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A third synaptotagmin gene, *Syt3*, in the mouse

(calcium/central nervous system/exocytosis/synaptic vesicles/PC12 cells)

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ABSTRACT **The synaptotagmins are integral membrane proteins of synaptic vesicles thought to serve as Ca^{2+} sensors in the process of vesicular trafficking and exocytosis. Results from antibody microinjection and gene-disruption experiments have led to a controversy over whether synaptotagmins are essential for neurotransmission. However, the studies casting doubt on the role of synaptotagmins have assumed that no further isoforms of these molecules exist. Here we report the isolation of a third member of the synaptotagmin family (*Syt3*) from mouse brain. Although retaining the characteristic five-domain structure of the other synaptotagmins, SYT3 is considerably more divergent at the level of amino acid sequence. In the most highly conserved C2 domain, the mammalian synaptotagmins, SYT1 and SYT2, share 88% sequence identity, whereas SYT3 has only ≈45% identity to either. Overall, SYT3 has the greatest sequence identity with rat SYT2 and marine ray p65A (both 37%), although homology to all of the known synaptotagmins is >30%. However, SYT3 is most like p65C when comparing domain structure. *Syt3* is expressed in many regions of the nervous system but is undetectable in extraneural tissues. The three murine synaptotagmins have differential expression patterns in the brain. Furthermore, in PC12 cells, *Syt3* is coexpressed with *Syt1* and is more abundant than the latter. This result suggests that individual neurons may have specific combinations of synaptotagmins that could provide for diversity in vesicular release.**

The Ca^{2+}-dependent release of neurotransmitters and neuropeptides from the presynaptic nerve terminal occupies a central role in synaptic transmission (1). The basic events involved in neurotransmitter release include the mobilization, docking, and fusion of synaptic vesicles with the presynaptic membrane. Although these steps use many molecules and processes that are ubiquitous components of general membrane trafficking, the mechanism underlying the Ca^{2+}-evoked release characteristic of neurons is incompletely understood. However, at least some of the molecules that provide for Ca^{2+}-dependent vesicular release are thought to be located on the surface of the synaptic vesicle, where they might mediate membrane fusion after stimulation (2).

Attractive candidates for the Ca^{2+} sensor are members of the synaptotagmin (p65) family (3–6). The synaptotagmins are integral membrane proteins of synaptic vesicles and chromaffin granules and are found only in neural and neuroendocrine tissues (3, 6). The hallmark of these proteins is a large cytoplasmic domain containing two internal repeats that have homology to the C2 regulatory region found in several isoforms of protein kinase C (5, 7) and phospholipase A_2 (8). The C2 domains of these proteins constitute a Ca^{2+}- and phospholipid-binding motif that in synaptotagmin are thought to function both in Ca^{2+} sensing and as a component of the fusion machinery (9). Several studies suggest that the C2 repeats of synaptotagmins are essential for its function. (*i*) Microinjection of antibodies directed against the cytoplasmic domains of synaptotagmins significantly reduces depolarization-induced catecholamine release in PC12 cells (10). (*ii*) Injection of C2 domain-derived peptides into squid giant presynaptic nerve terminals inhibits evoked neurotransmitter release (11). Although these studies established that synaptotagmins can play a role in neurosecretion, there has been a debate as to whether their function is indispensable for neurotransmission. For example, clones of PC12 cells that are deficient for synaptotagmins still release catecholamines (12). Furthermore, mutations in *Drosophila* (13) and *Caenorhabditis elegans* (14) that perturb the synaptotagmin (*Syt*) locus result in a compromise of neural function, although some neurotransmission still occurs. However, in other genetic studies in *Drosophila* where the *Syt* locus was disrupted, synaptotagmin was essential for Ca^{2+}-dependent release (15). One explanation for these discrepancies may reside in the distinction between spontaneous and evoked release of neurotransmitter. However, another explanation would be the existence of additional synaptotagmin family members.

