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Amphiphysin, a novel protein associated with synaptic vesicles

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To obtain access to novel proteins of the neuronal synapse, we have raised antisera against proteins of synaptic plasma membranes and used them for immunoscreening brain cDNA expression libraries. One of the newly isolated cDNAs encodes an acidic protein of 75 kDa with a distinct architecture of structural domains and multiple potential phosphorylation sites. Light and electron microscopy employing monospecific antisera raised against the expression product indicate a synapse-specific, presynaptic localization of this protein in many synapses of the chicken and rat nervous system. Its overall distribution in brain is very similar to that of synaptophysin, a ubiquitous protein of synaptic vesicles. In addition to brain, the protein or its mRNA is expressed in adrenal gland and anterior and posterior pituitary, but was not detected in a variety of other tissues. In controlled pore glass chromatography the native protein copurifies with synaptic vesicles and largely remains associated with them under various washing conditions. However, its amino acid sequence is very hydrophilic and it segregates into the aqueous phase in detergent phase partition. An earlier step of synaptic vesicle purification, sucrose cushion centrifugation, separates a vesicle-bound fraction of this protein from an unbound fraction. This seems to be a new, perhaps peripheral, protein of synaptic vesicles for which we propose the name, amphiphysin.

Key words: brain/immunoscreening/neuron/neurotransmitter vesicle/synapse

Introduction

Chemical synapses are the main channel of communication between neurons and hence of fundamental importance for nervous system function. In presynaptic terminals, classical neurotransmitters are stored in small electron-translucent vesicles. Upon stimulation they are released into the synaptic cleft by exocytosis, followed by retrieval and reloading of the empty vesicles. However, the molecular machinery responsible for events like the immobilization of the vesicles in the presynaptic cytoskeletal meshwork, their translocation to the presynaptic plasma membrane, their docking at the active zones, their fusion with the plasma membrane and their eventual retrieval from it are only beginning to be understood. It can be expected that some mechanisms are shared with other forms of exocytosis and endocytosis while Jahn, 1991; Trimble *et al.*, 1991). Proteins involved in these events are likely to play a role in mechanisms of neuronal plasticity, they are potential targets for toxins and drugs affecting nervous system function, and they may be involved in inherited or acquired disorders of the human nervous system.

A major obstacle to the identification of such proteins seems to be the fact that, unlike enzymes or ion channels, they play their role only as part of a very complex and dynamic supramolecular system. Therefore, functional assays for individual proteins that can serve as a criterion for purification or expression cloning are difficult and only beginning to be developed (Thomas and Betz, 1990). Another approach is to try to define the molecular architecture of the synapse and its compartments, i.e. to identify the proteins that are their essential and possibly specific components, and subsequently investigate their function. Synapsin I, a peripheral protein of synaptic vesicles, was the first protein involved in the life cycle of synaptic vesicles that was molecularly characterized. It was discovered as the main protein substrate for cAMP-dependent phosphorylation in membrane preparations from brain (Ueda *et al.*, 1973), and in the course of two decades evidence has accumulated that it modulates the availability of synaptic vesicles for exocytosis (reviewed by Bähler *et al.*, 1990; and Südhof and Jahn, 1991). The synapsins may also be involved in the morphogenesis of synapses (Han *et al.*, 1991). During the last few years, several of the main protein components of neurotransmitter vesicle membranes have been characterized (see Südhof and Jahn, 1991), and investigation into their functions is proceeding (Thomas *et al.*, 1988; Johnston *et al.*, 1989; Leube *et al.*, 1989; Perin *et al.*, 1990; Thomas and Betz, 1990; Fischer von Mollard *et al.*, 1991; Petrenko *et al.*, 1991).

To obtain access to new synaptic proteins, we raised antisera against proteins of synaptic plasma membranes and used them to immunoscreen brain cDNA expression libraries. One of the cDNAs isolated in these experiments encodes a protein which (i) is expressed in brain, adrenal gland and anterior and posterior pituitary but not in a variety of other tissues, (ii) is found in many presynaptic terminals of chicken and rat brain and (iii) is associated (perhaps peripherally) with synaptic vesicles.

Results

Preparation of antibodies and immunoscreening

Synaptic plasma membranes were purified from chicken forebrains and their proteins were used to immunize rabbits. As a first fractionation step, membrane proteins were separated into a hydrophilic and a hydrophobic fraction by Triton X-114 phase partition. Both fractions were used to raise antisera, which were characterized for specificity by immunoblot analysis and immunofluorescence microscopy. Figure 1 demonstrates that on Western blots of chicken brain

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synaptic plasma membrane proteins, the antisera against the hydrophobic and the hydrophilic fractions (α-D and α-A respectively) label spectra of antigens that are largely complementary to each other and brain specific. The major band of ~50 kDa found in both fractions and labelled by both sera was subsequently identified as GAP-43 (see Materials and methods), which is known to distribute into both phases. In the lanes carrying liver membrane proteins, only very weak bands are labelled that do not correspond to the main bands formed with brain antigens. Immunofluorescence microscopy of cerebellar sections produced a picture compatible with a localization of the main antigens of both sera in synapses and some small neuritic processes (not shown). Both sera were used to immunoscreen chicken forebrain cDNA libraries in the expression vector, λgt11. Screening of 270 000 recombinant plaques yielded 69 immunoreactive clones.

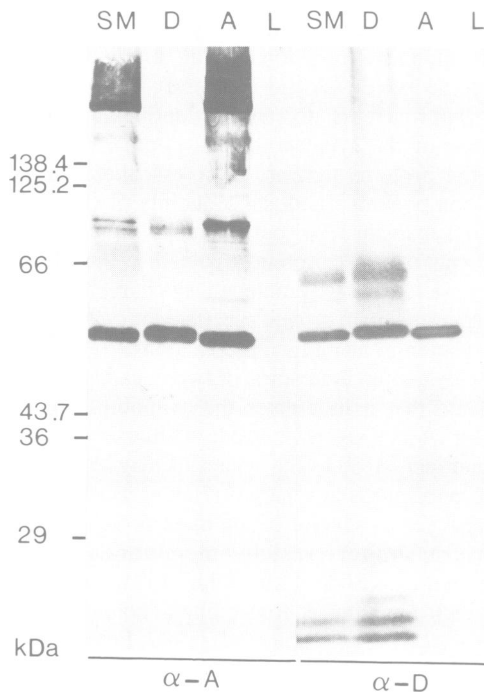

Fig. 1. Immunoblot characterization of antisera against synaptic plasma membrane protein fractions. 15 μg of protein per lane were resolved on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were probed with polyclonal antisera (dilution 1:400) against synaptic plasma membrane proteins of the aqueous phase (α-A) and the detergent phase (α-D) of Triton X-114 phase partitioning. (SM), synaptic plasma membranes from chicken forebrain. (D), detergent phase and (A), aqueous phase of synaptic plasma membranes after Triton X-114 phase partitioning. (L), chicken liver membrane proteins (fraction P_2''', prepared according to Babitch *et al.*, 1976). Positions of molecular size standards are indicated (left).

