar bodies within degenerating pyramidal neurons were strongly immunoreactive for annexin VI. The vacuolar membranes of these bodies were consistently labeled, whereas the granular bodies were more variably stained (Figure 3E). In contrast, annexin VI was never detected in neurofibrillary tangles or neuritic plaques.

Each case of acute hypoxic ischemic injury displayed a unique pattern of annexin VI immunoreactivity within neurons. The first was a concentration of immunoreactivity within proximal dendritic segments in CA2–4 (Figure 3C) or within the somal cytoplasm in CA1–2 (Figure 3D). The other was a markedly heterogeneous distribution of immunoreactivity within pyramidal neurons, including focal staining of plasma membrane and vesicular structures within the cytoplasm.

In contrast to annexin VI, annexin I immunoreactivity in neurons showed no consistent pattern of alteration in the various disease states. In Alzheimer's disease, staining within neurons was excluded from intracellular neurofibrillary tangles and granulovacuolar bodies in degenerating neurons.

Glial Annexins

Reactive astrocytes were identified by prominent cytoplasm and processes with strong GFAP immunoreactivity. Astrocytes in the endfolium region adjacent to the granular cell layer appeared to be the most sensitive to developing reactive changes in response to hypoxic ischemic injury and seizures. In more severe cases reactive astrocytes were more widely distributed, particularly in areas of neuronal loss or infarction. In Alzheimer's disease, reactive astrocytes were diffusely distributed, often in association with senile plaques and degenerating neurons.

Reactive astrocyte populations displayed variable expression of annexins I, II, and IV. In general, annexin immunoreactivity was most prominent within cells and the surrounding neuropil in regions of more severe gliosis, eg, adjacent to an infarct (Figure 2A). The intracellular pattern of annexin I immunoreactivity was always diffuse (Figure 3F). Annexin IV was diffusely distributed within gemistocytic astrocytes (Figure 3, H) or in punctate, granular, or vesicular patterns within fibrillary astrocytes. In contrast, annexin II p11 and p36 were usually most strongly localized at the cell periphery. In Alzheimer's disease, astrocytic p11/p36 was also localized to discrete plaque-like areas (Figure 2B). Dual-label immunohistochemistry showed that p11 immunoreactive astrocytes, when present, were only in close proximity to diffuse and mature β-amyloid plaques and were not associated with either dystrophic neurites or extracellular neurofibrillary tangles. However, many β-amyloid plaques were not associated with p11/p36 immunoreactivity. In contrast to the focal pattern of p11/p36 expression, GFAP and S100β immunoreactive astrocytes were more widely distributed (Figure 3G). Annexin I or IV immunoreactive astrocytes did not show a plaque-like pattern and were distributed without any specific relationship to neuritic plaques, neurofibrillary tangles, or degenerating neurons.

Only annexin IV could be consistently identified in activated microglia with ramified, rod, and ameboid morphologies. These cell populations were also immunoreactive for the HAM-56 macrophage antigen but not GFAP or factor VIII. Annexin IV immunoreactivity was distributed in granular or vesicular patterns within the cytoplasm and processes.

Discussion

Previous studies have described the isolation and biochemical characterization of the major species of annexins in mammalian brain, which include annexins I, II, IV, and VI.[4,13] Little is known, however, about the specific cellular distribution or physiological roles of this family of proteins in the human nervous system. This study examined the comparative cellular localization of the major annexins in the human hippocampus, which contains physiologically distinct neuronal cell types and undergoes well-characterized pathological changes in response to injury and degenerative processes. Annexin VI was selectively distributed in neurons and annexins II and IV in glia, whereas annexin I was present in both neurons and astroglia. These data concur with previous findings in non-

