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Brain Ac39/physophilin: cloning, coexpression and colocalization with synaptophysin

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Abstract

Physophilin is an oligomeric protein that binds the synaptic vesicle protein synaptophysin constituting a complex that has been hypothesized to form the exocytotic fusion pore. Microsequencing of several physophilin peptides putatively identified this protein as the Ac39 subunit of the V-ATPase. Ac39 has recently been shown to be present in a synaptosomal complex which, in addition to synaptophysin, includes the bulk of synaptobrevin II, and subunits c and Ac115 of the V_0 sector of the V-ATPase. We have cloned physophilin from mouse brain and found a differential region of 12 amino acids when compared with the previously reported sequence of Ac39 from bovine adrenal medulla. RT-PCR cloning from the bovine adrenal medulla demonstrates that sequencing errors occurred in the previous cloning study, and shows that the amino acid sequences of physophilin and Ac39 are completely identical. *In situ* hybridization in rat brain reveals a largely neuronal distribution of Ac39/physophilin mRNA which spatio-temporally correlates with those of subunit c and synaptophysin. Immunohistochemical analysis shows that Ac39/physophilin is mostly concentrated in the neuropil with a pattern identical to subunit A and very similar to synaptophysin. Double-labelling immunofluorescence shows a complete colocalization of Ac39/physophilin with subunit A and a partial colocalization with synaptophysin in the neuropil. Our findings bring anatomical support for the *in vivo* occurrence of the synaptophysin-Ac39/physophilin interaction and further suggest a coordinated transcription of V-ATPase and synaptophysin genes. A putative role of Ac39/physophilin in the inactivation of the V-ATPase by disassembly of its V_1 sector is also discussed.

Introduction

In the last few years an increasing number of presynaptic proteins putatively involved in exocytosis have been isolated and cloned (Südhof, 1995). However, apart from those involved in the uptake of neurotransmitters (Schuldiner *et al*., 1995), very little is known about their specific function (Augustine *et al*., 1996). *In vitro* studies have also revealed that some of these proteins form a number of complexes in detergent extracts, but the occurrence and relevance of these complexes *in vivo* remains to be demonstrated.

The prevailing model for neuronal exocytosis, the so-called SNARE hypothesis, postulates the synaptic vesicle membrane protein synaptobrevin II (also called VAMP II), and the synaptic membrane proteins synaptosome-associated protein (SNAP)-25 and syntaxin I as membrane-specific receptors in the core of a docking/fusion machine (reviewed in Rothman & Warren, 1994). Recent evidence links the vacuolar ATPase (V-ATPase) to this machinery. In fresh synaptosomal extracts, the bulk of synaptobrevin II has been found associated with both synaptophysin (another synaptic vesicle membrane protein) and the membraneous portion of the V-ATPase (V_0 sector) in a complex that excludes the cytoplasmic portion of this enzyme (V_1 sector), as well as SNAP-25 and syntaxin I (Galli *et al*., 1996).

The V-ATPase (EC 3.6.1.34) is the large (600–750 kDa) hetero-oligomeric ATP hydrolase enzyme that generates the transmembrane proton gradient that supplies the driving force for the uptake and storage of neurotransmitters (reviewed in Nelson, 1991). The V-ATPase activity seems to be highly concentrated in the synaptic vesicles (Südhof & Jahn, 1991) where it accounts for about one-fifth of the total synaptic membrane protein (Moriyama & Futai, 1990; Floor *et al*., 1990). Like the well known F-ATPase, this enzyme also consists of two sectors, V_1 (a cytoplasmic globular domain responsible for nucleotide binding and ATPase catalytic activity) and V_0 (an intrinsic membrane domain). V_0 contains subunit c, a proteolipid thought to constitute the proton-conducting pore (reviewed in Nelson, 1992). This subunit has recently been shown to be identical to two independently identified proteins, the mediatophore (Brochier & Morel, 1993) and ductin (reviewed in Finbow *et al*., 1995). The mediatophore is a nerve-ending protein complex necessary for neurotransmitter release that was originally isolated from presynaptic plasma membranes of Torpedo electric lobe (Cavalli *et al*., 1993), while ductin is a putative subunit of the gap junction channels. Two additional subunits, Ac39 and Ac115, are also present in the V_0 (reviewed in Nelson, 1992). These two subunits were termed 'accessory', as they were thought not to be necessary for catalytic or proton pumping activities, but rather related with assembly, targeting or regulation of the V-ATPase complex. Moreover, their presence consti-

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tuted a major difference between V- and F-ATPases (Wang *et al*., 1988). The requirement of Ac39 for V-ATPase function and assembly of both V_0 subunits and V_0–V_1 sectors has been supported by recent genetic and biochemical studies (Bauerle *et al*., 1993; Myers & Forgac, 1993).

The direct binding of synaptobrevin II to synaptophysin suggests that the former protein may act as a double switch involved in both the clamping of the formation of the SNARE complex and the formation of the exocytotic fusion pore (Calakos & Scheller, 1994; Washbourne *et al*., 1995; Edelman *et al*., 1995). Synaptophysin was hypothesized to be involved in the formation of the fusion pore through its binding to physophilin, an oligomeric synaptic protein that behaves as an integral membrane protein. Although originally isolated from synaptic plasma membranes, physophilin seems to be enriched in synaptic vesicle membranes with only low amounts present in the plasmalemma (Thomas *et al*., 1988; Thomas & Betz, 1990). Recent microsequencing of several proteolytic peptides from this protein unexpectedly revealed its likely identity to Ac39 (Siebert *et al*., 1994), previously cloned from the adrenal medulla (Wang *et al*., 1988).

By virtue of its *in vitro* interaction with synaptophysin, Ac39 (putative physophilin) appears to be a good candidate to mediate the connection of the synaptobrevin II-synaptophysin complex to the V_0 sector of the V-ATPase. In this study, we report for the first time the complete sequence of physophilin and the corrected sequence of Ac39, demonstrating the total amino acid identity between these two sequences. We also analyse the distribution of its mRNA and protein in the rat brain, as the first step toward defining its biological function. Our results support a close functional relationship between Ac39/physophilin and synaptophysin, compatible with its direct interaction *in vivo*, and furthermore suggest the existence of a coordinated transcriptional regulation of their corresponding genes.

Materials and methods

Cloning and sequence analysis

Clone MBAc39 was previously isolated in our laboratory during the course of an unrelated immunoscreening of a mouse brain cDNA library using a polyspecific polyclonal antibody against bovine brain postsynaptic densities (Nieto-Sampedro *et al*., 1981). This clone was found to be strongly homologous to subunit Ac39 of V-ATPase (Wang *et al*., 1988; see also GenBank for a first correction, March 1993, of the original sequence regarding the assignment of the initiation codon). The library (a gift from Dr Wolfgang Wille) was constructed from brain (excluding cerebellum) of 2–6-day-old postnatal (P2–P6) mice (C57BL/6 strain) on the expression vector λgt11 as described by Barthels *et al*. (1987). Eco RI restriction fragments of the selected cDNA clones were subcloned into the plasmid pBluescript SK + (Stratagene Cloning Systems, La Jolla, CA, USA) for subsequent studies. Four subclones of the same size of ≈ 1.5 kb also displayed identical restriction maps. One of these clones designated as MBAc39 was further characterized.

Sequencing was carried out by the dideoxy chain termination method (Sanger, 1977) using T7 DNA polymerase (Pharmacia, Biotech Inc., Piscataway, NJ, USA) and the recA minus strain JM109 as a host. Hydropathy plots were done using the HIBIO DNASIS program package for microcomputer systems (Hitachi America Ltd, San Francisco, CA, USA) according to Kyte & Doolittle (1982).

Data base searching and multiple sequence alignment were done using the Genetics Computer Group Sequence Analysis Software Package (Devereaux *et al*., 1984). Nucleotide and derived amino acid sequences of MBAc39 were compared with data bases in GenBank and EMBL Bank, using the TFastA, FastA and WordSearch programs. Multiple sequence alignment was created using the Pileup program (manufacturer default settings). The Prosite data base was used to search for specific sequence motifs.

Northern and Southern blot analyses

Radioactive cDNA probes were prepared from the subcloned inserts of the cDNA library and used to probe Northern blots from brain and liver. Total RNA was isolated from forebrain and liver of Balb/c mice at postnatal day 15 (P15) using the acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi, 1987). Poly $(A)^+$ RNA was purified by chromatography with an oligo (dT) cellulose column. Denatured poly $(A)^+$ RNA samples (≈5 μg) were electrophoretically separated in a 1% agarose gel containing 6% formaldehyde and a MOPS buffer (400 mM MOPS, 100 mM Na acetate and 100 mM EDTA buffered at pH 7.0 with acetic acid). RNA was directly transferred using a Posiblot apparatus (Stratagene) to Nytran nylon membranes (Schleicher & Schuell Inc., Keene, NH, USA) and UV cross-linked using a UV-Stratalinker (Stratagene). Blots were prehybridized at 42 °C for 1–2 h in a buffer containing 50% formamide, 6 × standard saline citrate (SSC) (1 × SSC = 150 mM NaCl; 15 mM Na citrate), 2 × Denhardt's reagent, 0.5% sodium dodecyl sulphate (SDS), and 1 mg/mL herring sperm DNA. Hybridization was carried out overnight in the same buffer with the addition of 0.1 mg/mL tRNA and denatured [^{32}P]dATP-labelled cDNA probe (2×10^6 c.p.m./mL). The DNA probe was randomly primed from an *Eco*RI insert (specific activity of 0.5–1×10^9 c.p.m./μg DNA). Blots were washed twice in a 1 × SSC/0.1% SDS solution for 20 min at room temperature followed by three washes (20 min each) in 0.2 × SSC/0.1% SDS at 68 °C. Then the membranes were exposed to Agfa Curix RP2 film at –80 °C with two High Speed HG intensifying screens (Dupont, NEN, San Mateo, CA, USA). rRNAs of 28s and 18s were used to estimate RNA size (Noller, 1984).