Molecular cloning has identified two synaptotagmin genes in mammals [(3, 5), referred to here as *Syt1* and *Syt2*], although a third gene is known in the marine ray (6). Synaptotagmin homologs have been identified in species from *C. elegans* (14) to human (16), and sequence comparisons show that the domain structure of all synaptotagmins has been conserved, especially within the C2 region. For example, the rat *Syt1* and *Syt2* genes encode proteins that share an 88% identity at the amino acid level across both C2 repeats (3), and rat and *Drosophila Syt1* homologs maintain a 78% identity in this region (16). The high degree of conservation of the synaptotagmin structure throughout evolution indicates that members of this gene family perform fundamental and conserved functions in neurosecretion.

Here we report the cloning of a cDNA encoding a third synaptotagmin isoform, *Syt3*, in mouse.† The deduced amino acid sequence of this member shows that SYT3, while retaining the domain structure of all previously characterized synaptotagmins, is the most divergent synaptotagmin known. The identification of a more structurally divergent synaptotagmin has a number of important implications for the possible mechanism of action of this class of synaptic protein in neurotransmitter release.

MATERIALS AND METHODS

Northern Blot Analysis. Total RNA was prepared from frozen tissues or cells using the RNAzol B method (17). RNA samples (5 μg) were electrophoresed in 0.8% agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized with ^{32}P-labeled cDNA probes, as described (18).

Probe Preparation. Probes for murine *Syt1*, *-2*, and *-3* were generated by PCR and subcloned into Bluescript II KS+

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Abbreviation: CNS, central nervous system.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U10355).

(Stratagene). Adult mouse brain cDNA was obtained by reverse transcription of RNA using oligo(dT) as primer employing the Superscript system (GIBCO/BRL). Nondegenerate primer pairs were used to amplify a portion of each *Syt* cDNA corresponding to the amino acid positions as follows: *Syt1*, 55–61 and 261–267; *Syt2*, 108–115 and 293–299, and *Syt3*, 212–218 and 362–368. Oligonucleotide primers (100 pmol) were used in a final reaction volume of 100 μl containing an aliquot of cDNA following the recommendations of the manufacturer. Cycling conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec (25 times) with a final 72°C for 10 min using the Perkin–Elmer system 9600 thermal cycler.

PCR Amplification of *Syt3*. Degenerate oligonucleotide primer pairs were designed to amplify cDNAs encoding potential C2 domains present in synaptotagmins by PCR using 100 pmol in a reaction volume of 100 μl: C2.1, 5′-ATACGGATCCA(T/C)(A/C)CNTA(T/C)(T/C)T; C2.2, 5′-ATAGGAATTCCA(A/T)(A/G)GGACC(A/C/G)AAC(A/G)TC. Restriction sites for *Bam*HI and *Eco*RI were included to facilitate subcloning and are underlined; degenerate positions are in parentheses. Cycling conditions used were 94°C for 5 min, 42°C for 1.5 min, and 72°C for 1.5 min (once); 94°C for 1 min, 45°C for 1.5 min, and 72°C for 1.5 min (three times); and 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min (25 times).

Library Screening. cDNA was prepared from 5 μg of poly(A)$^+$ mRNA isolated from adult mouse brain and introduced into the Uni-ZAP XR vector (Stratagene). Approximately 5×10^4 recombinants were screened using the *Syt3* PCR probe and two related clones, p122 (1.5 kb) and p171 (4.0 kb), were isolated and sequenced on both strands. Comparison of the nucleotide sequences of these clones revealed an exact match in the overlapping region. DNA sequencing reactions were done with the Prism cycle sequencing kit (Applied Biosystems) and electrophoresed and analyzed on an Applied Biosystems 373 DNA sequencer. The p171 clone contained a single open reading frame and coupled transcription/translation reactions with this clone resulted in the synthesis of a 47.5-kDa protein, as predicted from the proposed initiator methionine (data not shown).

Data Base Searches and Protein Alignments. Data base searches were done by using the TFASTA algorithm (19), and analysis of sequence relatedness and protein alignments were done with the GAP and PILEUP programs (version 7.3, Genetics Computer Group, Madison, WI). The symbol comparison table used for assigning amino acid relatedness was as described in ref. 20.