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CCCTCCCTCCCTCCATCCATCCCCGCTCTCCGCCGCTCGCCGCCGCCGGGCTCTGCCTCGCCCGCCTTC     69
CCCCACCCCGCTCCCCCTGCCGCCAGCCATGGCCGACATGAAGACGGGCATCTTCGCCAAAAACGTCCA    138
                            M  A  D  M  K  T  G  I  F  A  K  N  V  Q     14

GAAACGCCTCAACCGCGCCCAGGAGAAGGTACTTCAAAAACTAGGAAAAGCAGATGAGACAAAGGATGA    207
 K  R  L  N  R  A  Q  E  K  V  L  Q  K  L  G  K  A  D  E  T  K  D  E     37

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 Q  F  E  E  Y  V  Q  N  F  K  R  Q  E  A  E  G  S  R  L  Q  R  E  L     60

GAGAGCATATCTAGCAGCCATCAAAGGGATGCAAGATGCCTCAAAGAAGCTTACAGAGTCTCTCCATGA    345
 R  A  Y  L  A  A  I  K  G  M  Q  D  A  S  K  K  L  T  E  S  L  H  E     83

AGTGTATGAGCCAGATTGGTATGGACGAGAAGATGTGAAAATGATTGGTGAGAAATGTGATGAACTCTG    414
 V  Y  E  P  D  W  Y  G  R  E  D  V  K  M  I  G  E  K  C  D  E  L  W    106

GGAAGACTTCCATCAAAAACTTGTGGATGGGTCGTTGTTGACTTTGGATACATATCTAGGACAATTTCC    483
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 D  I  K  T  R  I  A  K  R  S  R  K  L  V  D  Y  D  S  A  R  H  H  L    152

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TTTACTATGTCACAAACTGTATGAAGTGATGACGAAGCTGGGAGATCAGCATGCAGACAAGGCCTTCAC    828
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CTCTCCCCTTCCTAGCCCTACTGCTAGTCCCAATCACATGCTGGCACCAGCATCTCCAGCACCAGCCCG    966
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 P  K  S  P  T  Q  L  R  K  G  P  P  V  P  P  L  P  K  L  T  P  T  K    313

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TCCATCACAGAATGAAATTCCTGAAACTAAGAAAGTGGAATCTTTGCTAGATCTGGACTTCGACCCTTT   1173
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CAAGCCAGAAGTTGTATCAACAGGAGTAACTCATTCACCCATGTCTCAGACATTGCCATGGGATCTGTG   1242
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TGTACTAGTAATTCCGTCTGAGACCACAGCTGATCAGGAGGCTGGCTGGTTGACAGGCATTAAAGAATC   2070
 V  L  V  I  P  S  E  T  T  A  D  Q  E  A  G  W  L  T  G  I  K  E  S    658

TGAGTGGCTTCAGTACAGAGATGCAAATAGTTATAAAGGACTTTTCCCAGAGAACTTCACACGCCATCT   2139
 E  W  L  Q  Y  R  D  A  N  S  Y  K  G  L  F  P  E  N  F  T  R  H  L    681

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 E  •                                                                    682

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CTGTGTCATTAAAAATAAACCTTTGATCCAAAGAAAAAAAAAAAAAA                        3220
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Fig. 2. Nucleotide and deduced amino acid sequence of amphiphysin cDNA. The four clones whose 5′ ends were sequenced begin at nt 1 (two clones), 14 and 46. Triangles mark potential phosphorylation sites of proline-directed protein kinases (serine or threonine residues followed by a proline). Squares mark potential protein kinase C sites (serines or threonines with a lysine or arginine residue in position +2). Circles mark potential casein kinase II sites (serines or threonines with an acidic residue in position +3 plus at least one additional acidic residue between positions −1 and +5). The EMBL Data Library accession number for this sequence is X60422.

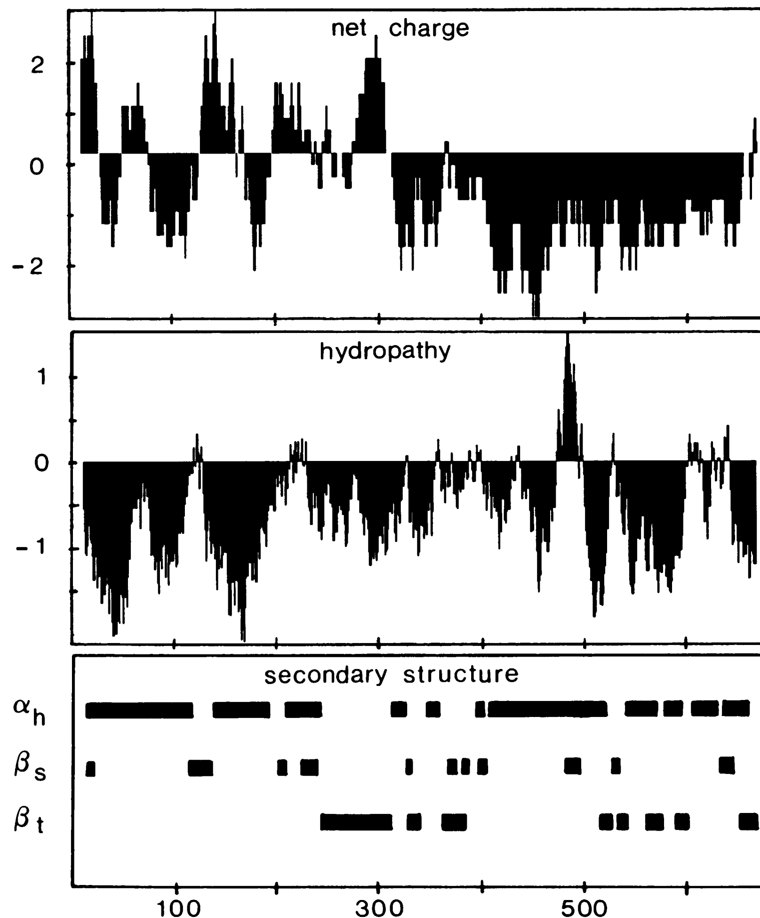

Fig. 3. Distribution of charged amino acids, hydropathy and predicted secondary structure (windows: 20 residues each) in amphiphysin. Horizontal bars indicate regions were α-helix (α_h), β-sheet (β_s) and β-turn (β_t) potential is predicted to be >1. The number of amino acids is given on the abscissa.