Southern blot analysis was carried out on Balb/c mouse genomic DNA isolated according to Arribas *et al*. (1986). Restriction enzyme-digested DNA was electrophoresed on a 0.8% agarose gel, ethidium bromide stained, depurinated, denatured and neutralized as described (Sambrook *et al*., 1989). DNA transfer to a Duralon nylon membrane (Stratagene), cross-linking, hybridization with the appropriate labelled cDNA probe, and autoradiography were done as described above for Northern blots.

RT-PCR

The 20-mers primers used for amplification were: 5′-CAGATGGAGGCTGTGAACA-3′ [forward] (nucleotides 408–426)and 5′-AGCTCTGTGCCGAAAGAGTT-3′ [reverse] (nucleotides 674–693). These primers flank a stretch of 285 bp between nucleotides 408 and 693 of Ac39 sequence (accession number U21549 of GenBank/EMBL/DDBJ data banks).

Total bovine adrenal medulla RNA was extracted using the Micro RNA Isolation kit following the method described by the vendor (Stratagene). Reaction conditions for the synthesis of the first-strand cDNA and PCR amplification were as described in the StrataScript RT-PCR kit (Stratagene). Briefly, the annealing was conducted at 54 °C and 30 reaction cycles were performed. Each cycle consisted of incubations at 91 °C (1.5 min), 54 °C (1.5 min) and 72 °C (2.5 min).

The poly $(A)^+$ RNA from mouse hippocampal formation was prepared using the Quick Prep Micro mRNA Purification kit (Pharmacia) and then reverse transcribed by using the First-Strand cDNA

Synthesiz kit (Pharmacia). For PCR, the annealing was done at 58 °C and 30 reaction cycles were performed under the following conditions: 95 °C (1 min), 58 °C (1 min) and 72 °C (2 min).

The amplified DNA was separated on an agarose gel, the DNA bands were subcloned into pCRTMII using a TA Cloning kit (Invitrogen, San Diego, CA, USA) and then recombinant clones were sequenced as described above.

In situ *hybridization*

Sense and antisense cRNA probes were synthesized by *in vitro* transcription of the 1.5-kb *Eco*RI fragment of MBAc39 cDNA, subcloned into pBluescript SK^+. ^{35}S-UTP (1000 Ci/mmol; Amersham Corp., Arlington Heights, IL, USA) or digoxygenin-UTP (Boehringer Mannheim Corp., Indianapolis, IN, USA) were used to generate the riboprobes. The template plasmid for the antisense and sense riboprobes was linearized with *Sal*I and *Sma*I, respectively. The antisense and sense transcripts were obtained by using T3 RNA polymerase and T7 RNA polymerase (Boehringer Mannheim), respectively. The transcription reaction was carried out by incubating ≈1.2 μg of linearized plasmid with 7.5 U of T3 or T7 RNA polymerase for 4 h at 30 °C; after 2 h, 50% of the enzyme was added to the reaction mixture. Alkaline hydrolysis of the probe was carried out for 35 min at 60 °C (Pardue, 1985). The specificity of the *in situ* hybridization technique was confirmed by the absence of labelling of adjacent brain sections incubated with sense riboprobes used under identical conditions and specific activity.

In situ hybridization with radiolabelled riboprobes was carried out as described by Mellström *et al*. (1993) using male Wistar (Charles River Laboratories, Wilmington, MA, USA) rats (P15 and P60). The labelled probes were applied to the tissue sections at a concentration of ≈1 pg cRNA/μL (specific activity: 10^9 c.p.m./μg RNA). The hybridization solution contained 50% (v/v) formamide and 10% (w/v) dextrane sulphate. Hybridization was carried out overnight at 42 °C. The sections were washed at a stringency of 0.1 × SSC at 50 °C for 3 h, dehydrated, dried under vacuum and exposed to β-max film (Amersham) for 10 (P15) and 30 (adult) days.

Digoxygenin-labelled riboprobes (antisense and sense) were prepared following the manufacturer's instructions (Boehringer Mannheim) with the inclusion of an additional alkaline degradation step as described above. *In situ* hybridization was done as previously described (Chowen *et al*., 1993). The hybridization and stringent wash were conducted at 45 °C and 60 °C, respectively. Sections were processed for digoxygenin detection as described by the manufacturer (Boehringer Mannheim) with the chromogen reaction carried out for 20 h. The reaction was stopped and then the slides rinsed in distilled water and mounted with Mowiol for microscopy.

Anatomical loci were assigned using the atlases of Paxinos & Watson (1986), Paxinos *et al*. (1991) and Kaufman (1992). Preparations were analysed with a computer-assisted image analysing system using online image analysis software ('Morpho' from IMSTAR/Starwise, Paris, France), and relative optical densities were recorded for the different brain areas.

Immunohistochemistry

Adult male Wistar rats (Charles River) of ≈250 g weight were transcardially perfused with saline (0.9% NaCl) followed by 400 mL of fixative [4% paraformaldehyde, 0.03% $CaCl_2$ in 0.1 M phosphate buffer (PB), pH 7.4]. Perfused brains were removed, postfixed for 4 h (4% paraformaldehyde in 0.1 M PB) at room temperature and cryoprotected by immersion in a solution containing 30% sucrose in 0.1 M PB at 4 °C. Sagittal frozen sections, 20 μm thick, were cut with a cryostat and then washed in phosphate-buffered saline (PBS) containing 0.3% H_2O_2 to block endogenous peroxidase (30 min at room temperature). Sections were then rinsed in PBS and processed for free-floating immunocytochemistry. The primary antibodies used were: the antisera to Ac39/physophilin (Wang *et al*., 1988; 1 : 500) and to subunit A, obtained from Dr N. Nelson (Moriyama & Nelson, 1989; 1 : 500); the monoclonal antibody to synaptophysin SY38 (Boehringer Mannheim Biochemicals, Wiedenmann & Franke, 1985; 1 : 50); and the antiserum G-96, obtained from Dr P. DeCamilli (Jahn *et al*., 1985; 1 : 500). All the antibodies used in the immunohistochemical procedures were diluted in PBS containing 0.2% Triton X-100 and 1% BSA blocking solution. Serial sections were incubated overnight at 4 °C with the primary antibodies, and processed using the ABC method (Vector Laboratories Inc., Burlingame, CA, USA) and incubated overnight at 4 °C with the primary antibodies. The rest of the incubations were done at room temperature. Controls carrying normal IgG at comparative dilutions, omitting the primary antibody, produced no immunostaining or light homogeneous background. Biotinylated anti-IgG and streptavidin-peroxidase complex solution were from Vectastain ABC kit (Vector Laboratories). 3-3′ Diaminobenzidine was used as the chromogen of the reaction. For light microscopy analysis, low magnification pictures were taken with a stereomicroscope (Wild Photomicroscope M400).

Sections for double indirect immunofluorescence were treated sequentially with the primary antibodies for 2 h at room temperature and visualized using Fluorolink-Cy2-labelled goat antirabbit IgG and Fluorolink-Cy5-labelled donkey antimouse IgG (1/1000; Amersham). Secondary antibodies were preincubated with different brain sections to remove unspecificities. For anti-Ac39/physophilin and antisubunit A (both antirabbit sera), the protocol was modified as follows. After doing regular immunofluorescence with anti-Ac39, the antibodies were crosslinked by incubating for 30 min in 4% paraformaldehyde/PBS, then thoroughly washed. The second immunofluorescence, with antisubunit (A), was done by using a goat biotinylated antirabbit IgG (1/250; Vector) followed by Fluorolink-Cy5-labelled streptavidine. Control sections, prepared either by omitting the primary antibody or by replacing it with an equivalent concentration of non-immune IgG, gave a homogeneous background where no structures were discernible. Immunolabelled sections were mounted in PBS–glycerol (9 : 1). Preparations were viewed under standard epifluorescence with a Leika TCS 4D laser confocal system (equipped with an argon–krypton laser) coupled to a Leitz DM IRB microscope.

