Antibodies to Synaptic Vesicles Purified from *Narcine* Electric Organ Bind a Subclass of Mammalian Nerve Terminals

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ABSTRACT Antibodies were raised in rabbits to synaptic vesicles purified to homogeneity from the electric organ of *Narcine brasiliensis*, a marine electric ray. These antibodies were shown by indirect immunofluorescence techniques to bind to a wide variety of nerve terminals in the mammalian nervous system, both peripheral and central. The shared antigenic determinants are found in cholinergic terminals, including the neuromuscular junction, sympathetic ganglionic and parasympathetic postganglionic terminals, and in those synaptic areas of the hippocampus and cerebellum that stain with acetylcholinesterase. They are also found in some noncholinergic regions, including adrenergic sympathetic postganglionic terminals, the peptidergic terminals in the posterior pituitary, and adrenal chromaffin cells. They are, however, not found in many noncholinergic synapse-rich regions. Such regions include the molecular layer of the cerebellum and those laminae of the dentate gyrus that receive hippocampal associational and commissural input. We conclude that one or more of the relatively small number of antigenic determinants in pure electric fish synaptic vesicles have been conserved during evolution, and are found in some but not all nerve terminals of the mammalian nervous system. The pattern of antibody binding in the central nervous system suggests unexpected biochemical similarities between nerve terminals heretofore regarded as unrelated.

Synaptic vesicles from the electric organ of *Narcine brasiliensis* can be purified to homogeneity (8). The vesicles obtained have a quite simple lipid (11) and protein composition (55, 56). Antibodies raised to the pure vesicles, after suitable adsorption, bind specifically to synaptic vesicles in crude homogenates of electric organ (7) and do not bind to homogenates of nonneural *Narcine* tissues. By immunofluorescence techniques it can be seen that the antiserum directed against fish synaptic vesicles cross-reacts with some antigen or antigens in the frog, rat, and chick neuromuscular junction (46). Thus, antigenic determinants present in electric organ synaptic vesicles are also found in some nerve terminals of higher species.

What is the nature of these conserved antigenic determinants? Because the electric organ is embryologically derived from striated muscle, the antiserum could be recognizing antigens specific for neuromuscular junctions. Alternatively, because both tissues are cholinergic, the shared antigens may be involved in acetylcholine (ACh) metabolism. The shared antigens might be common to all nerve terminals, irrespective of transmitter type. Conceivably, the common element between the two tissues could be exocytosis, and, if so, the shared antigens should be present in all secretory tissue. The experiments outlined in this paper, using indirect immunofluorescent procedures, were designed to distinguish among such possibilities.

The data presented here indicate that the conserved antigens are present in many but not all of the nerve terminals of the mammalian nervous system. It appears that antibodies raised to electric organ synaptic vesicles recognize some common feature of a subclass of mammalian nerve terminals not apparent by other techniques. As antibodies to defined neural elements become increasingly available, it is likely that they will offer classifications of cells of the nervous system different from and complementary to the morphological and biochemical (i.e., transmitter) criteria already available.

MATERIALS AND METHODS

Animals/ Surgery

All surgery and histology involved Sprague-Dawley rats of either sex, aged 2

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mo to adult (150–300 g). Animals with unilateral splanchnic nerve section, unilateral ciliary ganglionectomy, combined unilateral ciliary and superior cervical ganglionectomies, pituitary-stalk section, and sham pituitary-stalk section were obtained from Zivick-Miller, Allison Park, Pa. All other animals were obtained from Simonsen Laboratories, Gilroy, Calif. Successful operations were confirmed as follows: splanchnic nerve section by loss of acetylcholinesterase (AChe) histochemical staining in adrenal medulla, ciliary ganglionectomy by pupillary dilation, superior cervical ganglionectomy by loss of catecholamine (CA) histofluorescence in the iris, and pituitary stalk section by antidiuretic hormone radioimmunoassay of the pituitary (performed by Dr. L. Keil, Ames Research Center, Moffett Field, Calif.). Dual ciliary and superior cervical ganglionectomies were confirmed only by the loss of CA histofluorescence; the ciliary portion of the operation could not be independently confirmed because pupillary dilation is not seen with concurrent sympathetic and parasympathetic denervation.

Superior cervical ganglion (SCG) deafferentation (by removal of several millimeters of the preganglionic nerve trunk) and superior cervical ganglionectomies were performed in our laboratory. Successful deafferentations were confirmed by depletion of AChe histochemical staining in the ganglia.

Animals with fimbrial and entorhinal cortical lesions, both medial and lateral, were a gift of Dr. Carl Cotman. Extent of the lesions was confirmed by AChe histochemistry on hippocampus sections.

Histology

Rats were killed with ether overdose and appropriate structures were dissected and rinsed in phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM sodium phosphate, pH 7.4.) Blocks of <5 mm in largest dimension were cut and frozen in liquid nitrogen. In the case of the iris, SCG, and pituitary, convenient blocks were constructed by sandwiching the tissue between strips of diaphragm muscle. Animals from which whole brain was taken were perfused with 5% dimethyl sulfoxide in rat Ringer's solution and the brains were removed and then frozen in dry ice. In all cases, unfixed tissue was used because even mild fixation with paraformaldehyde and/or glutaraldehyde greatly reduced specific antibody binding and increased background. 4–10-μm cross sections were cut in a cryostat, mounted on ethanol-washed glass slides, air-dried, and stored at −20°C with desiccant until needed (up to 4 wk). Loss of antigenicity was minimal even for as long as 2 mo in the freezer. Sections intended for CA fluorescence were used immediately after sectioning.

AChe Histochemical and CA Histofluorescent Staining

Unfixed sections on slides identical to those used for immunohistochemical procedures were stained for AChe with either the Karnovsky and Roots (22) or the Koelle histochemical method as modified by Lynch et al. (31). Tetraisopropylpyrophosphoramide, Sigma T-1505 (Sigma Chemical Co., St. Louis, Mo.) at 10^{-5} M was used to inhibit nonspecific cholinesterases.

Freshly sectioned unfixed tissue was stained by the formaldehyde vapor technique of Falck et al. (13).

Antibodies

Rabbit antiserum raised against highly purified vesicles from *Narcine* was prepared as described by Carlson and Kelly (7). When adsorbed against a nonsynaptic vesicle fraction of electric organ, the resulting antiserum (RASVA) is specific for synaptic vesicles in crude homogenates of the electric organ (7). This vesicle-specific derivative of the crude antiserum was used in all experiments. As controls, both preimmune serum adsorbed against the nonsynaptic vesicle fraction and RASVA adsorbed against sonicated vesicles (7) were used. In all cases, both control sera gave similar results.