Primary structure of amphiphysin

Figure 2 gives the nucleotide sequence and deduced amino acid sequence of one of the cDNAs isolated in this screen. An open reading frame of 2046 nucleotides (nt) encoding a polypeptide of 75 204 Da begins with the first methionine codon and is followed by 1077 nt of 3'-untranslated sequence terminating in a tract of 13 adenyl residues. There is no other open reading frame of any significant length in this cDNA (not shown). The sequence surrounding the first ATG codon conforms excellently with the extended translation initiation consensus, GCCGCC(A/G)CCATGG (Kozak, 1987). Although the putative 5'-untranslated sequence does not contain stop codons that would unequivocally define the 5' end of the reading frame, the following evidence suggests that this is a practically complete copy covering the whole coding sequence. First, the total length of 3220 nt agrees very well with the mRNA size of 3400 nt estimated from Northern blots if a poly(A) tail is taken into account. Moreover, a hybridization rescreen of the libraries with a 5'-terminal fragment of the first clone isolated by immunoscreening (amphy-11.3) resulted in the isolation of 15 additional clones. Using an oligonucleotide primer derived from a 5'-terminal sequence, eight of them gave rise to polymerase chain reaction (PCR) products whose size indicated 5' ends between the putative translation start and ~100 nt further upstream. The three longest were 5'-terminally sequenced and found to have 5' ends only a few nucleotides apart (see legend to Figure 2). Finally, the putative 5'-untranslated sequence is very G+C-rich, a feature found in the corresponding sequences of many mRNAs.

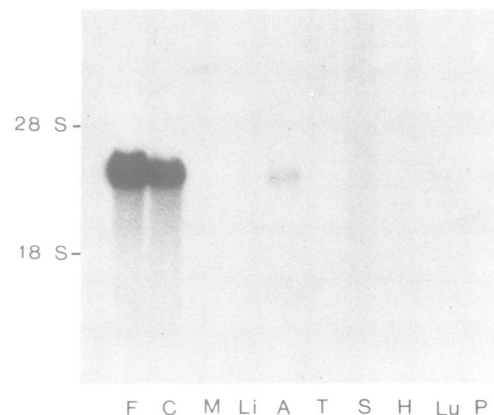

Fig. 4. Amphiphysin mRNA expression in various chicken tissues. Ten micrograms of poly(A)$^+$ RNA per lane were denatured by glyoxylation, resolved on a 1% agarose gel, transferred to a nylon membrane and hybridized with a probe comprising nt 46–3189 of the amphiphysin cDNA sequence. Exposure was for 7 days without an intensifying screen. RNAs were derived from the following tissues: F, forebrain; C, cerebellum; M, skeletal muscle; Li, liver; A, adrenal gland; T, testis; S, spleen; H, heart; Lu, lung; P, pancreas. Exposure for 7 days in the presence of an intensifying screen did not reveal additional bands (not shown).

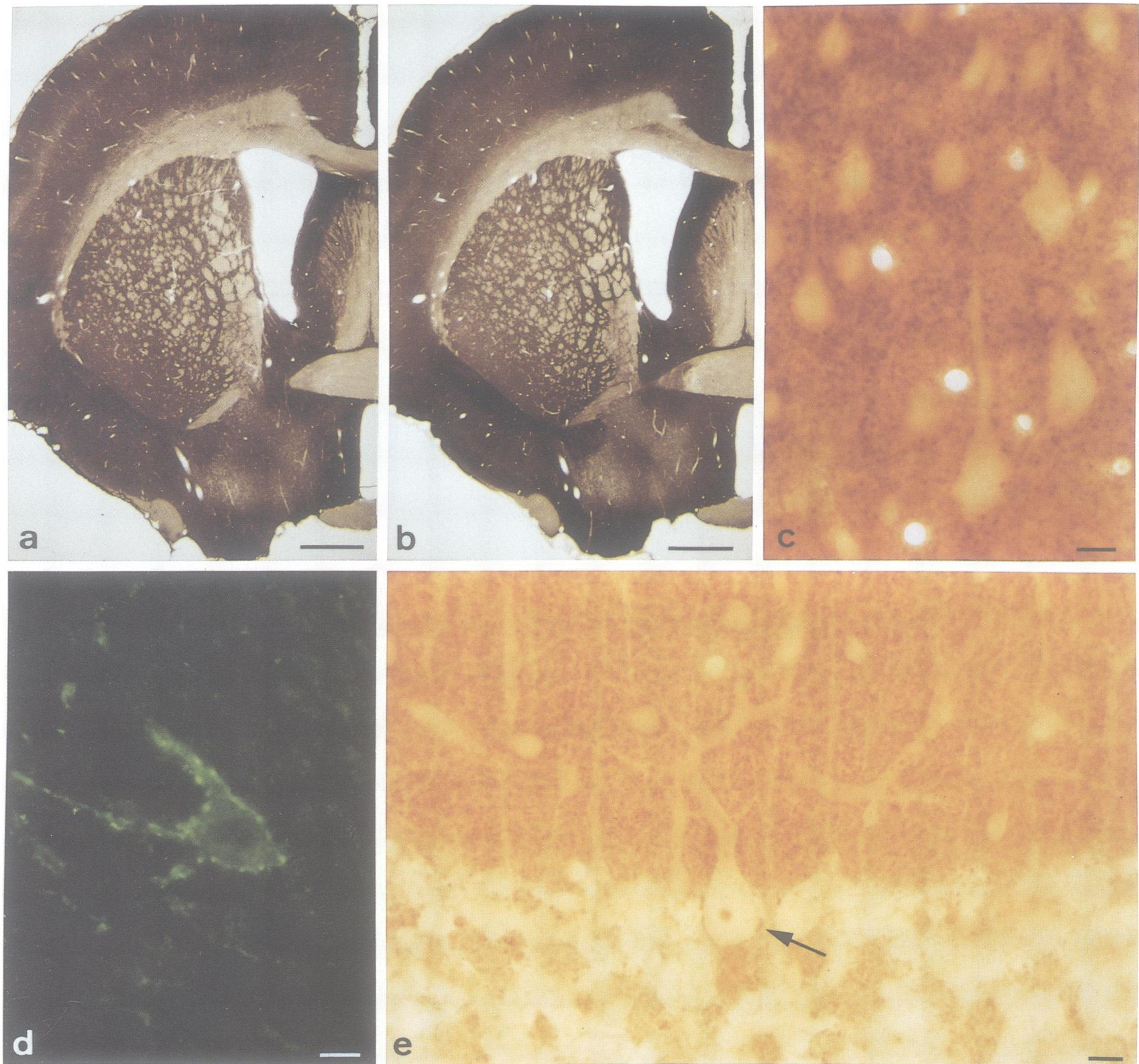

Fig. 5. Immunocytochemical localization of amphiphysin at the light microscopic level. The figure shows 50 μm vibratome sections from rat forebrain (coronal) (**a**–**c**) and rat cerebellum (sagittal) (**e**), and a parasagittal 7 μm frozen section from chicken cerebellum (**d**). Sections (b)–(e) were stained with anti-amphiphysin, and section (a) with anti-synaptophysin for comparison. Immunoreactivities were visualized by immunoenzymatic (a–c, e) or immunofluorescence (d) cytochemistry. Note the close similarity of the overall distribution of synaptophysin-like (a) and amphiphysin-like (b) immunoreactivities. At higher magnification, amphiphysin-like immunoreactivity gives a punctate pattern suggestive of a synaptic localization (c–e). The arrow in section (e) indicates the immunopositive dot examined by electron microscopy in Figure 6. Bars correspond to 1 mm (a, b) or 10 μm (c–e).