Results

Physophilin presents a region that differs from the reported Ac39 sequence

Ac39/physophilin identity was previously assumed based on the microsequencing of several proteolytic peptides from physophilin which were found to be identical to portions of the deduced sequence of Ac39 (Siebert *et al*., 1994). However, since physophilin (the brain Ac39) had not been cloned, its complete sequence was not available. We therefore undertook the sequence analysis of physophilin by first sequencing a mouse brain cDNA, clone MBAc39, strongly homologous to Ac39 from bovine adrenal medulla (Wang *et al*., 1988; see Materials and methods section). Its nucleotide and deduced sequences have been introduced in GenBank/EMBL/DDBJ data banks. The identity of both sequences was 81.7% at the nucleotide level; but surprisingly, at the amino acid level was not as complete as expected, but 96.9% (see below). The comparison of the deduced amino acid sequences of both cDNAs indicates that the first nucleotide

of MBAc39 corresponds to the third nucleotide of the initiation codon Met-1 in the adrenal medulla Ac39. The resulting single open-reading frame encodes a protein of 350 amino acids that only lacks the first two nucleotides of the initial Met to constitute the full-length protein. The predicted molecular mass of the protein is 40 168, which is in good agreement with its apparent molecular mass (i.e. 39 kDa). The complete 3′ non-coding region (432 bp) includes a consensus polyadenylation signal, AATAAA, located 12 bp upstream from the poly (A) tail. Since the total length of MBAc39 cDNA (1504 bp) is quite close to the size of the mRNA detected by Northern blots (about 1.6 kb; see below), it appears that only a very short 5′ non-coding region remains to be cloned to complete the full length message. A search for specific sequence motifs in MBAc39 revealed the presence of 12 casein kinase II phosphorylation sites, one consensus sequence for tyrosine-kinase phosphorylation and one tyrosine sulphation site.

Ac39 was originally identified by immunoblotting in all mammalian organelles exhibiting V-ATPases, but not in lower organisms like plants or yeast (Moriyama & Nelson, 1989); therefore, it was originally assumed to be restricted to mammals. However, the recent cloning of a yeast homologue refuted this idea and strongly suggested that Ac39 is a universal component present in most, if not all, organisms (Bauerle *et al*., 1993). Our search of the data banks also revealed the existence of Ac39 homologues in invertebrates (70.7 amino acid identity in a 92 amino acid stretch from *Caenorhabditis elegans*; accession number Z14621) and plants (44.6 identity in a 137 amino acid stretch from *Arabidopsis thaliana*; accession number Z26026), showing the broad phylogenetic distribution of this subunit.

V-ATPases share a common evolutionary origin with F-ATPases, are extraordinarily conserved and display a wide phylogenetic distribution, ranging from Archaebacteria to humans. The conservation of MBAc39 (46% amino acid identity between mammals and yeast; Fig. 1A) was found to be intermediate between that of the catalytic subunits (70–80% identity) and that of the more poorly conserved subunits of the V-ATPase (e.g. subunit C, 37%; Nelson, 1992). No significant homology was found to any subunit of F-ATPases, suggesting that Ac39 is a comparatively late addition to the V-ATPase complex. Data banks searches also revealed that Ac39 is nearly identical to several peptides of a putative subunit from the gap junction of insects (Ryerse, 1995). If the microsequencing data from this preliminary report receive further confirmation, this would be the second subunit of the V-ATPase (together with subunit c/ductin) to be identified as a putative subunit of the gap junctional complex.

Comparison of the deduced Ac39 sequences from mouse, human, bovine and yeast showed a high degree of conservation of this protein and also revealed some remarkable differences (Fig. 1A). The major difference between the mammalian and yeast sequences is a 7 amino acid insertion (between residues 287 and 293 of MBAc39) in the mammalian sequences (Fig. 1A), which constitutes an imperfect ATP/

A

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          1                                                  50
MBAc39     SFFPELYFN VDNGYLEGLV RGLKAGVLSQ ADYLNLVQCE TLEDLKLHLQ
HAc39     M......... .......... .......... .......... ..........
bmAc39    M......... .......... .......... .......... ..........
YAc39        MEGV... ID..FI..V. ..YRN.L..N NQ.IT.T..D .......Q.S.

         51                                                 100
MBAc39    STDYGNFLAN EAS-PLTVSV IDDKLKEKMV VEFRHMRNHA YEPLASFLDF
HAc39     .......... .......... .......... .......... ..........
bmAc39    .......... .......... .......... .......... ..........
YAc39     ........SS VS.ES..T.L .QEYASS LY H..NYI.DQS SGSTRK.M.Y

         101                                                150
MBAc39    ITYSYMIDNV ILLITGTLHQ RSIAELVPKC HPLGSFEQME AVNIAQTPAE
HAc39     .......... .......... .......... .......... ..........
bmAc39    .......... .......... .......... .......... ..........
YAc39     .......... A.M....I.D .DKG.ILQR. ....W.DTLP TLSV.TDLES

         151                                                200
MBAc39    LYNAILVDTP LAAFFQDCI- SEQDLDEMNI EIIRNTLYKA YLESFYKFCT
HAc39     .......... .......... .......... .......... ..........
bmAc39    ........S. -GG..PGLH. L......... .......... ..........
YAc39     ..ETV...T. ..PY.KN.FD TAEE...... .....K.... ...D..N.V.

         201                                                250
MBAc39    -LLGGTTADA MCPILEFEAD RRAFIITINS F-GTELSKED RAKLFPHCGR
HAc39     .......... .......... .......... .......... ..........
bmAc39    .......... .......... .......... .......... ..........
YAc39     EEIPEPAKEC .QTL.G.... ..SIN.AL.. LQSSDIDPDL KSD.L.NI.K

         251                                                300
MBAc39    LYPEGLAQLA RADDYEQVKN VADYYPEYKL LFEGAGSNPG DKTLEDRFFE
HAc39     .......... .........K L......... .......... ..........
bmAc39    .......... .......... .......... .......... ..........
YAc39     ...IATFH.. Q.Q.F.G.RA ALANVY..RG FL.TGN---- ---...H.YQ

         301                                                350
MBAc39    HEVKLNKLAF LNQFHFGVFY AFVKLKEQEC RNIVWIAECI AQRHRAKIDN
HAc39     .......... .......... .......... .......... ..........
bmAc39    .......... .......... .......... .......... ..........
YAc39     L.ME.CRD.. TQQ.AISTVW .WM.S....V ...T...... ..NQ.ER.N.

          351        Identity respect MBAc39 (%)
MBAc39    YIPIF                100
HAc39     .....                 99.1
bmAc39    .....                 96.9
YAc39     ..SVY                 45.7
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B

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      D  S  P  G  G  F  F  P  G  L  H  L  E
     g--acagccctggcggctttttttccaggactgcatct-agag        bmAc39
     |  ||| ||||||||||||||||||||||||||||||| ||||
467  GACACACCCCTGGCGGCTTTTTTCCAGGACTGCATCTCAGAG   508  MBAc39
      D  T  P  L  A  A  F  F  Q  D  C  I  S  E

      D  T  P  L  A  A  F  F  Q  D  C  I  S  E
     GACACGCCCCTGGCGGCTTTTTTCCAGGACTGCATCTCAGAG        BMAc39
     ||||| ||||||||||||||||||||||||||||||||||||
467  GACACACCCCTGGCGGCTTTTTTCCAGGACTGCATCTCAGAG   508  MBAc39
      D  T  P  L  A  A  F  F  Q  D  C  I  S  E
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FIG. 1. (A) Multiple sequence alignment of the amino acid sequence of the Ac39/physophilin homologues. MBAc39 (accession number U21549), human osteoclastoma cell line (HAc39; merged sequence from two cDNAs expressed sequence tags with Embo Data Bank accession numbers X71490 and T09451), bovine (bmAc39; Wang *et al*., 1988, with first correction of N. Nelson in the GenBank), and yeast (YAc39, Bauerle *et al*., 1993) were aligned using the Pile-Up command in the GCG program. Dots indicate identical amino acid positions with respect to the MBAc39 sequence. The identity is indicated at the end of each sequence. Hyphens indicate gaps inserted to obtain the optimal alignment between sequences. (B) Sequence analysis of the Thr-81/Ser-92 region of Ac39/physophilin. Nucleotide and deduced amino acid Ac39 sequences of the region (nucleotides 467–508) found to be different from that previously reported by Wang *et al*. (1988). Upper-case letters: nucleotide sequence and the deduced amino acid sequence (single-letter code) of the cDNAs isolated in this study (MBAc39 and bmAc39); lower-case letters: original nucleotide sequence and corresponding amino acid sequence of Wang *et al*. (1988) (bmAc39). Nucleotide sequence alignment of MBAc39 with bmAc39 (top) and with the RT-PCR sequence obtained after six independent clones were isolated from bovine adrenal medulla (bmAc39, bottom). Alignment was done with the Bestfit program of the GCG package. Putative sequencing errors are highlighted in bold. This correction was communicated to Dr N. Nelson, who has updated (March 1995) the bovine sequence in the GenBank (accession number J04204).

GTP binding site (a P-loop with an additional Asp residue). This motif is found in the catalytic subunits of the V-ATPase (Saraste *et al*., 1990). The possibility of this insertion being related to the known differences between constitutive (yeast) and regulated (mammalian) secretion remains to be explored.