Immunofluorescent Staining

Antiserum was diluted in PBS containing 1% fetal calf serum (FCS) and applied to sections in ~30-μl drops. After 30-min incubation at room temperature, the sections were washed with PBS-FCS and incubated for 30 min with fluorescein-conjugated goat anti-rabbit IgG serum (Cappel Laboratories, Cochranville, Pa.) at a 1:60 dilution in PBS-FCS. Finally, the sections were washed in PBS-FCS, mounted under 90% glycerol–10% PBS, and examined with a Zeiss photomicroscope. Fluorescein was excited and viewed with Zeiss 48-77-09 filters (excitation, 455–490 nm; barrier 520 nm; reflector, 510 nm) and CA with 48-77-05 filters (excitation, 400–440 nm; barrier, 470 nm; reflector, 460 nm).

Peroxidase Antiperoxidase (PAP) Staining

We followed the method of Sternberger (50), except that for primary antibody incubation we used the same conditions as in immunofluorescent procedures rather than longer incubation at lower dilution. After being washed in PBS-FCS, sections were incubated 30 min with goat anti-rabbit IgG serum at 1:200 dilution in PBS-FCS. After a second wash in PBS-FCS, sections were incubated 45–60 min in a 1:500 dilution of rabbit PAP (Cappel Laboratories) in PBS-FCS. Finally, the sections were washed in PBS-FCS and incubated in 0.8 mg/ml 3-3′-diaminobenzedine tetrahydrochloride, Aldrich Chemical Co., Inc., Milwaukee, Wis., 0.015% H_2O_2 until sufficient reaction product was present. Sections were then washed several times in PBS, dehydrated, defatted, and mounted with Permount (Fisher Scientific Co., Pittsburgh, Pa.).

RESULTS

Antibodies raised against synaptic vesicles from *Narcine* electric organ bind to the motor nerve terminals of rat, chicken, and frog (46). Some insight into the nature of these conserved synaptic vesicle antigenic determinants (SVAD) can be obtained by examining their distribution in other parts of the mammalian nervous system. Especially useful are regions of well-established transmitter type, regions with layered cell types or synaptic inputs, or regions which can be readily denervated. We have chosen to look at the iris as a model of noncholinergic nerve-muscle junctions, the spinal cord to examine subcellular distribution, the SCG and the adrenal medulla as models of peripheral nerve-nerve synapses, the pituitary to compare endocrine and neuroendocrine cells, and the hippocampus and the cerebellum as central nervous system areas with defined layering.

Iris

The iris, a smooth muscle structure with dual autonomic innervation, offers a system to test whether the conserved SVAD are restricted to cholinergic neurons or may be associated with other transmitters such as norepinephrine. Cholinergic parasympathetic and adrenergic sympathetic fibers are found in both sphincter and dilator muscles in the rat iris. When the SCG is removed, all CA fibers disappear, leaving the majority of the cholinergic fibers intact. When the ciliary ganglion is removed, the majority of remaining fibers are adrenergic (12). By examining the antigenicity that remains after selective removal of one of the inputs, the transmitter specificity of the antiserum can be tested.

Immunofluorescent staining of the iris, sectioned perpendicular to its plane, reveals many fine fiberlike structures with varicosities both in the dilator and sphincter regions (Fig. 1 *a*). The fibers run radially in the dilator muscle and circumferentially in the sphincter. These results are consistent with the known structure and distribution of the autonomic fibers innervating the iris. This staining is specific because it is not seen when we use a control antiserum that has been preadsorbed with pure synaptic vesicles (Fig. 1 *b*). When both the ciliary and SCG are removed, almost all staining disappears (Fig. 1 *f*), confirming that only nerve fibers stain appreciably. The distribution of fluorescence that is seen when the cholinergic component is removed via ciliary ganglionectomy (Fig. 1 *d*) is similar to that seen in a normal iris with formaldehyde vapor-induced CA fluorescence (Fig. 1 *c*). After ciliary ganglionectomy, limited antibody fluorescence is seen in the sphincter, whereas fluorescence in the dilator region is identical in distribution, although perhaps less intense than in the normal iris (Fig. 1 *a*). Finally, when the adrenergic fibers are removed via cervical sympathectomy, little change is detected in the staining pattern (Fig. 1 *e*). Some change might be expected upon removal of the adrenergic fibers, especially in the dilator muscle because they are immunoreactive. However, because nonad-

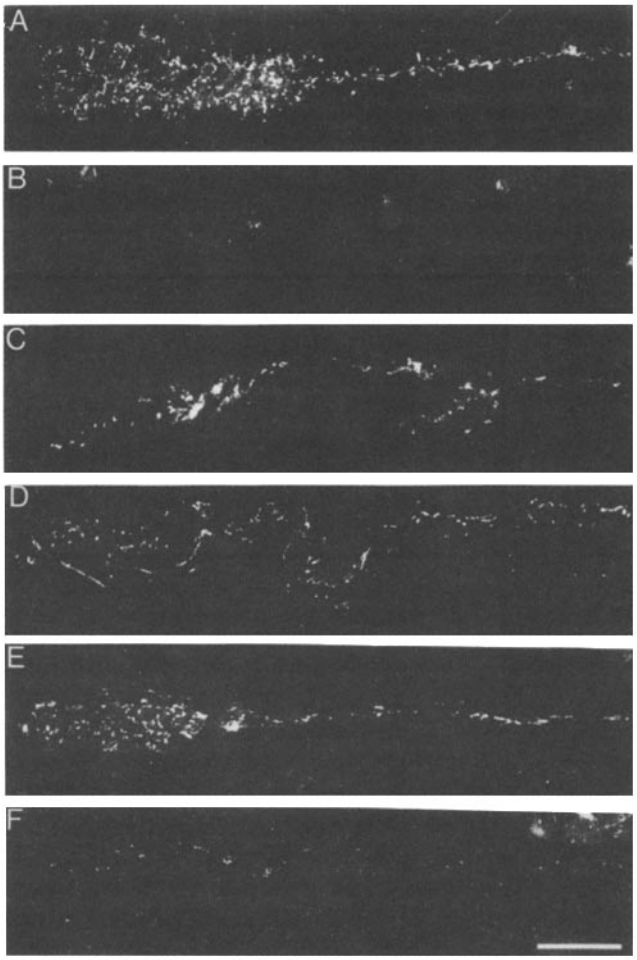

FIGURE 1 Conserved synaptic vesicle antigenic determinants in the iris of adult rat after the removal of the sympathetic innervation, parasympathetic innervation, or both. 4-μm cryostat sections of unfixed iris are cut perpendicular to the plane of the iris and through the pupil. Each micrograph shows a sperm-shaped section of iris with the "head" to the left being the sphincter and the "tail" to the right being the dilator muscle. The pupil would be found to the extreme left, beyond the sphincter. (*A*) Normal iris stained with RASVA and fluorescein-second antibody shows numerous fine fibers with varicosities; in the dilator they run in the plane of section, whereas in the sphincter they tend to run perpendicular to the plane of section. (*B*) Background staining in section identical to *A* but stained with control serum, that is, RASVA adsorbed with synaptic vesicles. (*C*) Formaldehyde vapor-induced CA fluorescence showing limited staining in the sphincter and staining in the dilator comparable to that seen in *A*, using RASVA. (*D*) Staining as in *A* of iris 1 wk after ciliary ganglionectomy, which removes parasympathetic inputs. (*E*) Staining as in *A* of iris 1 wk after superior cervical ganglionectomy, which removes sympathetic inputs. (*F*) Staining as in *A* of iris 12 d after combined ciliary and superior cervical ganglionectomies. Similar results are seen with 1 wk post operative survival (not shown). Bar, 100 μm.

renergic fibers are so numerous, even in the dilator (45) we would not expect to detect loss of the adrenergic component. We conclude that because in the iris both cholinergic and adrenergic fibers stain, the conserved SVAD are not restricted to cholinergic neurons.