The deduced polypeptide has no significant similarity to any sequences found in the EMBL (release 29) and SWISS-PROT (release 20) databases, in particular not with the synapsins and the EF hand calcium binding proteins. Overall, the molecule is very hydrophilic, with a single hydrophobic stretch spanning amino acids 478–499 (Figure 3). The sequence can be divided into three major structural domains. The N-terminal 36% of the sequence forms a domain of balanced charge with long stretches of high α helix potential, which is rich in charged amino acids (aspartate, glutamate, lysine and arginine make up 34% of all amino acids) but almost devoid of proline (1%). It is followed by a positively charged stretch (10% of the total sequence) with high β-turn potential highly enriched in proline (32%) and other small amino acids (glycine, alanine, serine and threonine make up another 32%). The C-terminal 54% of the sequence contains few basic amino acids, resulting in a pronounced negative charge of this domain (Figure 3). Consequently, the whole protein is rather acidic with a predicted pI of 4.39. Approximate domain borders are marked by arrows in Figure 2. Because the whole polypeptide is rich in hydroxyl amino acids (14%), a substantial number of consensus substrate sites for various protein kinases can be found (Figure 2). According to the marked differences in amino acid compositon between the three major domains, potential protein kinase C sites (indicated by squares) predominate in the N-terminal domain, potential sites for proline-directed kinases (triangles) cluster densely in the central domain and potential casein kinase II sites (circles) predominate in the C-terminal domain. Good consensus sequences for protein

kinase A phosphorylation sites are not apparent. The best potential candidates are Thr78 and Ser139. The central domain is reminiscent of the multiphosphorylation regions rich in hydroxyl amino acids, proline and other small amino acids that have been found in a number of proteins (reviewed by Roach, 1991). Sequences rich in proline and other small amino acids are also found in the synapsins, synaptophysins and VAMPs/synaptobrevins. However, as this structural feature is not restricted to synaptic vesicle proteins, it is unknown whether it has any specific significance.

Amphiphysin mRNA is expressed in brain and adrenal gland

Northern blot hybridization of the cDNA to poly(A)$^+$ RNAs from various chicken tissues demonstrates an mRNA species of ~3400 nt that is abundant in forebrain and cerebellum and detectable at lower levels in adrenal gland, but undetectable even after prolonged exposure in spleen, skeletal muscle, lung, liver, testis, pancreas and heart (Figure 4).

Amphiphysin is localized to synaptic terminals

To investigate the cellular and subcellular localization of the protein encoded by this DNA sequence, a monospecific antiserum was raised against the fusion protein of the original clone, amphy-11.3. The coding sequence of its cDNA insert is in-frame with the λgt11 β-galactosidase coding sequence, giving rise to a fusion protein of apparent molecular mass 230 kDa (the contribution of the β-galactosidase moiety being 114 kDa) that is readily separated from the other bacterial proteins in SDS–PAGE. Antiserum was raised against PAGE-purified fusion protein and affinity-purified by adsorption to nitrocellulose-bound fusion protein. It is found to react with an endogenous antigen of chicken and rat on Western blots and in immunocytochemistry.

In a survey of a variety of regions of the nervous system including sections from olfactory bulb, forebrain, hippocampus, mesencephalon, cerebellum, and spinal cord (to be published elsewhere), amphiphysin-like immunoreactivity exhibits a widespread distribution throughout the central nervous system. Comparing coronal sections from the forebrain at the level of the anterior commissure stained for amphiphysin (Figure 5b) with those for the ubiquitous synaptic vesicle protein, synaptophysin (Figure 5a), immunoreactivity for both antigens is found throughout the cortex, the striatum, the septal nuclei and the basal forebrain with a closely similar distribution. At higher magnification (Figure 5c), amphiphysin-like immunoreactivity in the cortex shows a punctate pattern, leaving the cytoplasm of pyramidal and other cells without staining. The somata and dendrites of the pyramidal cells are surrounded by amphiphysin-positive dots, presumably representing synaptic terminals. Similar immunoreactive puncta outline the contours of neurons in the cerebellar nuclei (Figure 5d). In the cerebellar cortex (Figure 5e) the molecular layer shows a densely punctate pattern similar to that of the cerebral cortex. Purkinje cells and their main dendrites are negative but, as in the case of the pyramidal cells, outlined by amphiphysin-positive dots, which are occasionally of considerable size (Figure 5e, arrow). In the granule layer, stained dots form a patchy pattern, suggestive of small terminals synapsing onto the glomeruli of mossy fibres. The medulla is almost devoid of antigen (not shown). In conclusion, the immunocytochemical distribution of amphiphysin-like immunoreactivity at the light microscopic level strongly suggests a synaptic localization of this new protein.

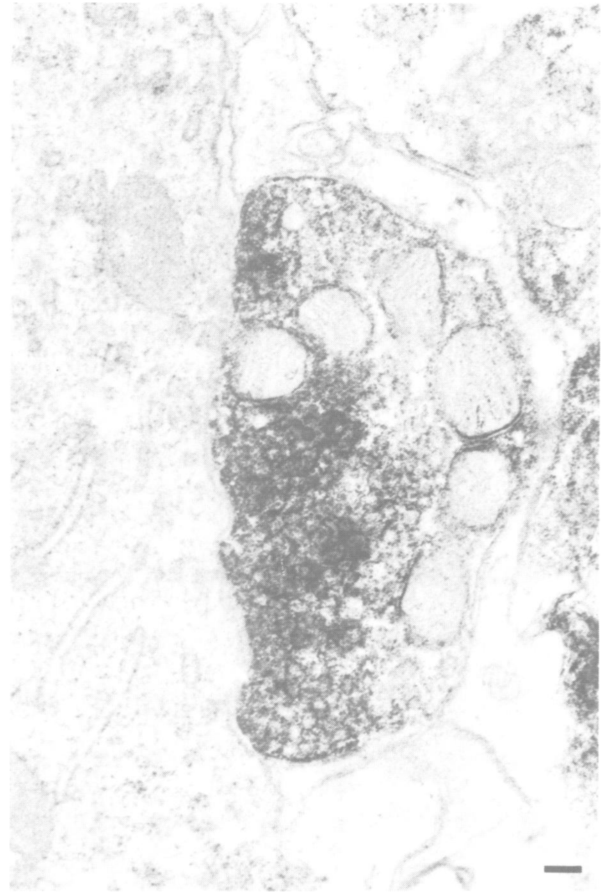

Fig. 6. Electron microscopy of an amphiphysin-positive synaptic terminal. The amphiphysin-positive dot marked by an arrow in Figure 5e is shown at high magnification in pre-embedding staining electron microscopy. It represents a large presynaptic terminal densely filled with synaptic vesicles. Amphiphysin-like immunoreactivity concentrates around these vesicles, while the postsynaptic cytoplasm of the Purkinje cell and the presumed glial process that envelopes the terminus are completely devoid of peroxidase reaction product. The bar represents 0.1 μm.

The immunopositive dot marked by the arrow in Figure 5e was further analysed at the electron microscopic level (see Materials and methods). It turned out to be a large presynaptic terminal (Figure 6), presumably of a basket axon. Besides several mitochondria, the synaptic cytoplasm is densely filled with synaptic vesicles. Amphiphysin-like immunoreactivity is predominantly localized around these vesicles, whereas the postsynaptic cytoplasm of the Purkinje cell and the structure enveloping the terminal (presumably a glial process) are completely devoid of peroxidase reaction product. Thus, electron microscopy confirms the suspected synaptic localization of amphiphysin and confines it to the presynaptic bouton.