The major differences within the mammalian Ac39 sequences occurred in a 12 amino acid stretch located between Thr-157 and Ser-168 of MBAc39 (Fig. 1A). Despite the high nucleotide homology (94%) within this region, the amino acid identity between MBAc39 and the bovine adrenal medulla Ac39 was only 25%. The bovine adrenal medulla sequence lacked three nucleotides (one amino acid in the deduced sequence) in this region. Furthermore, the sequence of this stretch was identical between MBAc39 and a sequence from a human osteoclastoma cell line and, surprisingly, it presented higher conservation in yeast than in bovine. Therefore, considering the extraordinary conservation of the V-ATPase subunits, the existence of these differences between the (mouse) brain Ac39 homologue (MBAc39, the putative physophilin) and the (bovine) adrenal medulla Ac39 strongly suggested the likely existence of tissue isoforms (as opposed to the total Ac39/physophilin identity, previously assumed), rather than differences among species (i.e. mouse versus bovine). Moreover, considering that the major organelles present in those tissues are synaptic vesicles and chromaffin granules, respectively, this differing region was likely to represent interorganellar differences.

RT-PCR cloning of Ac39 demonstrates the complete Ac39/physophilin identity

Since a brain-specific 18-bp insert has been reported for subunit Ac115 (Peng *et al*., 1994), it was important to discriminate between the existence of tissue-specific isoforms of Ac39 and the occurrence of sequencing errors in the original study. For this purpose we analysed the region bearing the differences by reverse transcriptase-PCR on bovine adrenal medulla (Fig. 1B). The nucleotide sequences of six independent clones were found to be identical, but different from the previously reported sequence. The deduced amino acid sequence of those clones was identical to that of MBAc39, showing only one conservative mismatch at the nucleotide level. Furthermore, four independent clones obtained by RT-PCR analysis of the same region from mouse brain were completely identical to MBAc39 (data not shown). Two sequencing errors in the original cloning study seem to be the likely explanation for the observed differences: (i) a two-nucleotide misreading at the beginning of the box (a GC-rich region) which led to a frame shift; and (ii) a one-nucleotide misreading at the end of the box, which restored the original frame. This resulted in a total of three nucleotides missing from the box (Fig. 1B). Therefore, it can be concluded that Ac39 is completely identical to physophilin (for simplicity Ac39/physophilin will be referred to hereafter as Ac39).

Distribution of Ac39 mRNA in brain is largely neuronal

Radioactive cDNA probes prepared from the MBAc39 clone and hybridized to blots of poly $(A)^+$ RNA isolated from brain and liver of adult mouse recognized a 1.6-kb message. This mRNA is expressed abundantly in brain and, at much lower levels (at least 20 times lower), in liver (Fig. 2A). This is in good agreement with previous reports showing that brain contains up to 20-fold higher V-ATPase activity than any other tissue (Perin *et al*., 1991; Südhof & Jahn, 1991) and that the mRNA levels of the corresponding subunits are higher than in any other tissue (Wang *et al*., 1988; Hirsch *et al*., 1988; Nelson *et al*., 1990; Nezu *et al*., 1992). In contrast with the results obtained by Wang *et al*. (1988), we did not detect additional transcripts in either brain or liver, despite the use of poly $(A)^+$ RNA for maximal sensitivity. A similar 1.6-kb band was also observed in Northern blots of brain RNA from postnatal mice of 5 (P5) and 14 (P14) days of age, indicating that the same message was expressed throughout postnatal development and in adulthood. Southern blot analysis of *Eco*RI-digested mouse genomic DNA probed with MBAc39 revealed a single band of $\approx$ 22 kb, which suggests that the corresponding gene is present in the mouse genome either as a single or low frequency copy (data not shown).

In order to know which cells and to what extent they are expressing the Ac39 message, we used *in situ* hybridization in rat brain sections. Subcloned MBAc39 cDNA was used to generate ^{35}S-labelled sense and antisense cRNA probes, which were hybridized to rat brain sections from adult (P60) and at P15, the approximate time when synapses are stabilized (Chun & Shatz, 1988). Binding of the antisense riboprobe was evident in many regions of the brain. Figure 2(B) shows the macroscopic distribution of the Ac39 transcripts in the adult brain. In the olfactory bulb, most binding occurred in the mitral cell layer. In the cerebral cortex, binding showed a laminated distribution in the frontoparietal, retrosplenial and pyriform areas. Hybridization was also evident in the pyramidal cell layer of the hippocampus, the granule cells of the dentate gyrus and cerebellum, and in the thalamic, pontine and deep cerebellar nuclei. Labelling intensity did not correlate with cell density. MBAc39 gene expression was undetectable in fibre tracts (e.g. corpus callosum, fimbria and deep cerebellar white matter).

To achieve cellular resolution in our analysis, we used digoxygenin-labelled riboprobes. They displayed a similar labelling pattern to that of the radiolabelled probes for the two ages studied, i.e. adult and P20 (Fig. 3). In the adult, labelling was moderate to strong in neurons and pinealocytes, but weak to very weak in some glial cells, including the ventricular ependymal cells (Fig. 3B), some hippocampal astrocytes, and corpus callosum oligodendrocytes. No binding was apparent in non-neural cells (e.g. capillary muscle cells or endothelial cells) except for the epidermal cells of the choroid plexus (Fig. 3B) and fibroblasts of the pia mater, which exhibited a moderate to low signal. Striking differences in labelling intensity were apparent in different neuronal types from various adult rat brain regions (Figs 2B and 3A–C, Table 1). The highest labelling occurred in the hippocampal areas CA2–CA3, dentate hilus, mitral cell layer of the olfactory bulb, pyriform cortex, latero-dorsal thalamic nucleus, brain motor nuclei and the deep cerebellar nuclei. Relatively high labelling was detected in the hippocampal subfield CA1, dentate gyrus granule cell layer, substantia nigra pars compacta, Purkinje cell layer, pontine nuclei, layers IV–V of the cerebral cortex, paraventricular nuclei, most of the superior colliculus, and the anterior olfactory nucleus. A moderate hybridization signal was detected in most of the thalamic nuclei, septal nuclei, subiculum, trapezoid body, most of hypothalamic nuclei, striatum, layers II–III and VI of the neocortex, and the granule cell layer of the cerebellum. Low labelling was observed in the amygdaloid nuclei, inferior colliculus, central grey, glomerular cell layer and internal granular cell layer of the olfactory bulb, as well as in stellate/basket interneurons of the molecular cell layer of the cerebellum. Similar results were obtained using both radiolabelled and digoxygenin-labelled probes, and with different animals. In any region that was examined in detail, Ac39 mRNA was found in essentially every neuron.

Expression of Ac39 precedes synaptogenesis

We have studied the developmental regulation of the Ac39 gene in rat brains from age E17 through to adult. Labelling in E17 brain