Spinal Cord

In the spinal cord the cell bodies of the motor neurons are large and easily recognizable. We have used them to determine whether the conserved SVAD are present in nerve cell bodies or are restricted to their terminals. Indirect immunofluorescent staining of sections through the lumbar region of the spinal cord (Fig. 2) shows no staining associated with the cytoplasm of motor neurons, even though their terminals are intensely fluorescent (46).

Although the cell bodies do not contain the conserved SVAD in detectable quantities, specific punctate immunofluorescence (1–3 μm in diameter) is seen throughout the grey matter, but not the white, of both ventral and dorsal horns. The coarse-grained rings that surround the soma of the motor neurons are reminiscent of the anti-substance P staining seen by Hokfelt et al. (19). The rather uniform distribution of this punctate immunofluorescence throughout the grey matter of the spinal cord is surprising because known transmitters such as substance P, somatostatin (19), gamma-aminobutyric acid (GABA) (3), and ACh, as monitored by AChe distribution, show marked regional localization. From the pattern of staining seen in the spinal cord, it is clear that the majority of conserved SVAD are found in terminal regions rather than in axons or inside

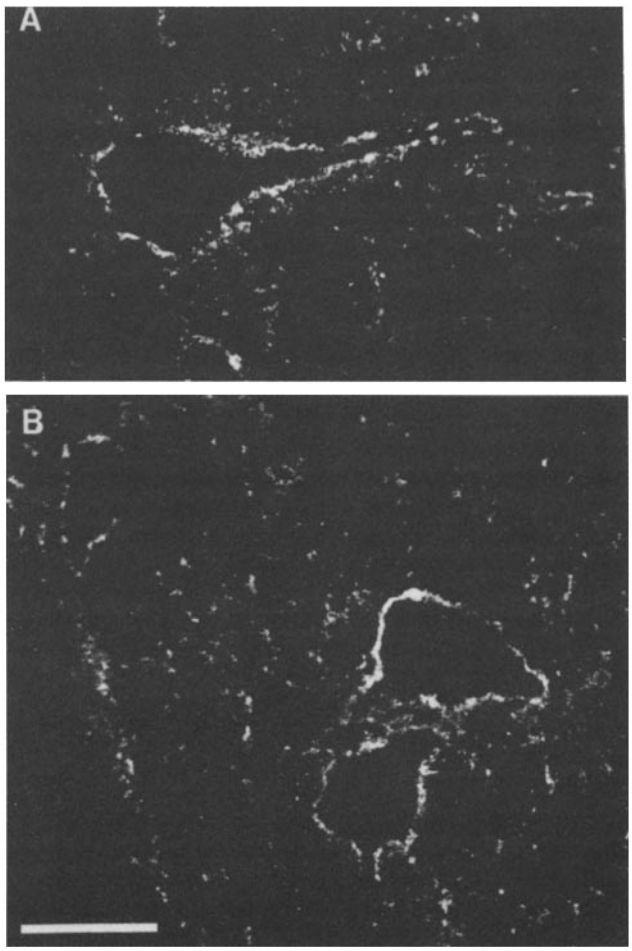

FIGURE 2 Immunofluorescence of two examples of unfixed spinal cord ventral horn cross sections (4 μm) incubated with RASVA followed by fluorescein-second antibody. Large motor neurons and some smaller cells (s) are defined by coarse punctate fluorescence around their periphery. Diameter of these punctate structures is ~3 μm. Background staining, when the primary antibody is RASVA adsorbed against synaptic vesicles, is virtually undetectable. Bar, 50 μm.

cell bodies, and they are unlikely to be associated with a single transmitter.

SCG

Synapses of sympathetic preganglionic fibers constitute a group of well-defined nerve-nerve cholinergic synapses whose morphology is quite different from that at the neuromuscular junction. In sections of the SCG, antibody fluorescence is primarily seen as small specks, from <0.8 to ~4 μm in diameter, between and around the edges of principal ganglion cells (Fig. 3*a*). In size and distribution these specks fit descriptions of preganglionic terminals in the rat SCG (15). Diffuse fluorescence is associated with the cytoplasm of both the small intensely fluorescent and principal ganglion cells, but, because it is only slightly more intense than background labeling seen in controls, (Fig. 3*c*) it is of questionable significance. Upon denervation, (Fig. 3*b*), the frequency of the "specks" is drastically reduced, indicating that they are of preganglionic origin. Also, cytoplasmic fluorescence of deafferented principal cells is significantly more intense than in controls or normal ganglia and often takes on a nonuniform distribution, clumped around the nucleus or at the edges of the cells. These distributions are similar to the distribution of synaptic vesicles in the principal ganglion cell soma described in normal ganglia by Richards and Tranzer (42). They did not report the distribution after deafferentation.

Whereas the high background fluorescence makes results in SCG cells more difficult to interpret than results in motor neurons, it appears that low levels of conserved SVAD may be found in the SCG cell bodies. A definite change in the level and distribution of these conserved antigens is found in the somata of ganglion cells after deafferentation. We conclude that antibodies to fish synaptic vesicles recognize some common antigenic determinants in preganglionic nerve terminals, and possibly sympathetic ganglion cells as well.