RESULTS

Cloning and Structure of Murine Synaptotagmin 3. Degenerate oligonucleotide primer pairs were designed to C2 domains to amplify cDNAs encoding unusual synaptotagmins by PCR. One primer pair (C2.1 and C2.2) amplified a 450-bp fragment that appeared to encode a C2 domain. This 450-bp cDNA was used as a probe to screen an adult mouse brain cDNA library (see *Materials and Methods*). Two clones were identified that contained significant homology to sequences encoding the C2 repeats present in synaptotagmin (5). The longest clone, p171 (4.0 kb), contained a single open reading frame predicted to encode a protein of 425 amino acids (Fig. 1).

The predicted amino acid sequence of the p171 clone and its overall structure identified the protein as another member of the synaptotagmin family, which we designate SYT3. Synaptotagmins are defined by a characteristic primary structure consisting of five domains (21). These include an N-terminal domain with low sequence conservation among members, a single hydrophobic stretch of 25–27 amino acids, a spacer region of variable length, the highly conserved C2 domain, and a C-terminal tail. Kyte–Doolittle analysis of SYT3 identified a single hydrophobic region of 25 amino acids near the N terminus (Fig. 2*A*). This potential transmembrane region in SYT3 is preceded by a short stretch of 13 amino acids with no significant homology to N-terminal sequences in other synaptotagmins. The hydrophobic domain is followed by a 109-amino acid stretch containing several serine/threonine-rich clusters. The C-terminal half of SYT3 contains two internal repeats that are highly homologous to the C2A and C2B repeats found in all synaptotagmins and rabphilin 3A, a rab3A-binding protein (22) and to the single C2 regulatory domain of several protein kinase C isoforms (7). Because SYT3 possesses a hydrophobic domain, it is unlikely to be an additional rabphilin member because rabphilin 3A lacks a transmembrane domain. Therefore, the overall domain organization characteristic of the synaptotagmins is conserved in SYT3.

Data base searches using the TFASTA algorithm (19) and subsequent protein alignments show that SYT3 is most closely related to synaptotagmins. The overall identity of the amino acid sequences composing the C2 repeats of SYT3 was highest when compared with other synaptotagmins (43–47%) than to either rabphilin 3A (37%) or protein kinase C isoforms (27–29%). The homology of SYT3 to the synaptotagmin family extends outside of the C2 domain, as significant identity was found in the conserved C-terminal tail region. Comparison of the amino acid sequence for the C2 domain and C-terminal tail of murine SYT3 to those of synaptotagmins from rat (SYT1 and -2) and marine ray (p65A, -B, and -C) is shown in Fig. 2*B*. Only limited sequence homology was detected outside of the C-terminal half of SYT3 when compared with other synaptotagmins. The overall sequence identity of SYT3 to these synaptotagmins is as follows: SYT1, 31%; SYT2, 37%; p65A, 37%; p65B, 34%; p65C, 34%. By comparison, SYT3 shares a 28% identity overall to rabphilin 3A. Sequence comparisons were also done between SYT3 and murine SYT1 and -2. cDNAs corresponding to portions of SYT1 and -2 were subcloned and sequenced from the mouse (see *Materials and Methods*). Murine SYT1 and -2 are highly homologous to their rat counterparts (>95% overall amino acid identity) and are clearly distinct from SYT3. Thus, in the absence of functional data, these results suggest that SYT3 represents a third, highly divergent member of the synaptotagmin family in rodents.

Expression of *Syt1*, *-2*, and *-3* mRNA in Mouse Tissue and PC12 Cells. The synaptotagmins are vesicle proteins that are expressed only in neural and neuroendocrine tissues (3, 4, 6). To determine whether *Syt3* is expressed in a similar manner, the distribution of *Syt* transcripts in various tissues was analyzed by Northern blot (Fig. 3). A cDNA probe specific for *Syt3* recognized a single transcript of ≈4.5 kb in brain tissue. *Syt3* mRNA was absent from all other tissues examined, including adrenal gland, but was present as an abundant transcript in PC12 cells, a cell type that harbors both catecholamine-containing dense-core vesicles and small synaptic vesicles. The brain-specific restriction of *Syt3* is similar to that found for *Syt2* (Fig. 3 and ref. 3), although no expression of *Syt2* could be detected in PC12 cells as reported (12). Analysis of *Syt1* expression revealed an abundant transcript in brain and a low level of expression in adrenal tissue (Fig. 3). Surprisingly, in comparison with *Syt3*, *Syt1* was expressed at very low levels in PC12 cells (Fig. 3). These data indicate that *Syt3* is expressed in a neural-specific manner in mouse tissue and represents the most abundant synaptotagmin mRNA in PC12 cells.