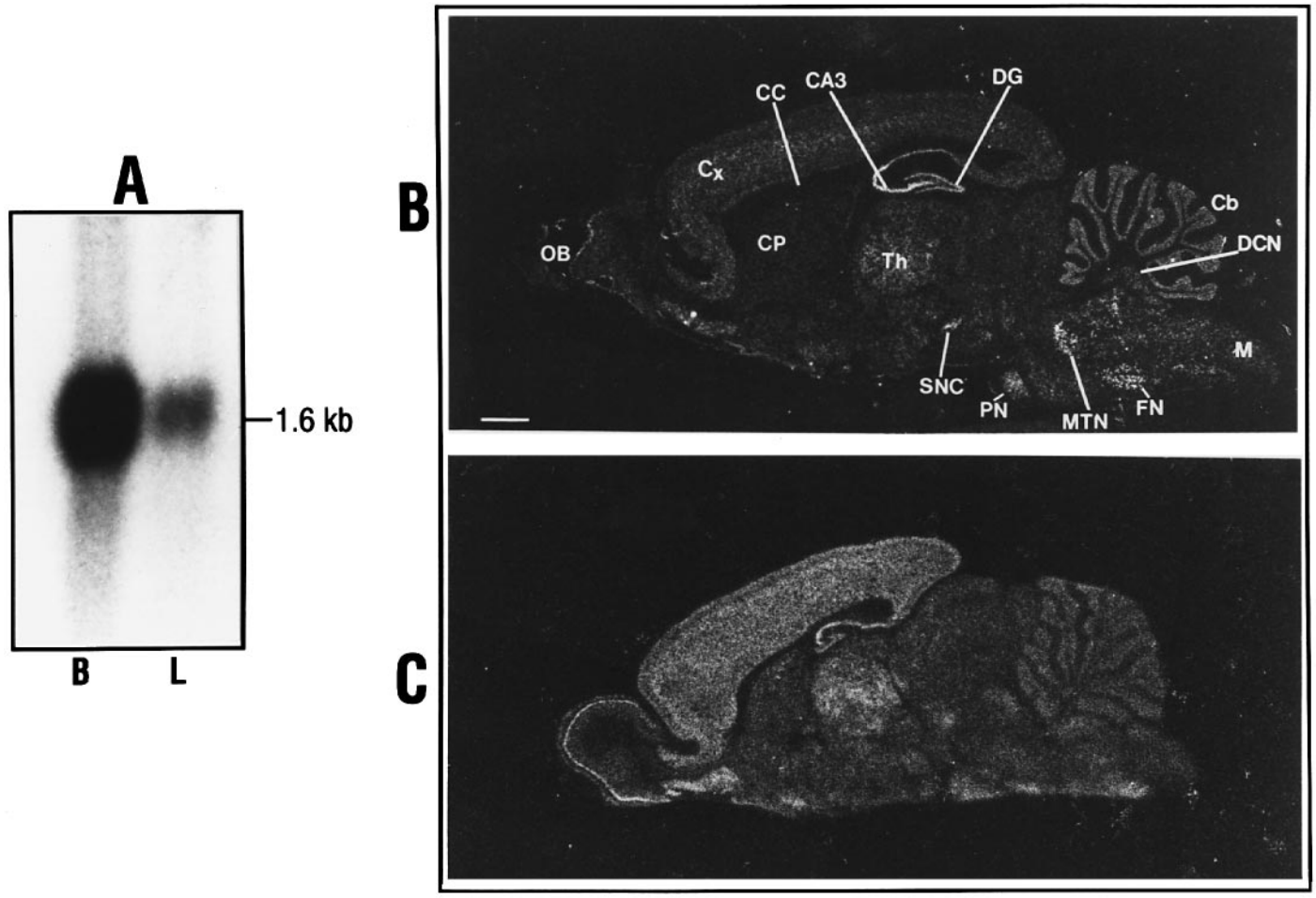

FIG. 2. Gene expression of Ac39/physophilin in rat brain. (A) Northern blot of adult rat. mRNA from brain (lane B) and liver (lane L) were hybridized with a MBAc39 ^{32}P-labelled probe. (B and C) Regional distribution of Ac39/physophilin mRNA in the rat brain. Parasagittal sections of rat brain from adult (B) and P15 (C) were *in situ* hybridized to an antisense ^{35}S-labelled riboprobe complementary to the MBAc39 cDNA and exposed to film for 30 and 10 days, respectively. Adjacent sections hybridized with the sense probe were always found to be below film background (negative control). The hybridization signal is white as the print was made directly from X-ray films. Representative sections are shown. Abbreviations: CA, pyramidal cell layer of Ammon's horn of hippocampus; Cb, cerebellum; Cx, cerebral cortex; CC, corpus callosum; CP, caudate-putamen; DCN, deep cerebellar nuclei; DG, dentate gyrus; FN, facial nucleus; M, medulla; OB, olfactory bulb; PN, pontine nucleus; SNC, substantia nigra compacta; Th, thalamus. Scale bar: 1.64 mm (both panels).

was weak and roughly homogeneous, with some exceptions, which included the mitral cells of the olfactory bulb and the left trigeminal ganglion, both of which display a strong intensity of labelling. Beginning at around E17, labelling in some brain areas became selectively higher, for example in the ventricular zone and the external granular layer of the cerebellum, both of which are germinal layers. By E20, the labelling of the mitral cells was still strong and it increased to moderate in several other regions that included: Ammon's horn of the hippocampus, ventricular zone, cortical plate, granular cell layer of the olfactory bulb, olfactory epithelium, diencephalon (thalamus), pontine nucleus, hypophysis and spinal cord. The final adult pattern of relative staining intensities was established by P5, except for the presence of hybridization in the external granular layer of the cerebellum, which remained until P20, and in the caudate-putamen which exhibited a relatively higher signal at P5 compared to the adult. Generally, Ac39 mRNA levels increased progressively with developmental age reaching a maximum about P15–P20. This increase was observed in all areas of the brain with the exception of the caudate-putamen where labelling at P20 decreased slightly relative to labelling at P5. Brain labelling decreased at ages older than P20, and in the adult it was several times weaker than at P15–P20 (Figs 2B,C and 3).

To study the temporal relationship between expression of Ac39 and synaptogenesis, we then focused in the cerebellum, a structure particularly suited to dissect developmental stages. During rat cerebellar development, the germinal cells of the external granular layer ('undifferentiated' neuroblasts) give rise from E16 to P20–P25 to granule cells that migrate afterwards to constitute the adult granular cell layer where they develop synaptic contacts (Jacobson, 1991). These germinal cells express synaptophysin (classically used as a marker of synaptic vesicles during synaptogenesis) and its mRNA from P1 to at least P15 (Marquèze-Pouey *et al*., 1991; Phelan & Gordon-Weeks, 1992), but conversely, they do not express other synaptic proteins like the GABA receptor (Meinecke & Rakic, 1990). The neuroblasts from the external granular layer express the Ac39 message clearly on E20 and throughout postnatal development until

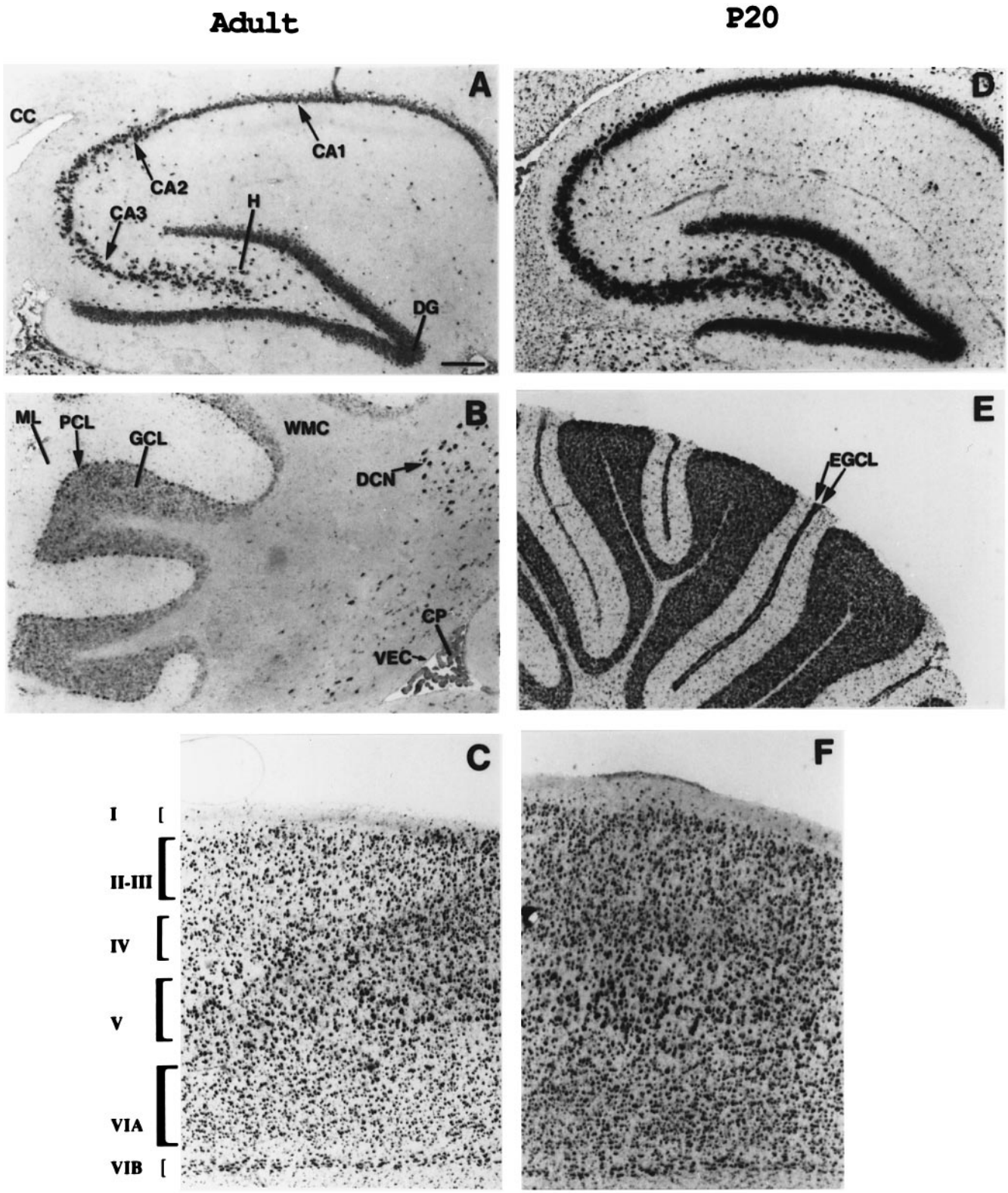

FIG. 3. Cellular distribution of Ac39/physophilin mRNA in rat brain. Parasagittal sections of adult male rat brain were *in situ* hybridized with an antisense digoxygenin-labelled riboprobe complementary to MBAc39 cDNA and developed as described in Experimental procedures. Low-power photomicrographs show cells containing Ac39 mRNA in the: hippocampal formation (A, D), cerebellum (B, E) and neocortex (C, F) for both adult and P20 rats. Adjacent sections hybridized with the sense probe were always found to be above the labelling in the corpus callosum which was considered as the background (negative control). Abbreviations: CA, regions of the pyramidal cell layer of Ammon's horn of hippocampus; CC, corpus callosum; CP, chroroid plexus; D, dentate gyrus; EGCL, external granule cell layer; GCL, granular cell layer; H, hilus of the dentate gyrus; ML, molecular layer; PCL, Purkinje cell layer; VEC, ventricular ependymal cells; WMC, white matter of cerebellum; I-VIB, layers of the cerebral cortex. Scale bar: 276 μm.