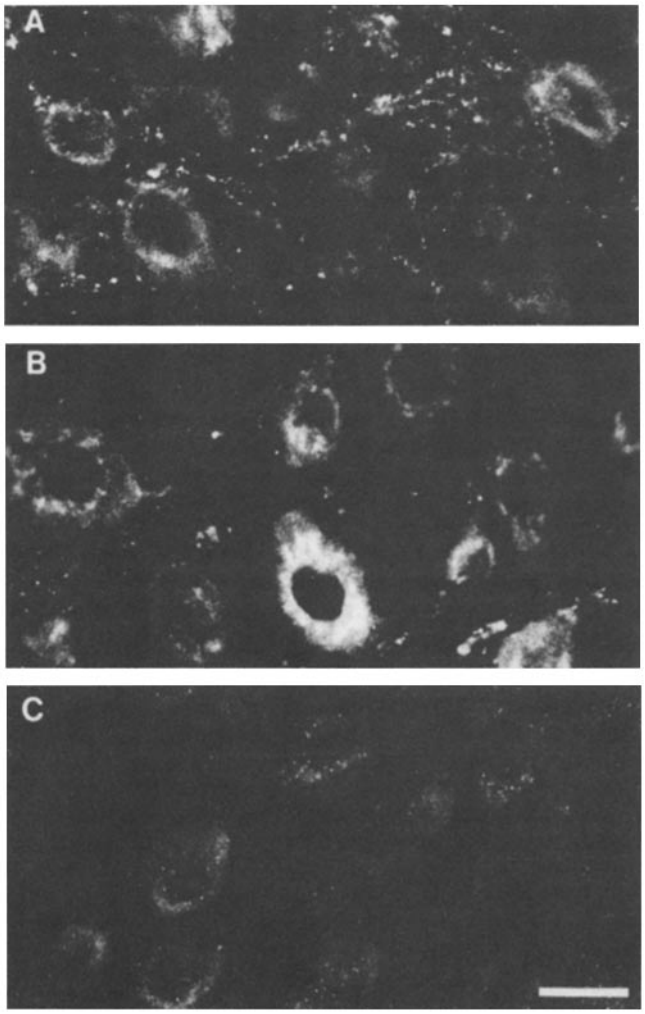

FIGURE 3 Conserved antigens in intact (*A*) or deafferented (*B*) SCG from adult rat. Longitudinal sections (4 μm) of unfixed ganglia were incubated with RASVA followed by fluorescein-second antibody. (*A*) Intact ganglion shows numerous small fluorescent structures ranging from 4 to <0.8 μm in diameter at the periphery of and in the spaces between cells. (*B*) 1 wk after deafferentation, most of these small structures are no longer seen. Instead, fluorescence within the cells becomes more pronounced, especially in the perinuclear regions. (*C*) Background staining in section identical to *A*, except with control serum, RASVA adsorbed with synaptic vesicles. Some nonspecific fluorescence is associated with the cytoplasm both of small intensely fluorescent and principal ganglion cells, although subjectively it is less intense than that seen in *A* or *B*. Bar, 20 μm.

Adrenal Gland

Chromaffin cells of the adrenal medulla, although technically endocrine cells, are developmentally and functionally closely related to sympathetic ganglion cells. Both are derived from the same precursor cells (1), are innervated by sympathetic preganglionic fibers, and secrete the same transmitter when stimulated. When stained with fluorescent antibodies, the major features of the adrenal medulla are numerous large diameter "bulbs" (~4 μm) on the basal and lateral aspects of the chromaffin cells (Fig. 4*a*). There is no binding to the adrenocortical cells, but dim smooth textured fluorescence is seen associated with the cytoplasm of the chromaffin cells. Because no staining is seen in controls, (Fig. 4*c*) binding to the bulbs and the chromaffin cell cytoplasm is specific.

Proof that the punctate fluorescence is associated with preganglionic terminals comes from denervation studies. When denervated adrenals are sectioned the fluorescent bulbs disappear, whereas the diffuse staining in the chromaffin cells remains (Fig. 4*b*). Furthermore, denervated chromaffin cells appear to stain more brightly than those from intact adrenals, as is the case with SCG cells. However, the labeling of cytoplasm is much less intense than that of nerve terminals.

Pituitary: Endocrine

The anterior and intermediate lobes of the pituitary consist of endocrine cells derived from the neural ridge (39) that secrete a variety of peptides. No antibody binding is detectable in the anterior lobe (Fig. 5*a*). Binding to the marginal cells between the two lobes is nonspecific because it is also seen when antiserum adsorbed against synaptic vesicles is used (Fig. 5*c*). Weak specific antibody fluorescence is evenly distributed throughout the cytoplasm of cells of the intermediate lobe (Fig. 5*a*). The intermediate lobe cells, therefore, resemble chromaffin cells and SCG cells in that they uniformly express low levels of the conserved SVAD throughout their cytoplasm, whereas the anterior lobe demonstrates that not all endocrine cells express the conserved SVAD.

Pituitary: Neural

The posterior or neural lobe of the pituitary is another region with defined transmitters. Neurons from the hypothalamus

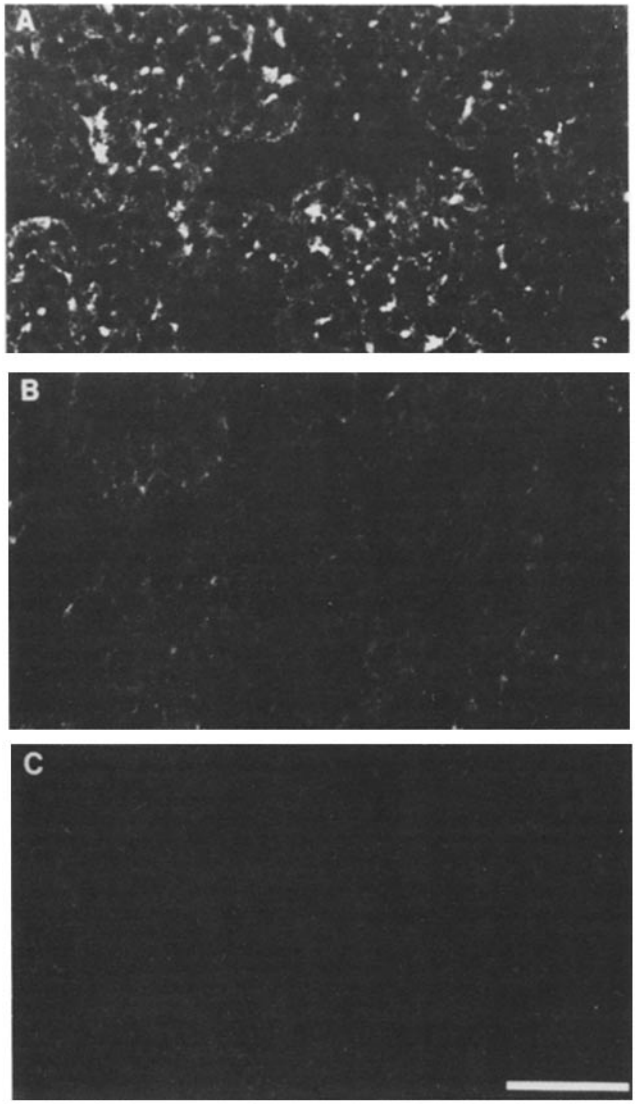

FIGURE 4 Conserved antigens in 4-μm sections of unfixed adrenal medulla from 150–200 g rats. (*A*) Normal medulla stained with RASVA and fluorescein-second antibody. Clumps of chromaffin cells with diffusely staining cytoplasm and vacant nuclei can be seen clustered around blood vessels and interstitial spaces. Along the basal and lateral aspects of these cells are numerous brightly staining bulbous structures, 3–6 μm in diameter. (*B*) Staining as in *A* of medulla 1 wk after denervation shows persistence of cytoplasmic staining, whereas the bulbous structures disappear. (*C*) Control using RASVA adsorbed against synaptic vesicles on section identical to *A*. Bar, 50 μm.