***Syt* Gene Expression in Mouse Central Nervous System (CNS).** The distribution of *Syt* transcripts in both the rat and marine ray nervous systems indicates that members of this gene family are expressed in a complementary and restricted manner (3, 6). To examine whether *Syt3* is expressed coor-

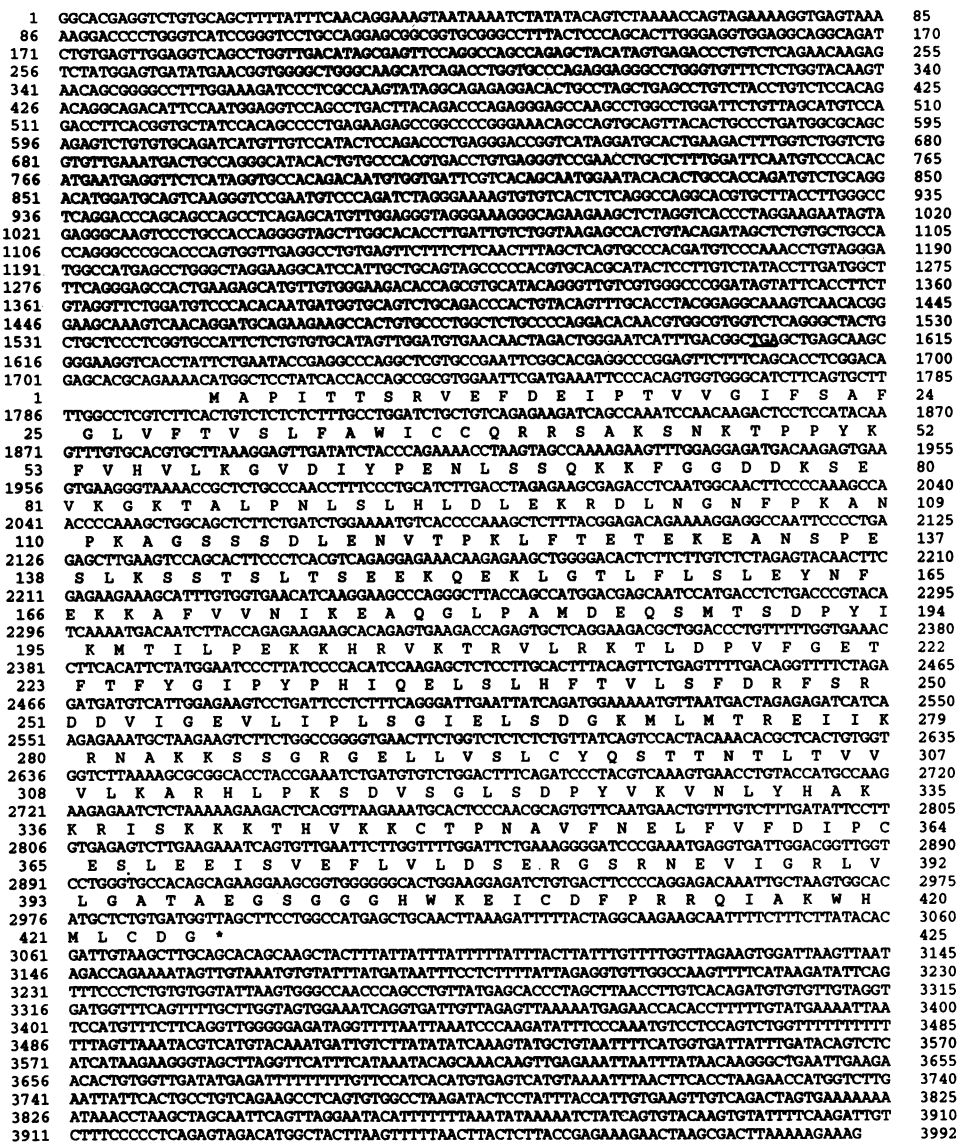

FIG. 1. Nucleotide and predicted amino acid sequence of murine *Syt3*. Nucleotides for the *Syt3* cDNA are numbered beginning with position 1, and the predicted amino acids are numbered beginning with the proposed initiator methionine codon. The first in-frame stop codon preceding the open reading frame in the 5′ untranslated region is underlined, and the 3′ terminator is indicated by a star.