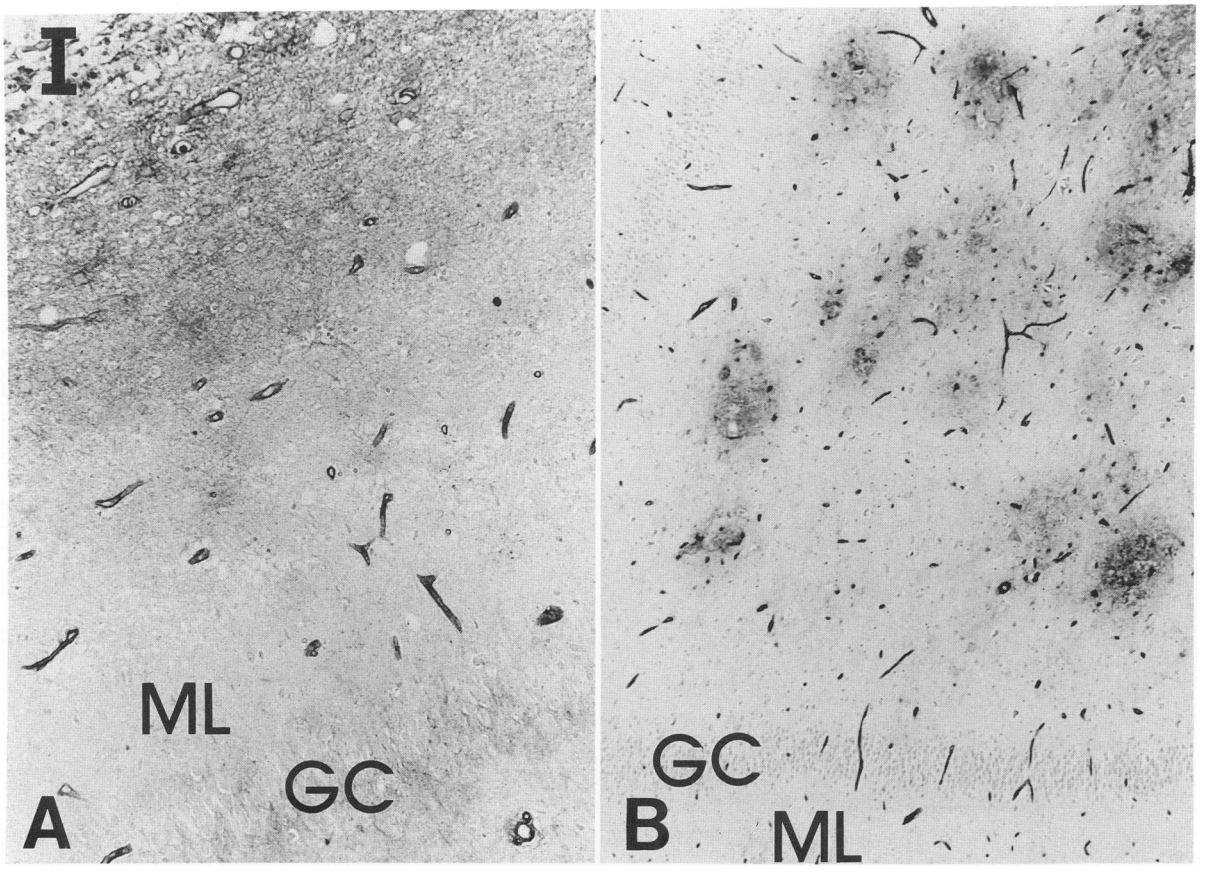
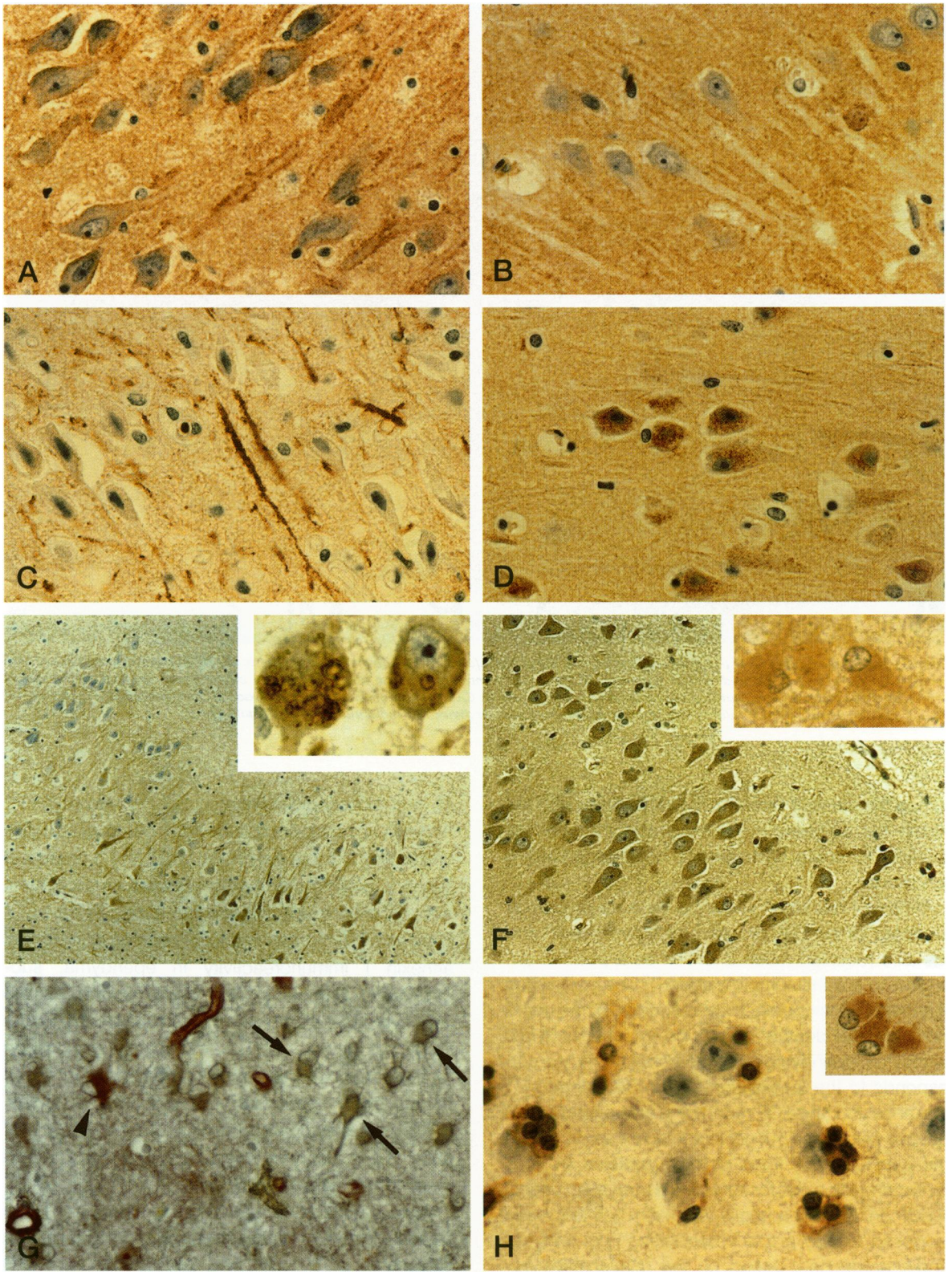