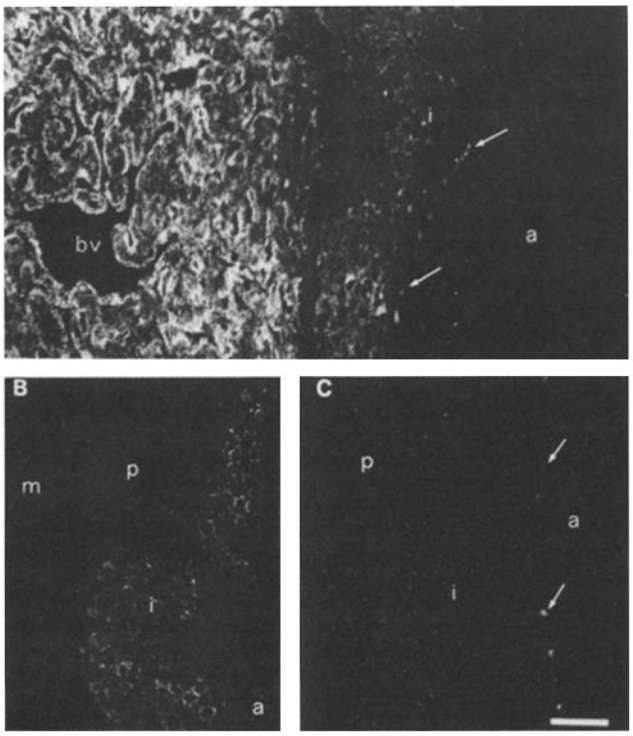

FIGURE 5 Conserved antigens in 4-μm sagittal sections of unfixed pituitary from adult rat. (*A*) Section of a normal gland incubated with RASVA and fluorescein-second antibody. In the posterior lobe (*p*), most staining is seen flanking the blood vessels (*bv*), although there is diffuse staining throughout the lobe. Cytoplasm of cells in the intermediate lobe (*i*) and the marginal layer (arrows) show diffuse fluorescence, whereas none is seen associated with the anterior lobe (*a*). (*B*) Staining as in *A* of the gland 10 d after pituitary stalk section. Upon denervation, staining of the posterior lobe (*p*) disappears, whereas that in the intermediate lobe (*i*) persists. Again, no staining is seen in the anterior lobe (*a*) or muscle (*m*) used in the sandwich procedure. (*C*) Control using intact gland incubated with RASVA adsorbed against synaptic vesicles shows only staining of the marginal layer (arrows) between the intermediate (*i*) and anterior (*a*) lobes. Bar, 50 μm.

project here and secrete oxytocin or vasopressin into the perivascular space. Several years ago it was suggested that, in addition to these peptides, the endings secrete ACh. This conclusion was based on AChe staining in both cell bodies and terminals, and the presence of small clear vesicles and large, dense-core peptide granules in the terminals (24). Later, these small clear vesicles were shown to be membrane recycling intermediates (37). Also, choline acetyltransferase (CAT) activity was shown to be much lower in the pituitary than elsewhere in the brain (25, 33), suggesting that the posterior pituitary does not have a cholinergic input.

The majority of antibody reactivity in the neural lobe of the pituitary is seen flanking the perivascular spaces, though some is scattered throughout the tissue (Fig. 5 *a*). To show that this fluorescence is caused by hypothalamic projections, we cut the pituitary stalk, leaving the hypothalamus and the pituitary intact. The surgery removed all antibody binding to the neural lobe, but that associated with the intermediate lobe remained (Fig. 5 *b*). The antibody binding was not eliminated as a result of surgical shock because the immunofluorescent staining of the pituitary from a sham-operated animal was indistinguishable from that of the unoperated controls. Therefore, we conclude that conserved SVAD seen in the neurohypophysis are associated with the noncholinergic endings of peptidergic neurons from the hypothalamus.

Hippocampus

Antibody binding to the rat spinal cord (Fig. 2) suggests that at least some central nerve terminals contain the conserved antigens. To determine whether all central nerve terminals bind antibodies raised to fish synaptic vesicles, we examined the hippocampus and the cerebellum, two regions of the brain in which synaptic endings are laminated.

The synaptic input to the various regions of the hippocampus, especially the dentate gyrus, is relatively well established. For example, the septal nuclei contribute all of the cholinergic

input to the dentate, except perhaps for a minor endogenous component in the hilus. The septal inputs as described by [^{3}H]leucine transport (53) or degeneration studies (e.g., 35) correspond well to CAT and AChe activities by microchemical determination (52) and AChe staining. Furthermore, these activities vanish upon removal of the septal inputs (27, 51). The septal fibers terminate primarily in three regions; a supragranule band, a band in the middle and outer zones of the molecular layer, and a diffuse zone in the hilus (Fig. 6*b*). Associational fibers from ipsilateral and contralateral CA3 and CA4 of the hippocampus proper form a second class of synapses, probably glutaminergic (52), primarily located in the inner zone of the molecular layer. Afferents from the entorhinal cortex form a third class, possibly glutaminergic, with dense termination in the middle and outer zones of the molecular layer (52). Glutamic acid decarboxylase, presumably marking interneurons, is found primarily in the granule cell layer and the outer zone of the molecular layer (3). In addition, there are minor exogenous monoaminergic, peptidergic, and perhaps other inputs.

To allow us to observe the distribution of conserved SVAD in the hippocampus at low magnification, we made use of the PAP method. Antigen distribution in the hippocampus (Fig. 6*a*) is remarkably similar to the distribution of septal inputs described by AChe histochemistry, although there are some differences. The hilus region stains more densely for AChe than with the antibody. The middle zone of the molecular layer shows a sharp band of antibody binding, whereas AChe has a diffuse distribution in the middle and outer zones. The inner zone of the molecular layer consistently shows very little antibody binding, although the AChe staining is variable.

The distribution of cholinergic terminals can be altered by two types of lesions. Septal lesions or fimbrial cuts, which destroy cholinergic input (35), remove almost all AChe histochemical staining. In contrast, entorhinal cortical lesions, which cause sprouting of cholinergic terminals (31), induce intensification of AChe staining in the outer and middle zones of the molecular layer. Surprisingly, neither fimbrial cuts nor entorhinal cortex lesions produce more than subtle changes in antibody staining patterns (Fig. 6*c* and *d*). 5 mo after a fimbrial lesion, the only detectable change is a minor decrease in intensity of the supragranule cell band. After lesions of medial or lateral entorhinal cortex, the only detectable change is a small increase in the width of the band in the middle zone of the molecular layer. In neither case are there any detectable changes in regions of the hippocampus outside the dentate.