TABLE 1. Distribution of Ac39/physophilin mRNAs in different regions of the adult rat brain

Structure	Signal
Telencephalon	
Olfactory bulb	
Mitral cell layer	+ + +
Periglomerular region	+
Internal granular cell layer	+
Neocortex	
Layers IV–V (pyramidal cells)	+ + +
Layers II–III and VI	+ +
Amygdalohippocampal area	+ +
Primary olfactory (piriform) cortex	+ + + +
Nucleus of the lateral olfactory tract	+ + + +
Anterior olfactory nucleus (lateral, dorsal and ventral)	+ + +
Amygdaloid nuclei	+ +
Taenia tecta	+ + +
Anterior commisure	+
Subiculum	+ +
Entorhinal cortex	+ +
Septal nuclei	+ +
Caudate-putamen	+ +
Globus pallidus	+ +
Hippocampal formation	
Hippocampus	
CA1 area	+ + +
CA2-CA3 area	+ + + +
Dentate gyrus	
Granule cell layer	+ + +
Hilus	+ + + +
Diencephalon	
Epithalamus	
Habenular nuclei	+ + +
Thalamus	
Mediodorsal thalamic nucleus	+ +
Anteromedial t.n.	+ +
Paratenial t.n.	+ +
Central medial t.n.	+ +
Reuniens t.n.	+ +
Gelatinous reticular t.n.	+ +
Ventromedial t.n.	+ +
Parafascicular t.n.	+ +
Anterodorsal t.n.	+ + +
Laterodorsal t.n.	+ + + +
Ventrolateral t.n.	+ +
Ventroposterior t.n.	+ +
Hypothalamus	
Medial preoptic area	+ +
Paraventricular nuclei, magnocellular	+ + +
Paraventricular nuclei, parvocellular	+ + +
Bed nucleus stria terminalis	+ +
Anterior hypothalamic area	+ +
Dorsal hypothalamic area	+ +
Dorsomedial hypothalamic nucleus	+ +
Dorsal hypothalamic nucleus	+ +
Arcuate nucleus	+ +
Mammilary nuclei	+ +
Posterior hypothalamic nucleus	+ +
Subthalamic nucleus	+ +
Mesencephalon	
Superior colliculus	
Superficial grey layer	+ +
Intermediate grey layer	+ + +
Deep grey layer	+ + +
Inferior colliculus	+
Central grey	+
Red nucleus	+ +
Ventral tegmental area	+ +
Interstitial magnocellular nucleus of posterior commissure	+ +
Laterodorsal tegmental nucleus	+ +
Substantia nigra, pars compacta	+ + +
Subthalamic nucleus	+ +
Nucleus of the mesencephalic tract of the trigeminal nerve	+ + + +
Rhombencephalon	
Cerebellum	
Cerebellar cortex (Purkinje cell layer)	+ + +
Granular cell layer	+ +
Molecular cell layer (stellate/basket interneurons)	+
Cerebellar nuclei	
Lateral cerebellar nucleus	+ + + +
Medial c.n.	+ + + +
Interpositus c.n.	+ + + +
Cochlear nuclei	+ + + +
Locus coeruleus	+ +
Pons and Medulla Oblongata	
Dorsal raphe nucleus	+ + +
Pontine nucleus	+ + +
Nucleus of Darkchewitsch	+ + +
Trochlear nucleus	+ + +
Pontine reticular nucleus	+ + +
Principal oculomotor nucleus	+ + +
Dorsal tegmental nucleus	+ + +
Gracile nucleus	+ + +
Nucleus of solitary tract	+ + + +
Dorsal motor nucleus of vagus	+ + + +
Hypoglosal nucleus	+ + + +
Gigantocellular reticular nucleus	+ + + +
Paramedial reticular nucleus	+ + +
Reticulotegmental nucleus of the pons	+ + +
Trapezoid body	+ + +
Inferior olive	+ +
Facial nucleus	+ + + +
Motor trigeminal nucleus	+ + + +
Other regions	
Piamatter (fibroblasts)	+ +
Pineal gland (pinealocytes)	+ +
Chroroid plexus (epidermal cells)	+
Ependymal cells	+

These data were compiled after evaluating parasagittal sections from autoradiographic X-ray films and digoxygenin-labelled slides. Based on relative optical density measurements, the hybridization signal above background was scored as low (+), moderate (+ +), high (+ + +), or very high (+ + + +).

P20 (Fig. 3E) in a way that parallels that of synaptophysin. In conclusion, the temporal expression of Ac39 in rat brain, like that of synaptophysin, is predominantly postnatal and slightly precedes synaptogenesis.

Ac39, like subunit A, shows a synaptic immunolocalization

The anatomical location of the protein Ac39 in the rat brain was examined by immunohistochemical analysis using both light microscopy and immunofluorescence. A previously characterized polyclonal antiserum generated against the Ac39 from bovine adrenal medulla was used (Wang *et al*., 1988). Adjacent sections were probed with antibodies against Ac39, synaptophysin and the subunit A of V-ATPase (V_0).

Consistent with our *in situ* hybridization results, anti-Ac39 staining was found in essentially all brain areas, being especially prominent in the olfactory bulb, neocortex, hippocampus and cerebellum. At low magnification, the Ac39 immunoreactivity was mostly concentrated in the neuropil with no appreciable binding to fibre tracts (e.g. corpus callosum, fimbria and deep white matter of the cerebellum) (Fig. 4). At higher magnification, punctate Ac39 immunoreactivity within the neuropil was the predominant pattern of staining, although immunoreactive perikarya were also detected in some neurons (e.g. Purkinje cells, mitral cells, deep cerebellar

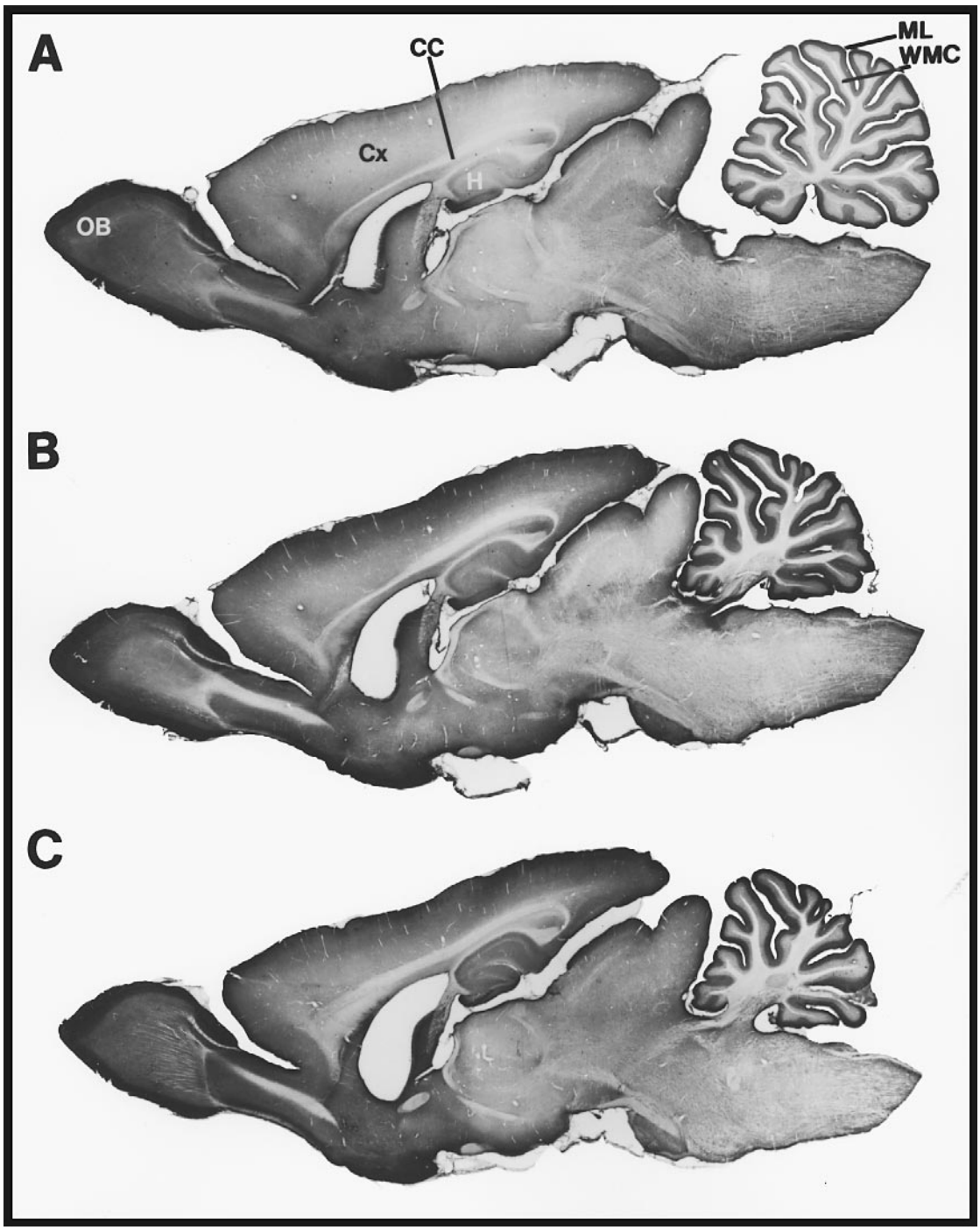

FIG. 4. Ac39/physophilin immunoreactivity in rat brain. Parasagittal sections probed with antibodies against Ac39 (A) were compared with those probed with anticatalytic subunit A (B) and synaptophysin (SY-38) (C). Abbreviations: Cx, cortex; H, hippocampus; ML, molecular layer; OB, olfactory bulb; WMC, white matter of the cerebellum. Scale bar: 1.81 mm (all panels).

nuclei and facial nucleus; data not shown). Some non-neuronal cells, known to exhibit considerable secretory activity, also displayed weak to moderate immunoreactivity (e.g. pinealocytes, epidermal cells of the choroid plexus, fibroblasts of the pia mater and ependymal cells), in good agreement with our *in situ* hybridization results. In the olfactory bulb, the higher immunoreactivity was concentrated in the glomeruli and the perikarya of the mitral cells. In the cerebellum, most of the immunoreactivity was concentrated in the molecular layer and the perikarya of Purkinje cells which occasionally displayed staining of some dendritic shafts. In the granule cell layer of the cerebellum, the overall immunoreactivity was weaker and exhibited a less homogeneous distribution of staining when compared with the molecular layer. In the forebrain, staining also occurred in the neuropil with slightly higher immunoreactivity found in the neuronal perikarya.