Figure 2. *Pathological patterns of p11 immunoreactivity.* **A:** *Subacute infarct in CA1. The region adjacent to the infarct was densely stained. Annexins I, II (p36), and IV showed similar patterns. (Biotin-avidin immunoperoxidase with hematoxylin counterstain, original magnification ×150.)* **B:** *Alzheimer's disease. Staining was associated with plaques in CA4. (Biotin-avidin immunoperoxidase with hematoxylin counterstain, original magnification ×60.) I, infarct; GC, granule cell layer of dentate gyrus; ML, molecular layer of dentate gyrus.*

human mammalian brain.[3–5,13] Our findings also implicate the annexins in neuronal and astroglial responses to acute and chronic neurodegenerative conditions.

Annexin I has been shown to be a major cellular substrate for the EGF receptor tyrosine kinase[14] and has also received much attention as a putative endogenous anti-inflammatory agent.[15] The localization of annexin I immunoreactivity in neurons of the cornu ammonis and dentate gyrus and in subependymal astrocytes, ependymocytes, and choroid plexus corresponds to the localization of the EGF receptor.[16] Like annexin I, the EGF receptor is also expressed in reactive astrocytes but not in queiscent astrocytes.[17] The parallel distributions of these proteins are thus consistent with a role for annexin I in mediating effects of EGF on neurons and glia. Annexin I has also been proposed to play a role in regulating glial prostaglandin production, based on the relationships between annexin I expression, phospholipase A_2 activity, and eicosanoid release in cultured astrocytes.[18] Annexin I in rat hippocampus has a neuronal and glial distribution like that described here[5] and is present in synaptic plasma membrane fractions as a covalent dimer,[19] presumably due to cross-linking by transglutaminase.[20] In human brain, Johnson et al[8] detected annexin I immunoreactivity in ependyma, subependymal astrocytes, choroid plexus, and reactive astrocytes associated with brain injury, but unlike this study, not in neurons. The most obvious explanation for this variance would be differences in the epitopes recognized by the antibodies used in these studies; it is possible that posttranslational modifications of annexin I in neurons, such as cross-linking, could mask immunoreactive epitopes.