If AChe staining is taken as a measure of cholinergic terminal distribution, conserved antigens are not restricted to cholinergic terminals because there is little change in antibody patterns after drastic manipulations of the cholinergic system. On the other hand, we cannot conclude that cholinergic terminals in the hippocampus do not contain conserved SVAD. Septal nerve terminals comprise as little as 1% of the total nerve terminals of the dentate. Disappearance of cholinergic terminals might be masked by the continued presence of noncholinergic terminals that contain conserved SVAD.

The entorhinal input to the hippocampus is a major one and is thought to use glutamate as neurotransmitter (52). Removal of this major input also has little effect on antibody binding. It is reasonable to conclude that this class of terminals contains little, if any, conserved antigens. The argument that a class of nerve terminals exists that lacks conserved antigens is additionally bolstered by the absence of antibody binding to the inner zone of the molecular layer, a region rich in synapses.

Cerebellum

The majority of synapses in the cerebellar cortex is thought to be GABAergic and glutaminergic, although cholinergic and other types are also present. Glutamic acid decarboxylase immunohistochemistry has shown that basket, stellate, and Purkinje cells are GABAergic (34), whereas granule cells have been identified as glutaminergic (43). These cerebellar cells synapse with each other in an orderly manner, primarily in the molecular layer of the cerebellum. Little is known about the inputs to the cerebellum, the mossy and climbing fibers. A subset of mossy fibers are thought to be cholinergic because CAT has been localized in the glomeruli of mossy fibers in the granule cell layer by subcellular fractionation (2) and immunocytochemistry (21). In addition, section of the cerebellar peduncles destroys mossy and climbing fibers and drastically reduces the levels of both CAT and ACh in the cerebellar cortex (14, 23).

Conserved SVAD in cerebellar cortex sections are limited to globular structures (~10 μm in diameter) interspersed between granule cells. They are not seen in white matter, the molecular layer, or the cytoplasm of granule or Purkinje cells (Fig. 7*a* and *c*). There is no noticeable difference in the distribution of these structures between the depth and crest of the folia or between folia. Cholinergic glomeruli are found primarily in the archicerebellum, folia IX and X, by AChe histochemistry (23). The density and distribution of AChe-staining glomeruli in the archicerebellum correspond well to the fluorescent globular structures (Fig. 7*b*). Because globular structures with conserved SVAD are seen in regions that do not show AChe staining, they may represent both cholinergic and noncholinergic glomeruli. Alternatively, they may represent some other synaptic element of the granule cell layer. The important finding, however, is the dramatic lack of conserved SVAD in the entire neuropile of the molecular layer.

Other Tissue

We have also screened liver, kidney, and sperm of the rat, using immunofluorescent techniques and found that they do not express detectable levels of conserved SVAD. A summary of the conserved SVAD distribution is given in Table I.

DISCUSSION

To understand the properties of a tissue as complex as brain, it is first necessary to understand the properties of individual cell types. This requires ways of identifying the individual cell types and possibly isolating them in pure form. Classically, morphology and transmitter type have been used as criteria for identifying cell types. Antibodies that recognize neurotransmitters (e.g., 19) or enzymes involved in their biosynthesis (e.g., 3, 9, 10, 21, 40) have been especially useful. A more recent approach takes advantage of other biochemical differences which can be recognized with specific antibodies. For example, anti-galactocerebroside binds selectively to oligodendrocytes (29, 41), whereas anti-phosphorylcholine binds selectively to neurons (20). Antibodies have also been raised to whole cells, tumor cells, primary cell lines, or dissociated cells. From these studies, antisera are now available that distinguish neurons from chromaffin cells (26), one neuronal cell line from another (49), and white matter from grey matter (47), and that bind to Schwann cells (6) or to cerebellum in preference to cortex (48, 57). This approach requires, as immunogen, cell populations that are free from contamination by other cell types and that