dinately with either *Syt1* or *Syt2*, the distribution of *Syt* transcripts in the mouse CNS was determined by Northern analysis. As shown in Fig. 4*A*, the *Syt1* probe hybridized to a major transcript of 5.0 kb and, to a lesser extent, to a transcript of ≈7.0 kb, which is most likely due to cross-hybridization to *Syt2* (compare Fig. 4 *A* and *B*). *Syt1* expression was highest in forebrain and diencephalon and was detectable in all brain regions examined, as reported previously in the rat CNS (3). The *Syt2* probe hybridized to a 7.0-kb transcript that was expressed predominantly in caudal brain regions, with highest levels being found in brain stem (Fig. 4*B*). An additional transcript of ≈3.5 kb was detected in cerebellum, which did not correspond to either *Syt1* or *Syt3* and may represent either a cerebellar-specific alternatively spliced form of *Syt2* or a closely related transcript. In contrast to the differential pattern of expression observed for *Syt1* and *Syt2*, *Syt3* transcripts were detected in most brain regions and at rather uniform levels (Fig. 4*C*). The global distribution of SYT3 mRNA demonstrates that the expression of this divergent synaptotagmin homolog overlaps with that of SYT1 and SYT2 and implies a more general utilization of the *Syt3* gene product in the CNS.

DISCUSSION

Our results establish that the murine synaptotagmins are encoded by a multigene family consisting of at least three members, *Syt1*, *-2*, and *-3*. The identification of a third gene in rodents demonstrates that the level of complexity of this gene family has been maintained during the evolution of marine vertebrates to mammals because three synaptotagmin homologs have been described in the marine ray *Discopyge ommata* (6). The degree of sequence similarity between synaptotagmins at the amino acid level, especially in the region encompassing the Ca^{2+} and phospholipid-binding C2 domain, suggests that this family consists of two closely related members (SYT1/SYT2 in mammals and p65A/p65B in the marine ray) and a third, more distantly related member (SYT3 in mammals and p65C in the ray). Based on overall amino acid sequence conservation, SYT3 is more related to p65A than it is to p65C. However, the domain structure of SYT3 is more like p65C than any other known synaptotagmin. For example, compared with other synaptotagmins, both SYT3 and p65C contain a long cytoplasmic loop separating the C2 domain from the hydrophobic region (109 and 149 amino acids for SYT3 and p65C, respectively, vs. 57 and 51