The immunoreactivity pattern displayed by a previously characterized antibody against the catalytic subunit A of the V-ATPase (Moriyama & Nelson, 1989), was identical to that of Ac39 at all

magnifications used and in all brain regions (Fig. 4A,B). This result is consistent with mounting evidence which strongly suggests that Ac39 is a constitutive subunit of the V-ATPase complex. An identical punctate pattern was also observed with this antibody at high magnifications. A sharper punctate pattern was observed when anti-synaptophysin antibodies were used (data not shown). The immunostaining pattern of synaptophysin, in contrast with the immunostaining patterns of Ac39 and subunit A, was restricted to the neuropil with no staining detected in the neuronal perikarya (Fig. 4C), which is in agreement with previous reports (Wiedenmann & Franke, 1985; Jahn *et al*., 1985; Navone *et al*., 1986).

Ac39 completely colocalizes with subunit A and partially colocalizes with synaptophysin

To study the possible colocalization of Ac39 with subunit A and synaptophysin we have performed double indirect immunofluorescence (Fig. 5). In comparison with the immunoperoxidase technique, described in the previous section, immunofluorescence resulted in higher background for anti-Ac39 and antisubunit A. In agreement with the light microscopy results, the Ac39 pattern of immunofluorescence is concentrated in the neuropil and exhibits moderate labelling within the neuronal perkarya. This pattern is also consistent with the pattern observed in our *in situ* hybridization experiments. Ac39 antigenicity completely colocalized with that of subunit A (Fig. 5A,B), and a partial colocalization of these two proteins and synaptophysin was observed in all the brain regions (Fig. 5C–F). Consequently, synaptophysin pattern appears as a subset of that of Ac39 (or subunit A), which is restricted exclusively to the neuropil with no appreciable signal in the perikaryon. In summary, for all regions of the brain examined, we found that the Ac39 immunoreactivity completely colocalized with that of subunit A and presented a partial colocalization with synaptophysin. These results are consistent with a selective concentration of Ac39, subunit A and probably the whole V-ATPase in the synaptic terminals.

Discussion

Sequence of the synaptic vesicle Ac39

The Ac39/physophilin identity was recently assumed based on microsequencing data (Siebert *et al*., 1994). Nevertheless, our results show that the sequence of physophilin from mouse brain contains a region of 12 amino acids which differs from that of the Ac39 sequence previously reported from bovine adrenal medulla (Wang *et al*., 1988). Based on RT-PCR cloning of the bovine adrenal medulla homologue, we correct the sequence of this region and demonstrate the complete identity of both proteins. Therefore, there is no evidence at present for the existence of Ac39 isoforms, in contrast with the situation for other V-ATPase subunits (reviewed by Kibak *et al*., 1992) and most of the synaptic vesicle membrane proteins (reviewed by Jahn & Südhof, 1994). Furthermore, considering that the bulk of brain V-ATPase activity seems to be concentrated in synaptic vesicles (Perin *et al*., 1991; Südhof & Jahn, 1991), and since we found only one sequence of Ac39 in the brain, it is likely that this sequence represents the Ac39 of the synaptic vesicle V-ATPase.

Coexpression of Ac39 with subunit c and synaptophysin

The distribution of Ac39 mRNA in brain does not correlate with the pattern of glucose utilization (Sokoloff *et al*., 1977), or with the distribution of known neurotransmitters (Emson, 1983). We found that the postnatal expression of Ac39 matches the time course of appearance of Mg^{2+}-dependent proton-pumping ATPase activity in brain (Kish *et al*., 1989) and is virtually identical to that of subunit c (Numata *et al*., 1995). Therefore, this expression pattern is most likely to represent that of the V-ATPase as a whole and, moreover, suggests a coordinated transcription of the V-ATPase subunits, which would assure the availability of the different subunits during the assembly of the complex.

The gene expression of both Ac39 and subunit c is widespread over the rat brain and largely neuronal, with nearly no detectable amounts of mRNA present in glial cells. However, since glia also possess V-ATPase (Pappas & Ransom, 1993), as expected for any eukaryotic cell, it follows that neurons must contain much higher levels of V-ATPase mRNA than glia. Therefore, considering that neurons are very active secretory cells which use the bulk of their V-ATPase to energize the uptake/storage of neurotransmitters in their synaptic vesicles (reviewed in Nelson, 1991), the expression pattern of Ac39 is likely to reflect differences in the secretory activity between different neurons of the brain.

Moreover, we found that the spatio-temporal expression of Ac39 is also strongly correlated with that of synaptophysin (Table 2, Marquèze-Pouey *et al*. 1991). Since both messages are present in essentially every neuron of the brain, it follows that Ac39 and synaptophysin genes must coexpress within the same neurons. This correlation between the *in situ* hybridization patterns of Ac39, subunit c and synaptophysin is also remarkably strong during the brain synaptogenesis. In particular, the expression of these proteins in the external granular layer of the cerebellum (Numata *et al*., 1995; Marquèze-Pouey *et al*., 1991) is consistent with mounting evidence for the existence of a vesicular system in immature neurons that is ready to store and release neurotransmitter, and to undergo exo-endocytotic cycles, independently of extracellular calcium (Young & Poo, 1983; Matteoli *et al*., 1992). The presence of Ac39, subunit c and synaptophysin, in every neuron surveyed, at those presynaptogenic stages suggests that these are very basic components of the vesicular machinery, and it is also consistent with the hypothesis that these proteins are components of the exocytotic fusion pore. To our knowledge, this is the first report of a strong correlation between expression patterns of synaptic vesicle proteins (see Südhof, 1995). Future research may identify common transcription factors that might coordinate the spatio-temporal expression of V-ATPase and synaptophysin genes, which would be a mechanism to ensure the concomitant availability of basic components, functionally related, of the vesicular machinery.

No similar matching is observed with the expression pattern of any other synaptic vesicle protein including the synaptophysin homologue synaptoporin (Marquèze-Pouey *et al*., 1991), synaptobrevins I and II (Trimble *et al*., 1990), rab3 A (Stettler *et al*., 1992), SNAP-25 (Oyler *et al*., 1989), syntaxin 1B/GR33 (Smirnova *et al*., 1993), SV2 (Bajjalieh *et al*., 1994), chromogranin A (Lahr *et al*., 1990), synapsin I (Melloni *et al*., 1993), synaptotagmin/p65 (Mahata *et al*., 1992; Marquèze *et al*., 1995), β-SNAP (Kato, 1990; Whiteheart *et al*., 1993), secretogranin II and chromogranin B (Mahata *et al*., 1992). Although the bulk of synaptobrevin II is likely to be associated with synaptophysin and V_0 in synaptic terminals (Galli *et al*., 1996), its gene expression appears not to be coordinated as tightly as that of the other elements of the complex, i.e. synaptophysin and V_0 (Ac39, subunit c).

Distribution of Ac39 immunoreactivity in brain: comparison with previous studies

In contrast to a previous study (Galli *et al*., 1996), we found anti-Ac39 (and anti-subunit A) immunoreactivity in rat brain sections