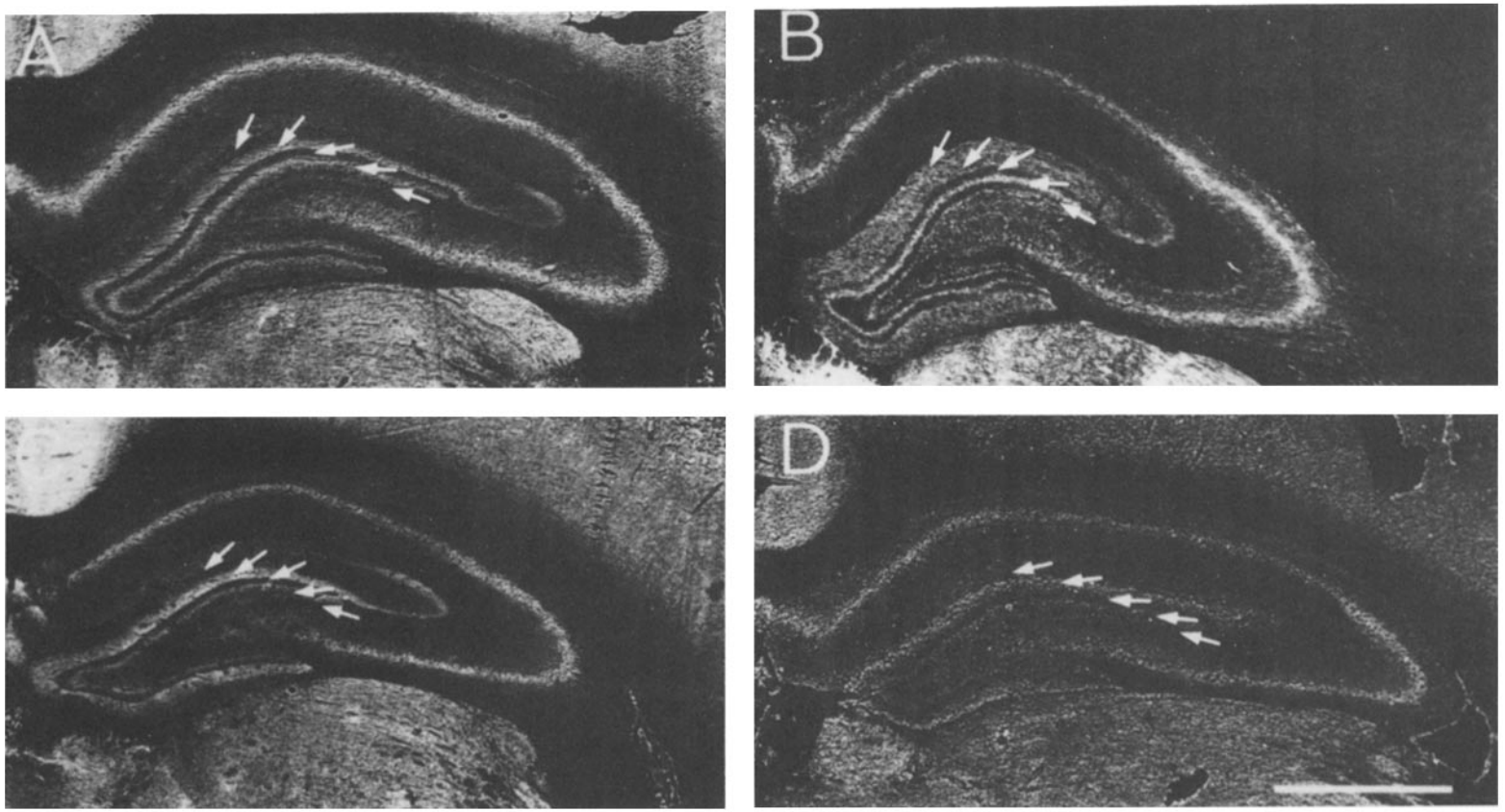

FIGURE 6 Photoreversals of 10-μm frontal sections of unfixed whole rat brain showing hippocampus. Arrows indicate laminae of the dentate gyrus, which are from top to bottom: outer zone of the molecular layer, middle zone of the molecular layer, inner zone of the molecular layer, supragranule band, and granule cell layer. The hilus, the innermost part of the dentate, is unmarked. (*A*) Normal hippocampus incubated with RASVA, using Sternberger's PAP procedure (see Materials and Methods). Three prominent bands of staining are seen; the supragranule cell band, the middle zone of the molecular layer of the dentate, and surrounding pyramidal cells of the hippocampus proper. (*B*) AChe stain of normal hippocampus. Staining in the dentate is seen in the hilus, the supragranule band, and the middle and outer zones of the molecular layer. In addition, staining is seen surrounding the pyramidal cells of the hippocampus proper. (*C*) Staining as in *A* of hippocampus from animal 5 mo after complete bilateral fimbria lesion. The change here from the normal seen in *A* is an increase in the intensity of the inner zone of the molecular layer relative to the supragranule cell band. (*D*) Staining as in *A* of hippocampus 10 d after lesion of the medial ipsilateral entorhinal cortex. Whereas this lesion causes shrinkage of the outer part of the molecular layer, the band staining here in the molecular layer is actually broader than that seen in *A* and extends into the outer zone. Lesion of the lateral ipsilateral entorhinal cortex gives similar results (not shown). Bar, 1 mm.

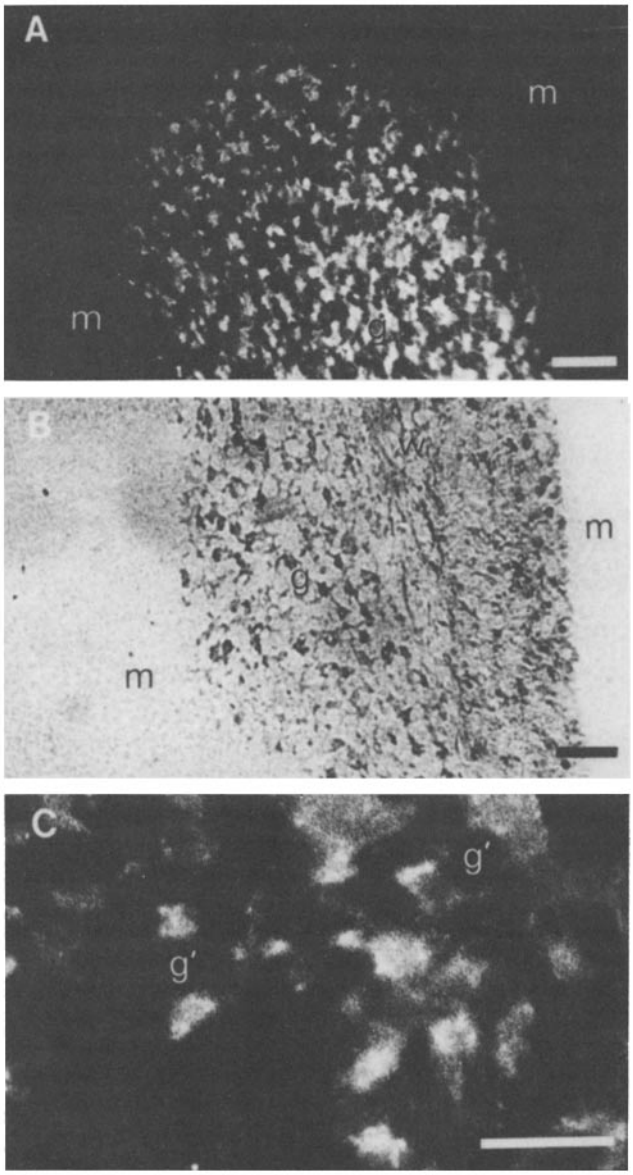

FIGURE 7 Conserved antigens in 6-μm sagittal sections of unfixed cerebellar cortex. (*A*) Low magnification of section incubated with RASVA and fluorescein-second antibody showing molecular (*m*) and granule cell (*g*) layers. The only fluorescence seen here is in the spaces between granules cells. Bar, 50 μm. (*B*) AChe stain (Koelle method) of section as in *A*, showing distribution of reaction product similar to that of the fluorescence in *A*. It is seen primarily in glomeruli between the cells of the granule cell layer (*g*) and to a lesser extent in the white matter (*w*). Bar, 50 μm. (*C*) A higher magnification of the section seen in *A*, showing these fluorescent structures between granule cells (*g'*). Bar, 20 μm.

retain differentiated characteristics. The resulting antiserum must then be extensively adsorbed with other cell types to remove antibodies against nonspecific determinants. Because of these difficulties, antibodies specific for neuronal types are not readily available.

An alternative way to increase specificity is to reduce the complexity of the immunogen. Obviously, whole cells contain a great many potential antigens. This number can be significantly reduced if a subcellular fraction is used to generate antibodies instead of the entire cell. Brain synaptosomes (17, 36), synaptic vesicles (5, 54, 58), and synaptic plasma membranes (4, 32, 44) have all been used as immunogens in a search for synapse and cell-type specific markers. In one case, binding to regions rich in nerve terminals has been observed (30), but white matter was also labeled, perhaps because of contamination of the immunizing fraction by myelin. Cell-type specificity has not yet been observed by using brain synaptosomes to generate antibodies, a finding that is to be expected because synaptosomes are derived from many cell types. More promising is the application of the monoclonal antibody procedure (18) to the isolation of nerve cell specific antibodies after immunizing with complex antigens (38).

Our approach utilizes as immunogen a simple subcellular fraction from a single cell type; cholinergic synaptic vesicles free of major contamination isolated from the electric organ of *Narcine brasiliensis* (8). Because these have a very simple composition (e.g., they have only eight polypeptides present in one or more copies per vesicle [55]) they presumably have a limited number of antigenic determinants. At least some of these determinants appear to be unique to synaptic vesicles and are not found in other membranes in electric organ homogenates (7). One or more of these determinants are also found in the mammalian nervous system, despite the evolutionary distance between elasmobranchs and mammals. The biochemical nature of the cross-reacting antigens in mammals is not yet known. Nor can we be certain that the same cross-reacting antigens are represented in different regions of the mammalian nervous system. Nonetheless, the presence of conserved SVAD in several distinct neural regions and its absence in others allow us to conclude that one or more subclasses of neurons are labeled.