Amphiphysin binds to synaptic vesicles and is expressed in neuronal cell lines and anterior and posterior pituitary

Immunoelectron microscopy suggests that the antigen is localized predominantly in the cytoplasm of the synaptic terminals or associated with the synaptic vesicles, rather than

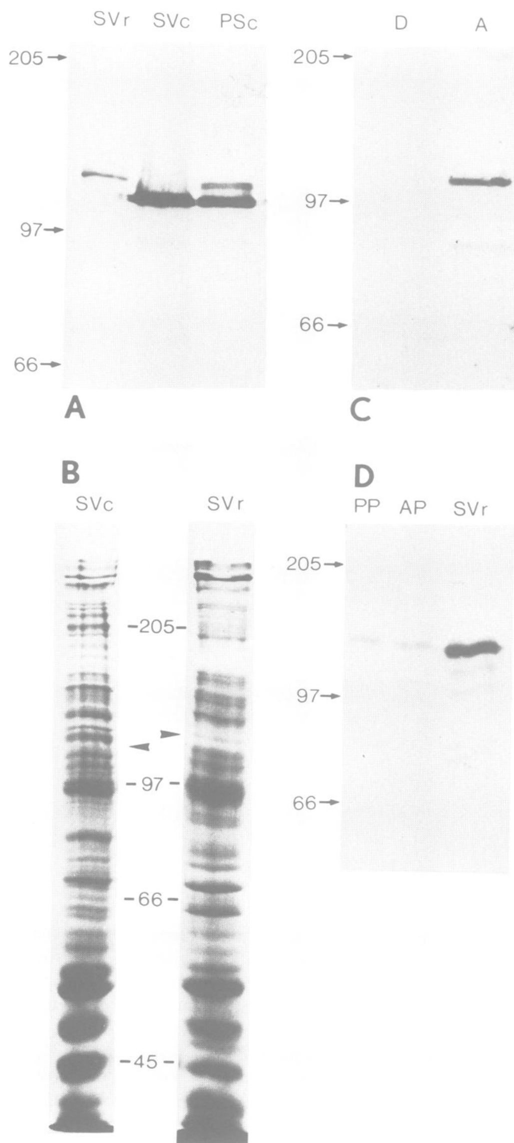

Fig. 7. Western blot analysis of amphiphysin. (**A**) Apparent molecular size of anti-amphiphysin immunoreactive material from rat synaptic vesicles (SVr), chicken synaptic vesicles (SVc) and chicken synaptosomes (PSc). In each lane, 50 μg protein from fractions $P_{II}P$ (synaptic vesicles) or fraction P_s (synaptosomes) were resolved on a 7.5% polyacrylamide gel. Positions of molecular size markers (in kDa) are given on the left. (**B**) Coomassie blue stain of synaptic vesicle proteins. Purified synaptic vesicles from chicken (SVc, 37.5 μg protein) and rat (SVr, 50 μg) were resolved on a 7% polyacrylamide gel. In parallel lanes, equivalent samples were blotted to nitrocellulose. Before immunostaining, the blot was stained for protein with Ponceau S, and the characteristic protein bands were marked with pencil. The positions of the immunopositive amphiphysin bands relative to the protein bands are indicated by arrowheads. Positions of molecular size markers (in kDa) are also indicated. (**C**) Triton X -114 phase partition of amphiphysin. Chicken synaptic plasma membranes were prepared and subjected to phase partitioning as described in Materials and methods, and aliquots (15 μg protein each) of the detergent (D) and aqueous (A) phases were resolved on a 10% polyacrylamide gel. The same result (distribution of amphiphysin into the aqueous phase) was obtained with chicken synaptic vesicles (not shown). (**D**) Amphiphysin expression in pituitary gland. Rat posterior (PP) and anterior pituitary (AP) were freshly removed, minced and immediately dissolved in PAGE sample buffer. About 40 μg protein was resolved on a 7% polyacrylamide gel and subjected to Western blot analysis. (SVr), 50 μg of rat synaptic vesicle proteins.

with the presynaptic plasma membrane. Immunoblotting analysis of subcellular fractions was carried out to pursue the question of subcellular localization further.

On Western blots, the affinity-purified serum specifically stains an antigen of apparent mol. wt 115 000 in chicken synaptosomes and purified synaptic vesicles, and of apparent mol. wt 125 000 in rat synaptic vesicles (Figure 7A). An additional, weaker band of mol. wt 125 000 is observed in chicken synaptosomes (Figure 7A) and some synaptosomal subfractions (not shown). On more intensely stained blots like Figure 9B, rat amphiphysin displays a second, weaker band that migrates slightly below the main band of chicken (not shown). When, after lysis of chicken synaptosomes, synaptic plasma membranes are prepared by density step centrifugation, Western blot analysis of the fractions demonstrates that part of the antigens is indeed found in the plasma membrane fraction that was used for immunization, but most of it accumulates in low-density fractions that presumably contain cytoplasmic proteins and synaptic vesicles (not shown).

The bands stained with anti-amphiphysin do not comigrate with any of the main protein bands of chicken or rat synaptic vesicles (Figure 7B; in each case, they may correlate with a faint protein band). Therefore, amphiphysin does not seem to be one of the major protein components of purified synaptic vesicles.

Phase partition with Triton X-114 separates hydrophobic proteins into the detergent phase and hydrophilic proteins into the aqueous phase. As predicted from its strongly hydrophilic primary structure, amphiphysin distributes into the aqueous phase (Figure 7C).

Figure 8 demonstrates that during the purification of rat synaptic vesicles by controlled pore glass (CPG) chromatography, amphiphysin comigrates with the vesicle peak, in parallel with the elution of the vesicle marker protein, synaptophysin. The affinity of the bound amphiphysin to the vesicles seems to be high as no trailing of the protein behind the optical density/synaptophysin peaks (Figure 8) or elution with the total column volume (not shown) was observed. Because our anti-amphiphysin antibody cross-reacts with rat, while the anti-synaptophysin antibodies available to us did not stain chicken synaptophysin, this experiment was carried out with rat vesicles. An identical amphiphysin CPG elution profile was obtained with vesicles from chicken (not shown).

To investigate further the binding of amphiphysin to synaptic vesicles, CPG-purified vesicles from rat were incubated under various buffer conditions and spun down in an ultracentrifuge, and aliquots of the pellet and the supernatant were analysed by Western blotting for amphiphysin and synaptophysin (Figure 9A). As expected, the integral vesicle protein, synaptophysin, sediments with the vesicles into the pellet at 150 mM KCl, at 1 M KCl and at pH 3, whereas only a very small fraction remains in the supernatant. The same is observed for amphiphysin, although it appears that the fraction of the antigen remaining in the supernatant is somewhat larger than with synaptophysin. Treatment of the vesicles with detergent, 1% Triton X-100, solubilizes both proteins.