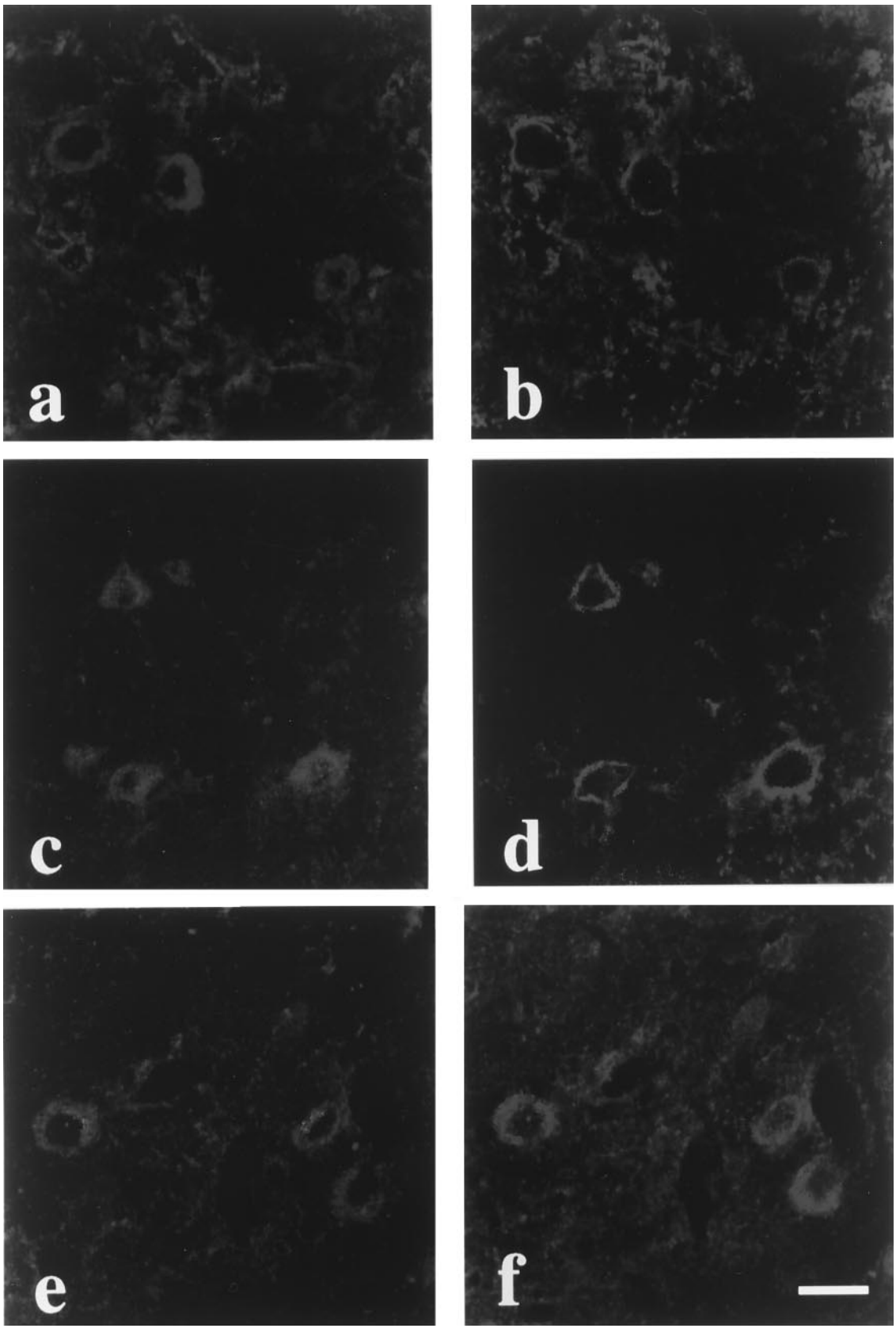

FIG. 5. Immunofluorescence localization of Ac39/physophilin in rat brain. Double immunofluorescence micrographs comparing the distributions of Ac39, catalytic subunit A and synaptophysin in parasagittal sections of cerebellar deep nuclei. Brightly immunoreactive puncta that outline perikarya and dendrites represent nerve terminals positive for both antigens. Double labelling for Ac39 (A) and synaptophysin (SY-38) (B). Double labelling for subunit A (C) and synaptophysin (SY-38) (D). Part of the Ac39 and subunit A immunoreactivity colocalized with synaptophysin immunoreactivity. Double labelling for Ac39 (E) and subunit A (F) showing a complete colocalization of both antibodies. Scale bar, 29 μm.

TABLE 2. Comparative location of Ac39 and synaptophysin mRNAs in adult rat brain

Structure	Ac39/physophilin	Synaptophysin
Olfactory bulb		
Periglomerula	+	+ +
External plexiform layer	+	+ +
Mitral cell layer	+ + +	+ + +
Internal granule cell layer	+	+
Neocortex		
Layers II–III and VI	+ +	+ +
Layers IV–V (pyramidal cells)	+ + +	+ + +
Primary olfactory cortex	+ + + +	+ + + +
Hippocampus		
Pyramidal cell layer		
CA1	+ + +	+ + +
CA2-CA3	+ + + +	**+ + + +**
Dentate gyrus		
Granule cell layer	+ + +	+ + +
Hilus	+ + + +	+ + + +
Amygdala	+ +	+ +
Amygdalohippocampal area	+ +	+ +
Striatum		
Caudate-putamen	+ +	+
Globus pallidus	+ +	+
Septum	+ +	+ +
Thalamus	+ + +	+ + +
Hypothalamus	+ +	+ +
Epithalamus		
Medial habenula	+ + +	+ + +
Superior colliculus	+ +	+ +
Inferior colliculus	+ +	+ +
Substantia nigra compacta	+ + +	**+ + +**
Cerebellum		
Molecular cell layer	+	**+**
Purkinje cell layer	+ + +	+ + +
Granular cell layer	+ +	**+ +**
Deep cerebellar nuclei	+ + + +	+ + + +
Pontine nucleus	+ + +	+ + +

Data compiled for Ac39 mRNA (this work) and for synaptophysin mRNA (Marquèze-Pouey *et al*., 1991). In order to do comparative analysis, data on relative levels of synaptophysin mRNA were re-scaled to four levels of signal intensity based on data and pictures from the original paper. Symbols are the same as in Table 1. Bold is used to indicate data coming from the reinterpretation of the original pictures, either when the brain structure was not explicitly specified or when our assigned relative value was in slight discrepancy with the original interpretation.

using both immunoperoxidase and immunofluorescence techniques. The reasons for this discrepancy are unclear.

Ac39 immunoreactivity partially colocalized with that of synaptophysin being concentrated in the neuropil with an additional presence of Ac39 in the neuronal bodies. This is consistent with a predominant location of Ac39 (subunit A and likely the whole V-ATPase) in the synaptic terminals. Our results agree with several previous observations which include: (i) V-ATPase activity of the brain is mostly concentrated in the synaptic vesicles (Perin *et al*., 1991; Südhof & Jahn, 1991); (ii) Ac39 is predominantly found in the synaptic vesicles after subcellular fractionation, with a lower concentration observed in the synaptic plasma membrane (Siebert *et al*., 1994); and (iii) Ac39 and synaptophysin are major components of the synaptic vesicle membranes, where they are found in approximately equimolar amounts (Floor & Leeman, 1988; Floor *et al*., 1990).

The partial colocalization of Ac39, and subunit A, with synaptophysin is similar to that of subunit B and synaptobrevins (I + II) recently reported (Galli *et al*., 1996). Subunit c has also been shown to display a similar distribution in Torpedo brain with prominent immunoreactivity in synaptic vesicles and a minor labelling in the synaptic plasma membrane (Brochier & Morel, 1993). Our immunohistochemical findings are also in good agreement with a recent report showing the existence of a synaptobrevin II–synaptophysin-V_0 complex (Galli *et al*., 1996). It must be considered that, although subunits A and B (from the V_1 sector) do not belong to this complex, at the level of resolution of the light microscope no difference in immunolabelling pattern is expected between the whole (V_0-V1) and the dissociated (V_0, V_1) V-ATPase at the nerve terminals, since the two V-ATPase sectors are supposed to remain in the terminal upon its hypothetical dissociation (Nelson, 1993; see below). Future electron microscopy may provide more insight on this issue.

A putative role for Ac39

Our findings that Ac39 is nearly identical to some putative peptides from the gap junctions and the previously reported subunit c/mediatophore/ductin identity suggest the interesting possibility of an additional role for some of the V_0 sectors of the V-ATPase in the formation of a channel-like fusion pore. This additional role of V_0 sectors in neurotransmitter release has been already hypothesized based on the reported excess of dissociated V_0 sectors in synaptic vesicles (Nelson, 1993). However, there are two pieces of evidence against this model. First, specific V-ATPase inhibitors did not affect membrane fusion or short-term transmitter release in mast cells (A. Oberhauser and J. M. Fernandez, personal communication). Second, the synaptobrevin–synaptophysin-V_0 complex is much more abundant than the calculated abundance of docked vesicles (De Camilli, 1995).

Our *in situ* hybridization findings suggest that the V_0 sector and synaptophysin work in coordination, in a complex linked by Ac39, to perform basic functions of the vesicular machinery. We propose that an alternative role for the synaptobrevin–synaptophysin-V_0 complex is the inactivation of the V-ATPase prior to exocytosis, which appears to be a prerequisite based on cell economy. We speculate that synaptophysin and V-ATPase may form a basic scaffold in the synaptic vesicle, and the binding of synaptobrevin to synaptophysin would trigger a conformational change in Ac39 resulting in the detachment of the V_1 sector from the V_0 sector with the concomitant inactivation of the V-ATPase, prior to the opening of the vesicle to the extracellular space.

The regulation of the V-ATPase by its dissociation has been already described (Sumner *et al*., 1995). Such a regulatory mechanism may prevent the V-ATPase from wasting ATP hydrolysis energy since, upon exocytotic membrane fusion, the vesicle would no longer be a closed compartment and then the generation of a proton gradient across the membrane would be virtually impossible. Ac39 is a good candidate to mediate this process through the dissociation of V_1 and V_0 sectors, which is the reverse reaction of its postulated assembling role during V-ATPase biogenesis (Myers & Forgac, 1993). In order to be compatible with the protein abundance of the synaptobrevin I–synaptophysin-V_0 complex, one must assume the occurrence of this inactivation in a predocking step.

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Abbreviations

PB	phosphate buffer
PBS	phosphate buffered saline
SNAP	synaptosome-associated protein
V-ATPase	vacuolar ATPase

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