Although electron microscopy will be required to verify that the conserved antigens are only on vesicles, this conclusion is suggested by several lines of evidence. In the motor neuron, where synaptic vesicles are concentrated in nerve terminals, the nerve terminal labels much more intensely than either the axon (46) or the cell body (Fig. 1*a*). Similarly, in the cerebellum, vesicle-rich glomeruli of the granule cell layer contain the conserved determinants, whereas adjacent vesicle-poor cell bodies do not. Weak specific labeling of cell bodies is found in the adrenal medulla (Fig. 4*b*), the SCG (Fig. 3*a*), and the intermediate lobe of the pituitary (Fig. 5*a*). These may also represent binding to secretory granules because both intermediate lobe and chromaffin cells are known to contain secretory granules, and synaptic vesicles have been reported in the soma of SCG cells (e.g., 42). Finally, preliminary experiments in this laboratory have shown that the conserved SVAD are not present on the outside of the nerve terminals of the resting frog cutaneous pectoris muscle. After stimulation, however, antibodies to the conserved SVAD bind to the nerve terminal, which is consistent with exocytotic transfer of vesicle antigens to the external surface.[1]

The antibodies raised to cholinergic synaptic vesicles of *Narcine* do not bind exclusively to cholinergic nerve terminals of the rat. Conserved SVAD have been found in all cholinergic endings examined, both in the peripheral and central nervous systems. However, they are also found in the posterior pituitary and the adrenal chromaffin cells, which are not cholinergic. They are also found in the iris and the hippocampus, even after all known cholinergic inputs are cut.

The conserved SVAD are not universal elements of the

[1] von Wedel, R. J., S. S. Carlson, and R. B. Kelly. Manuscript in preparation.

TABLE I

Narcine Synaptic Vesicle Antigen Distribution in the Rat

	Intense	Moderate	No staining
Diaphragm	Endplates	Axons	Muscle
Iris	Sympathetic and parasympathetic fibers		Muscle
Spinal cord	Neuropil		White matter Cell bodies
SCG	Preganglionic terminals	Cell bodies	
Adrenal	Preganglionic terminals	Chromaffin cells	
Pituitary	Neural lobe	Intermediate lobe	Anterior lobe
Cerebellum	Globular structures (perhaps glomeruli)		White matter Cell bodies Molecular layer
Hippocampus	Dentate outer molecular layer infrapyramidal cell band supragranule cell band		Dentate inner molecular layer Cell bodies
Other			Sperm, liver, kidney

molecular machinery involved in exocytosis because they were not found in all secretory cells that operate by exocytosis. For example, the anterior pituitary does not contain detectable antigen (Fig. 5*a*). In addition, both the molecular layer of the cerebellum (Fig. 7*a*) and the inner zone of the molecular layer of the dentate gyrus (Fig. 6*a*) have nerve terminals that presumably release transmitter by exocytosis yet do not bind antibody.

The conserved antigens might correspond to molecules present in developmentally related cells. This does not seem to be the case. Cells that express the antigens have very different developmental histories. They may be derivatives of neural tube or neural crest. Closely related cells do not necessarily express the same complement of conserved SVAD. Anterior and intermediate lobe cells of the pituitary are developmentally closely related, but one expresses the antigens and the other does not. What developmental step could be shared by the pituitary intermediate lobe cell and the motoneuron, but not by the pituitary anterior lobe cell or the cerebellar Purkinje cell? We feel that the cross-reactivity is more likely to be associated with a functional, rather than developmental similarity.

The functions presently attributed to the *Narcine* synaptic vesicle include recognition and fusion with the presynaptic plasma membrane, packaging of vesicle contents, and perhaps ATP-dependent calcium uptake. In addition, each of these functions may be regulated. At present we have no knowledge of which, if any, of the mechanisms for these functions are conserved during evolution. The following possibility intrigues us. The regions of the hippocampus and the cerebellum, which do not bind antiserum, are low in cholinergic or adrenergic synapses but rich in GABAergic and glutaminergic synapses (3, 23, 52). Perhaps the nerve terminals that bind antibody contain positively charged transmitters, whereas those that do not bind antibody have neutral or negatively charged transmitters. Packaging of positively charged transmitters might involve some mechanism, an ATP-driven proton pump, for example, which is shared by all vesicles that utilize positively charged transmitters. Hypotheses of this type are readily testable by biochemical techniques.

When immunohistochemistry techniques are used, it is possible that apparently specific staining patterns are actually caused by differential penetration of antibodies. In this particular case, lack of penetration is not a problem. Freeze-thawing and drying of unfixed tissue efficiently disrupts membranes. Acid-ethanol treatment, a standard permeabilization procedure, does not significantly change staining patterns of the iris or the neuromuscular junction. Under the standard conditions of this paper, two monospecific antibodies directed against different intracellular synaptic components intensely stain synaptic layers of both the hippocampus and the cerebellum, which were unlabeled by the antibodies directed against *Narcine* synaptic vesicles. Therefore, we feel that differences in staining are caused by differences in antigen distribution, not differences in antibody accessibility.

There are some obvious limitations to our work which should be noted. As mentioned before, the antiserum used here was not raised to a single molecular species but to an entire organelle, albeit a simple one. Although polyspecific antibodies are valuable in showing that cross-reactivity occurs, monospecific antisera derived either by the monoclonal antibody procedure or from isolated vesicle components would be immensely useful in identifying which synaptic vesicle antigens cross-react. Until such monospecific antisera are available, we cannot say whether the same antigenic determinants are expressed in all of the different antibody binding regions studied. Secondly, although the immunofluorescent techniques of the present study are sensitive, they are not quantitative. The antigen concentration can be shown to be much lower in the motoneuron cell body than in the terminal, but the ratio cannot be determined. The lack of quantitation is especially a problem when deciding whether, for example, the weak binding to SCG cells is greater than the binding observed with control sera, or whether changes in intensity seen upon denervation are significant. What is needed in this case is a sensitive quantitative biochemical assay of antigen content in these tissues. Unfor-

tunately, the radioimmunoassay that can detect the tiny amounts of vesicle antigen present in homogenates of electric organ (7) requires additional refining before it will detect the conserved SVAD in homogenates of mammalian brain regions.

At present, examples of cross-reactivity between synaptic elements of distantly related species are too few to permit generalization. Immunological cross-reactivity has been found between mammalian and elasmobranch ACh receptors (28) but not between their AChe (16). When conservation of antigenic determinants does occur, as it does here, it has the potential of being very useful. It should be possible to identify the molecules in the mammalian nervous system that have conserved SVAD, even if they are present in very tiny amounts. The biochemistry of these molecules, and even their function, might then be worked out using the abundant quantities of these molecules that are present in the electric organ.

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