Although amphiphysin was found to bind with considerable affinity to synaptic vesicles at the end of the purification procedure, it was noticed that in contrast to synaptophysin, no substantial enrichment of the protein

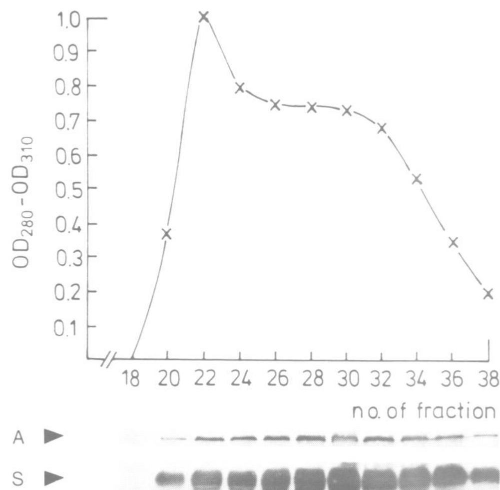

Fig. 8. Comigration of amphiphysin and synaptophysin in CPG chromatography of rat synaptic vesicles. The elution profile of a CPG column is shown at the top. 300 μl of the fractions indicated were precipitated with TCA, resolved on a 10% polyacrylamide gel and blotted to nitrocellulose. The upper part of the blot was developed with anti-amphiphysin (A), and the lower part with anti-synaptophysin (S).

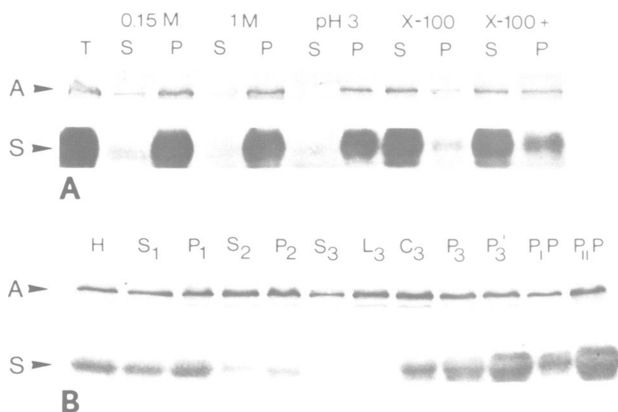

Fig. 9. Amphiphysin binding to synaptic vesicles during washing and purification. (**A**) Washing experiments with synaptic vesicle-bound amphiphysin. Rat synaptic vesicles were incubated in glycine buffer as described in Materials and methods, adjusted to the following additional conditions: 0.15 M KCl (0.15 M); 1 M KCl (1 M); pH 3 adjusted with HCl (pH 3); 1% Triton X-100 (X-100); 1% Triton X-100 + 0.15 M KCl (X-100+). Samples were spun at 260 000 *g* after incubation, and aliquots of the supernatant (S) and the pellet (P) were resolved on a 10% polyacrylamide gel. Lane (T) contains an aliquot of a control sample precipitated immediately from glycine buffer without incubation and centrifugation. The upper part of the blot was developed with anti-amphiphysin (A) and the lower part with anti-synaptophysin (S). (**B**) Distribution of amphiphysin and synaptophysin during purification of synaptic vesicles. 40 μg protein from each fraction was resolved on a 10% polyacrylamide gel. The upper part of the blot was developed with anti-amphiphysin (A) and the lower part with anti-synaptophysin (S). The nomenclature of fractions is as in Hell *et al.* (1988): H, homogenate; S_1 and P_1, 47 000 *g* supernatant and pellet; S_2 and P_2, 120 000 *g* supernatant and pellet; supernatant S_3, fluffy layer L_3, cushion C_3 and pellet P_3 from the 260 000 *g* spin; P_3', resuspended and cleared P_3 before CPG chromatography; P_IP and $P_{II}P$, pools from breakthrough peak and vesicle peak of the CPG chromatography.

during vesicle purification was observed (Figure 9B, compare lanes H and $P_{II}P$). A considerable quantity of amphiphysin is apparently lost during the purification procedure before CPG chromatography. Most strikingly, when the 120 000 *g* supernatant (fraction S_2) is centrifuged at 260 000 *g* through a sucrose cushion, only ~25% of the total amphiphysin follows the vesicle marker, synaptophysin, into the cushion and the pellet (fractions C_3 and P_3) whereas ~75% remains in the 260 000 *g* supernatant (S_3) and the fluffy layer (L_3), which are practically devoid of synaptophysin (Figure 9B) (this estimation is based on the relative band intensities in Figure 9B and analogous experiments, and the total protein content of these fractions; data not shown). Western blots reveal no difference in the electrophoretic behaviour of the light (S_3 and L_3) and heavy (C_3 and P_3) amphiphysin fractions, either in rat (Figure 9B) or in chicken (not shown).

Amphiphysin-like immunoreactivity is expressed not only in mature brain, but also in undifferentiated, mitotic neuroblastoma cells. The antibody detects a band comigrating with rat amphiphysin on immunoblots of homogenates from the mouse neuroblastoma cell lines, Neuro2a, NH15-CA2 and NS20Y. In NS20Y, band intensity is not altered if the cells are induced to differentiate morphologically by serum withdrawal. Amphiphysin-like immunorectivity was not detected on an immunoblot of *Torpedo* electric organ homogenate (data not shown), but was readily detectable on immunoblots of rat anterior and posterior pituitary (Figure 7D).

Discussion

The full-length chicken amphiphysin cDNA encodes a protein of 75 kDa, whereas immunoblotting labels a molecule with an apparent molecular mass (according to electrophoretic mobility) of 115 kDa in chicken brain. In good agreement with the electrophoretic behaviour of the brain protein, the fusion protein has an apparent size of 230 kDa, of which 114 kDa are contributed by the β-galactosidase moiety. Presumably, the discrepancy between the deduced molecular size and the electrophoretic mobility can be explained by aberrant migration in SDS−PAGE due to the acidic nature of amphiphysin (predicted pI 4.39) or to abnormal molecular shape. A dramatic reduction of electrophoretic mobility has also been found with other proteins like GAP-43 (size deduced from cDNA, 23 kDa; apparent size, 43−50 kDa; Karns *et al.*, 1987) or chromogranin B, which is also very acidic (size deduced from cDNA, 76 kDa; apparent size, 120 kDa; Benedum *et al.*, 1987). A weaker band with an apparent size of 125 kDa can be detected in some subcellular fractions from chicken, whereas rat displays a main band of apparent size 125 kDa and a minor band of 115 kDa. The relationship between the two bands (e.g. whether they are isoforms or derived by covalent modification or proteolysis) and the reasons for the differences between species and subcellular fractions (which were found very consistently in several preparations) remain to be clarified.