Annexin VI immunoreactivity was associated with neuronal cell membranes and processes in a pattern suggestive of terminal and perisynaptic locations and was the only annexin that was not detected in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reactive or quiescent macroglia. The choroid plexus secretory epithelium showed strong annexin VI staining concentrated in the subplasmalemmal region. One potential role for annexin VI in synaptic terminals and choroid plexus is regulation of clathrin-mediated membrane trafficking. Clathrin-coated vesicles are involved in the biogenesis of dense-core secretory granules and synaptic vesicles, and in the endocytotic recycling of vesicles after exocytosis.[21] Annexin VI is required for the budding of clathrin-coated endocytotic pits *in vitro*.[22] Although the clathrin heavy chain does not exhibit Ca^{2+}-dependent membrane binding properties, it co-purifies with annexins VI and IV prepared from brain using this technique, suggesting an association with the annexin proteins.[4] Annexin VI has also been shown to modulate the activity of a ryanodine-sensitive Ca^{2+} channel in sarcoplasmic reticulum.[23] Ryanodine receptors have been identified in neurons,[24] raising the possibility that annexin VI might modulate neuronal Ca^{2+} signaling mechanisms.

Expression of annexins II (p11/p36) and IV in neural cells was limited to glia. However, the glial cell populations expressing these annexins and the cytoarchitectural distribution of immunoreactivity within cells were quite different. Immunoreactivities for p11 and p36 were associated primarily with plasma membranes and processes of reactive astrocytes, whereas annexin IV staining was cytoplasmic in reactive astrocytes and was also present in some oligodendrocyte populations, activated microglia, and sometimes in the nuclei of glial (and endothelial) cells. These different patterns of annexin localization most likely reflect roles in different cellular activities, such as proliferation,[25] migration,[26] and extension of cytoplasmic processes.[25,27] The localization of annexin IV in nuclei has been previously reported in fibroblasts.[28] Intranuclear functions of annexins are suggested by the discoveries that annexin II is a component of the DNA polymerase α-primer recognition protein complex,[29] and of a novel annexin associated with calcyclin.[30]

Two patterns of annexin localization were characteristic of Alzheimer's disease: the association of annexin VI with granulovacuolar bodies in degenerating pyramidal neurons and expression of annexin II (p11/p36) by astrocytes closely associated with some β-amyloid plaques. Granulovacuolar bodies contain immunoreactivities for cytoskeletal epitopes similar to those found in neurofibrillary tangles and neuritic plaques, and thus seem to be related to neurofibrillary degeneration.[31] The presence of strong annexin VI immunoreactivity associated with the membranes of granulovacuolar bodies (but not with neurofibrillary tangles or neuritic plaques) suggests that annexin VI could be important in their formation, perhaps involving an aberrant vesicular trafficking pathway. The occasional association of p11/p36 immunoreactive astrocytes with extracellular β-amyloid deposits in Alzheimer's hippocampus suggests that some amyloid plaques may contain a component that stimulates annexin II expression. Plaques are heterogeneous in composition, containing variable amounts of amyloid precursor derivatives, neuritic components, microglia, and secondary substances such as immunoglobulins and complement factors.[32] It seems likely that plaque components other than amyloid and neurites are related to annexin II expression in plaque-associated astrocytes, because p11/p36 immunoreactivity was inconstantly present in mature or immature plaques and was not seen in reactive astrocytes associated with extracellular neurofibrillary tangles, which, like neuritic plaques, contain paired helical filaments.