According to immunomicroscopy and immunoelectron microscopy of chicken and rat brain, amphiphysin appears to be specifically localized to presynaptic terminals, or at least highly concentrated in these sites. Amphiphysin mRNA is expressed in brain and adrenal gland, but not in other excitable tissues like skeletal muscle and heart or in other exocytotically active tissues like liver and pancreas, or in any of the other tissues tested. Moreover, immunoblotting

demonstrates amphiphysin expression in the anterior and posterior pituitary (Figure 7D), and current immunocytochemical work (to be published elsewhere) localizes amphiphysin to the cell bodies of many if not all cells of the adrenal medulla and the anterior pituitary. These findings suggest that amphiphysin may be involved in molecular events specific to neuronal synapses and certain endocrine cells like those of the adrenal medulla and the anterior pituitary. Several other synaptic vesicle proteins like SV2 (Buckley and Kelly, 1985), synaptophysin (Wiedenmann and Franke, 1985; Leube et al., 1987), p65/synaptotagmin (Matthew et al., 1981) and p29 (Baumert et al., 1990) are also expressed in these two other tissues. In adrenal medulla, synaptophysin may be associated only with the small electron-translucent vesicles and not with the secretory granules (Navone et al., 1986; Cutler and Cramer, 1990) whereas synaptotagmin is associated with both classes of particles (Perin et al., 1991). The subcellular localization of amphiphysin within endocrine cells remains to be investigated.

The morphological data indicate that amphiphysin is present in many and diverse synapses of the central nervous system of rat and chicken, its overall distribution closely resembling that of synaptophysin, and the high abundance of amphiphysin mRNA in brain underlines this finding. More detailed studies are required to resolve whether it is a ubiquitous synaptic protein or whether there are types of synapses that are devoid of amphiphysin.

In CPG chromatography, amphiphysin copurifies with synaptic vesicles; this association is rather resistant to washing with isotonic, high-salt and acidic buffers, although it appears to be solubilized somewhat more easily than synaptophysin. The molecule is very hydrophilic and highly charged, and it segregates into the aqueous phase in detergent phase partition (in fact, its cDNA was isolated with the antiserum directed against the aqueous phase), but it does have a single hydrophobic stretch that would be suitable as a transmembrane helix. Alternatively, amphiphysin may be an addition to the as yet rather small group of peripheral proteins of synaptic vesicles like the synapsins and rab3A that mediate the interaction between vesicles and other structures of the synaptic terminal. The hydrophobic stretch may unilaterally insert into the vesicle membrane as was proposed for synapsin I (Benfenati et al., 1989; Bähler et al., 1990), and the many charged groups of amphiphysin and the high potential for amphiphilic helices particularly in its N-terminal domain could provide a basis for interactions with other vesicle and non-vesicle proteins. The pattern of immunoperoxidase staining in Figure 6, decorating the periphery of synaptic vesicles, suggests that the antigen is accessible at the vesicle surface and not confined to the vesicle lumen.

A phosphoprotein doublet, dephosphin, with an apparent molecular weight similar to that of amphiphysin has been described in rat synaptosomal cytosol (Robinson, 1991). However, the empirical pI of dephosphin (6.3–6.7) differs substantially from the deduced pI of amphiphysin (4.4), which would be even lower if amphiphysin were phosphorylated. A phosphoprotein doublet of 119 and 124 kDa (SVAPP-120) peripherally associated with rat synaptic vesicles has been described by Bähler et al. (1991). SVAPP-120 sems to differ from amphiphysin by several criteria. Enrichment of SVAPP-120 during vesicle purification was much higher (17-fold), and SVAPP-120 could be quantitatively washed off the vesicles under conditions (0.2 M NaCl) very similar to those (0.15 M KCl) where amphiphysin remains bound. Moreover, the upper band of rat SVAPP-120 is weaker than the lower band, whereas the opposite is true for rat amphiphysin. However, because the vesicle purification procedure employed by Bähler et al. (1991) differs from ours, it cannot be excluded at present that SVAPP-120 is rat amphiphysin or a related molecule.

Although amphiphysin copurifies with synaptic vesicles, it is enriched much less in the course of vesicle purification than is synaptophysin, indicating that a large part of it is separated from the vesicles before CPG chromatography. In particular, three-quarters of total amphiphysin remain in the low-density fractions and are separated from the vesicles and their marker protein, synaptophysin, during sucrose cushion centrifugation. This observation also suggest that amphiphysin is a peripheral rather than an intrinsic vesicle protein, and led us to coin the protein's name. It is tempting to speculate that there may be two, possibly interconvertible, subpopulations of amphiphysin with high and low binding affinity to the vesicles that might play a role in synaptic vesicle dynamics, and that some of its many potential phosphorylation sites may have functional relevance. Knowledge of the primary structure of amphiphysin and the availability of its cDNA and of monospecific antibodies now provide efficient tools for investigating this protein's function.

Materials and methods

Production of polyclonal antibodies

Synaptic plasma membranes were isolated from forebrains of 6–10 day old chickens (Tetra-Hybrid) essentially as described by Babitch et al. (1976). Material from the 0.6/0.8 M and the 0.8/0.95 M sucrose interphases contained synaptic plasma membranes as confirmed by enzymatic and electron microscopic analyses. Both fractions were combined and submitted to Triton X-114 phase partitioning according to Bordier (1981) with slight modifications. Briefly, 300 μg synaptic plasma membranes suspended in ~250 μl storage buffer (50 mM imidazole–HCl, pH 7.5, 0.1 mM EDTA, 100 mM KCl, 56 mM NaCl) were adjusted to a volume of 1 ml and final concentrations of 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-114, incubated on ice for 15 min, then centrifuged at 12 000 *g* for 5 min at 0°C. The small 'phospholipid pellet' was discarded and the supernatant was incubated for 5 min at 37°C to induce phase separation and then centrifuged at 12 000 g for 3 min at 25°C. Triton X-114 was added to the upper aqueous phase to 1%, while the lower detergent phase (~100 μl) was mixed with 900 μl of 10 mM Tris–HCl, pH 7.5/150 mM NaCl. Both phases were again subjected to phase partitioning as described. The aqueous phase originating from the repartitioning of the first aqueous phase, and the detergent phase derived from repartitioning of the first detergent phase, were saved and precipitated with acetone. Precipitates were resuspended in phosphate buffered saline with 0.05% SDS and used to immunize rabbits. Animals were injected subcutaneously with 500 μg detergent or aqueous phase protein emulsified with complete Freund's ajduvant followed by three boosts at 14 days intervals (250 μg protein emulsified with incomplete Freund's adjuvant). The antisera were tested for titre and specificity by ELISA, Western blotting and immunofluorescence of cryotome sections of chicken brain and liver.

Construction and screening of cDNA libraries

RNA was isolated from chicken forebrain according to Auffray and Rougeon (1980). Poly(A)$^+$ RNA was selected by one cycle of binding to oligo(dT)–cellulose, and cDNA was synthesized according to Gubler and Hoffman (1983), except that methylation was omitted. Both oligo(dT) and random hexanucleotide primers were used. Blunt-ended cDNA was ligated into λgt11 with *Eco*RI–*Not*I adaptors (Pharmacia), and recombinants were packaged using a commercially available extract. *Escherichia coli* strain Y1090 was infected with unamplified phage and plated using standard

procedures (Huynh *et al.*, 1985). Screening of duplicate nitrocellulose replica filters was carried out according to Sambrook *et al.* (1989). One duplicate filter was screened with the antiserum against detergent phase proteins and the other with the serum against aqueous phase proteins. Immunopositive plaques were identified with an affinity-purified, alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) and the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. Immunoreactive clones were plaque-purified, and the cDNA inserts were excised with *Eco*RI, subcloned into M13 vectors and terminally sequenced according to Sanger *et al.* (1977). The deduced protein sequences were compared with the sequences in the EMBL and SWISS-PROT databases. cDNAs encoding GAP-43 (Karns *et al.*, 1987), N-CAM (Cunningham *et al.*, 1987) and MARCKS (Stumpo *et al.*, 1989) were among the clones thus identified. To select sequences expressed specifically in the brain, anonymous cDNAs were labelled by nick translation and used to probe Northern blots carrying poly(A)$^+$ RNA from chicken forebrain, skeletal muscle and liver under standard, high stringency conditions (Sambrook *et al.*, 1989).

cDNA sequencing and sequence analysis

Among the novel clones identified by immunoscreening was a cDNA (amphy-11.3) comprising nt 46–3189 of the amphiphysin sequence of Figure 2. Restriction fragments were subcloned into M13 vectors and sequenced according to Sanger *et al.* (1977). Fifteen additional amphiphysin clones were isolated by a high stringency plaque hybridization rescreen of 10^6 recombinant plaques of chicken brain cDNA libraries produced as described above with a nick translated cDNA fragment encompassing nt 46–325. The lengths of the 5′ ends of the new clones were determined by PCR analysis of plaque eluates using a primer complementary to nt 188–205 of the amphiphysin sequence and primers complementary to vector sequences flanking the insert. The cDNA inserts of the three clones giving the longest 5′-terminal PCR products were subcloned into M13 and terminal sequenced, yielding nt 1–45 and 3190–3220. Processing of sequences and analysis of secondary structure [based on the predictions of Chou and Fasman (1978)], hydropathy (Kyte and Doolittle, 1982) and net charge of the deduced amino acid sequence was carried out with the GENEPRO software package (Riverside Scientific Enterprises).

Production of a monospecific antiserum against amphiphysin

A rabbit was immunized with the β-galactosidase–amphiphysin fusion protein derived from the cDNA clone (amphy-11.3) originally isolated by immunoscreening. Lysogens were prepared in *Escherichia coli* strain Y1089, and synthesis of fusion protein was induced according to standard procedures (Sambrook *et al.*, 1989). Fusion protein was purified by SDS–PAGE under reducing conditions; protein-containing slices were excised from the gel after staining with Coomassie blue, homogenized and used for immunization. Immunization was as described above for the production of antisera against membrane proteins, except that the animal had four booster injections, and 8 μg of fusion protein was administered at each injection. The antiserum was affinity-purified by incubating with nitrocellulose strips that were loaded with gel-purified fusion protein by Western blotting.

Western blot analysis

After separation by SDS–PAGE (Laemmli, 1970) proteins were transferred to nitrocellulose membranes according to Kyhse-Andersen (1984). After blocking in 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% BSA, blots were incubated in a dilution of the primary antibody in blocking buffer. Secondary antibody and colour detection system were the same as described for immunoscreening. To detect synaptophysin, a rabbit polyclonal antiserum (ASY 3, Biometra, Göttingen, FRG) against rat synaptophysin was used.

Immunocytochemical methods

For immunofluorescence, chicken tissues were removed, embedded in tissue-Tek (Miles) and frozen in isopentane chilled with liquid nitrogen. Frozen sections (5–10 μm) were cut in a cryostat at −30°C and mounted on gelatin coated glass coverslips. After fixation in 4% formaldehyde in phosphate buffered saline (PBS) for 30 min at 25°C and three washes with PBS, sections were blocked with 1% BSA in PBS for 15 min. Incubations with dilutions of primary antibodies in blocking solution were done for 16 h at 25°C. After three washes with PBS, affinity-purified fluorescein-conjugated goat anti-rabbit IgG (Sigma) was incubated with the sections for 1 h at 25°C. After three washes with PBS, the slides were viewed under fluorescence optics.

For immunoenzymatic staining, Sprague-Dawley rats under deep anaesthesia were fixed by vascular perfusion with a solution of 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. Fixation was followed by perfusion with 0.15 M saccharose in 0.13 M phosphate buffer, pH 7.4 (SPB). The brains were dissected out and either frozen after 48 h pretreatment with 30% saccharose and stored for up to 3 months or directly sectioned at 50 μm on a vibratome in SBP. Sections were pretreated in 1% sodium borohydride in PBS for 15 min and subsequenty permeabilized with 0.1% Triton X-100 for 30 min. After incubations of 36 h with primary antibody in a cold room (mouse monoclonal anti-rat synaptophysin from Camon, Wiesbaden, FRG; rabbit polyclonal affinity-purified anti-chicken amphiphysin prepared as described above), 24 h with secondary antibody at room temperature (biotinylated goat anti-mouse IgG or anti-rabbit IgG, respectively, from Vector/Camon, Wiesbaden, FRG) and 6 h with the avidin–biotinylated peroxidase complex (Vector/Camon), peroxidase activity was visualized with 1.4 mM 3,3′-diaminobenzidine in a solution of 10 mM imidazole in 50 mM Tris–HCl pH 7.6, supplemented with 0.3% nickelous ammonium sulfate and 0.015% H_2O_2 for 4 min. For optimal correlation between light and electron microscopy, the sections were temporarily mounted in buffer, and areas of interest were photographed for light microscopical analysis.

For electron microscopy, immunoenzymatically stained sections were postfixed in OsO_4 and flat-embedded in araldite. Areas of interest were re-identified in the araldite-embedded vibratome section with a light microscope. The area was cut out and the surface was carefully sectioned on an ultramicrotome and re-identified on a semithin section. Adjacent ultrathin sections were viewed in a Philips EM 420 electron microscope.

For controls, sections were incubated either without primary antiserum, with 'affinity-purified' preimmune serum or with the monospecific anti-amphiphysin serum after preabsorption of this serum with nitrocellulose-bound amphiphysin fusion protein. All controls were negative.

Isolation and washing of synaptic vesicles

Synaptic vesicles were isolated essentially as described by Hell *et al.* (1988). For washing experiments, aliquots (200 μg protein) of synaptic vesicles were incubated in 1 ml of 300 mM glycine, 5 mM HEPES–KOH, pH 7.4, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg per ml pepstatin A, plus the conditions given in the legend to Figure 9A. After 20 min on ice, the suspension was centrifuged for 90 min at 260 000 *g* and 4°C. The upper 900 μl were precipitated with TCA and resuspended in 60 μl of reducing SDS–PAGE sample buffer, while the pellet was resuspended in 500 μl glycine buffer, precipitated with TCA (TCA pellets of samples with Triton X-100 were additionally extracted with 0.1 M HCl/90% acetone at −20°C overnight to remove the detergent) and dissolved in 60 μl of reducing SDS–PAGE sample buffer. 30 μl aliquots were resolved on a 10% polyacrylamide gel.

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