The increased expression of annexins in pathological states may represent a neural tissue response that serves to limit damage. Membrane degradation by phospholipases represents one mechanism underlying neuronal injury after hypoxia ischemia, excitotoxin exposure, seizures, and in chronic neurodegenerative diseases.[33] Annexins, which inhibit phospholipase activity *in vitro*, could thus act as endogenous neuroprotective agents. In subacute and chronic states (posthypoxic, seizures, and Alzheimer's disease) annexin VI immunoreactivity was consistently increased within the cytoplasm of pyramidal neuronal soma in CA2. Neurons in this region are more resistant to injury than those in other regions of the hippocampus, raising the possibility that an increase in annexin VI expression could represent a response promoting neuronal survival or recovery from injury. An alternative explanation is that annexin VI may accumulate in neuronal soma because of disruption of normal axonal-dendritic transport mechanisms, because an-

Figure 3. *Cellular localization of annexins.* **A–E:** *Annexin VI. Immunoreactivity in normal CA2* (**A**) *and CA1* (**B**) *was associated with neuronal membranes and neuropil. In acute hypoxic ischemic injury, annexin VI was concentrated within dendritic segments in CA2* (**C**) *and neuronal cell bodies in CA1* (**D**). *In Alzheimer's disease* (**E**), *annexin VI was selectively increased within CA2 neurons (lower right) but not in CA1 neurons (upper left) and was also associated with granulovacuolar bodies (inset).* **F:** *Annexin I immunoreactivity was present in both pyramidal neurons and reactive astrocytes (inset).* **G:** *Dual-labeling for p11 (AEC, red) and GFAP (DAB-Ni, black) demonstrated p11 immunoreactivity in an Alzheimer's plaque-associated astrocyte (arrowhead) and capillaries, whereas other astrocytes stained only for GFAP (arrows).* **H:** *Annexin IV was present in oligodendrocytes and reactive astrocytes (inset). Biotin-avidin immunoperoxidase with hematoxylin counterstain, magnifications:* **A–D,** *×338;* **E** *×52; inset, ×676;* **F,** *×180; inset, ×541;* **G,** *×226;* **H,** *×541; inset, ×609.*

nexin VI has been identified in the slow component of axonal transport in rat peripheral nerve.[34]

The presence of annexin I immunoreactivity in neurons is also compatible with the hypothesis that neuronal annexins could confer resistance to Ca^{2+}-induced neuronal injury. Although annexin I did not show patterns of expression in neuronal populations which clearly corresponded with their relative degrees of resistance or susceptibility to injury, recent studies have directly demonstrated neuroprotective properties of endogenous annexin I. Surprisingly, the active protein appears to reside in the extracellular space, because intraventricular administration of annexin I in live rats decreased the sizes of cerebral lesions induced by ischemia[9] or glutamate receptor agonist,[35] whereas injection of neutralizing antibody had the opposite effect. Potential sources of endogenous extracellular annexin I could include local release by neurons and reactive astrocytes or secretion into the cerebrospinal fluid by choroid plexus, because annexin I has been shown to be selectively secreted by other cell types.[36,37] Annexin I could also be released from damaged tissue or inflammatory cells. Although this study confirms previous observations that annexin I is increased in injured brain tissue,[8,9] we found that annexins II and IV were also increased. The role(s) of these annexins in neuroprotection has yet to be studied.

Annexins in the cerebral vascular endothelium are probably not related to special physiological functions, such as the blood-brain barrier, because relatively high levels of annexins I, II, IV, and VI are present in cultured human umbilical vein endothelial cells.[38] It is quite possible that we did not detect significant annexin VI immunoreactivity in the endothelium because of the relatively strong reaction in adjacent vascular smooth muscle and neuropil. Our data regarding annexin expression in cerebrovascular smooth muscle agree with a previous report that smooth muscle cells express annexin VI but not annexin II.[39]

Annexins are widely distributed throughout the body and therefore could be involved in pathophysiological processes in nonneural tissues and brain. Annexin I expression is increased in rat renal tubules during recovery from ischemia,[40] supporting a hypothesis that annexins may participate in general cellular and tissue mechanisms for limiting injury and promoting repair. Further studies of multiple annexins, as described here in the human hippocampus, may define patterns of expression that are unique or common to various types of cells and pathological states, suggesting links between specific annexins and cellular functions.

Acknowledgments

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