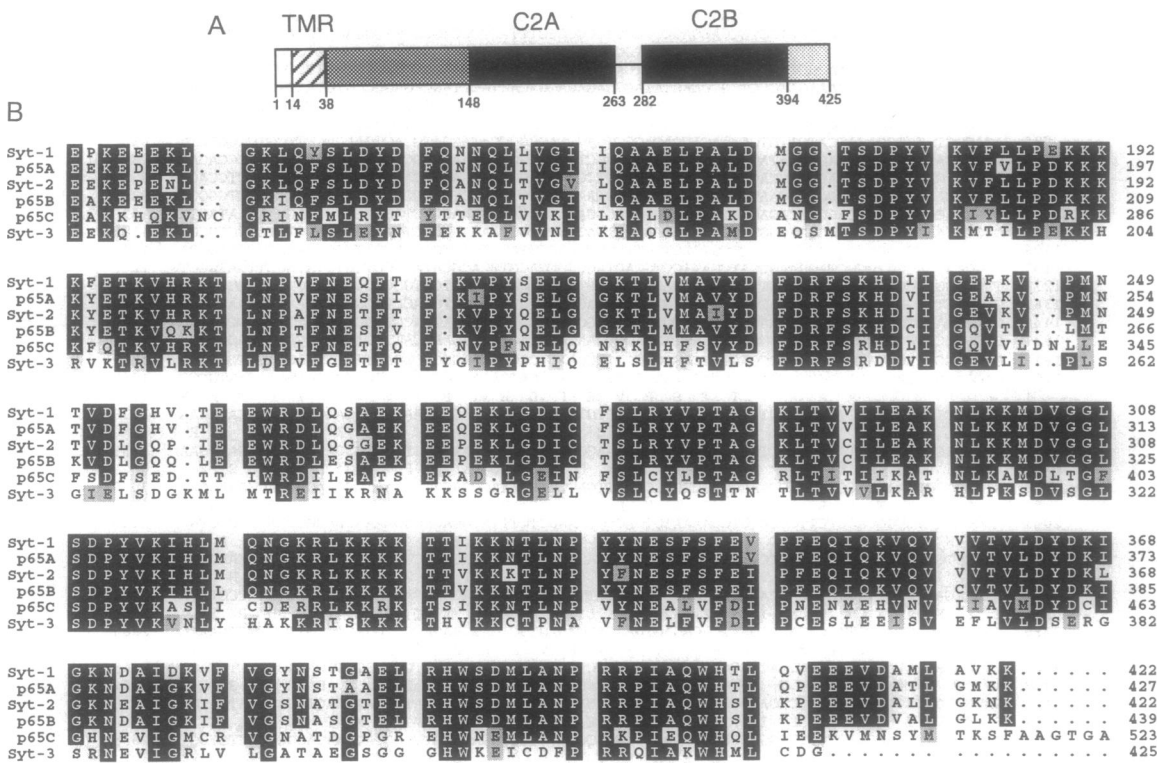

FIG. 2. Domain structure of murine SYT3 and alignment of C2 domain with rat and marine ray synaptotagmins. (*A*) Schematic representation of the overall structure of SYT3 is shown; boxes denote domains identified in synaptotagmins (21). The N-terminal region is shown as an open box followed by a proposed transmembrane region (TMR) indicated by diagonal bars. The two repeats (C2A and C2B) composing the C2 domain are shown as black boxes. An intervening region between the TMR and the C2 domain is indicated with a dark-stippled background, and the C-terminal domain is shown by a light-stippled background. Amino acid positions defining the domain boundaries are below. (*B*) Sequence comparison of C2 domains of rat and marine ray synaptotagmin isoforms with mouse SYT3. Alignment of amino acid sequences was generated by using PILEUP. Identical residues in four of six sequences are shown with black background; conservative amino acid changes are shown with dark gray shading, and moderately conservative amino acid changes are shown with light gray shading, using threshold values of 1.0 and 0.5, respectively (21).

amino acids for p65A and p65B, respectively). In addition, the hydrophobic region is highly conserved among SYT1/SYT2 and p65A/p65B homologs, whereas this conservation is not found in either SYT3 or p65C. Although the hydrophobic domain of SYT3 is most related to that of p65C, the level of amino acid identity in this region or in other conserved domains is not sufficient to establish whether *Syt3* is the mammalian homolog of p65C and leaves open the possibility that other synaptotagmin family members await identification.

The existence of multiple synaptotagmin isoforms raises a number of issues. The first is whether a given neuronal cell type contains a single, or several, form(s) of synaptotagmin. Analysis of grossly dissected brain regions reveals that while each synaptotagmin isoform has a distinctive pattern of expression in the CNS, the distributions clearly overlap. However, the resolution of Northern blot analysis is insufficient to determine whether individual neurons in the brain contain multiple synaptotagmins. To circumvent this limitation, synaptotagmin expression was determined in PC12 cells, which represent a homogeneous population of cells. Both *Syt1* and *Syt3* are coexpressed in PC12 cells, thereby suggesting that synaptotagmins can be present in particular combinations within a given cell type. Indeed, *Syt3* transcripts are more abundant than those for *Syt1* in PC12 cells, indicating further that one isoform may predominate over another. The prevalence of *Syt3* transcripts in PC12 cells also casts doubt on a recent study in which depolarization-induced transmitter release was assessed in SYT1-deficient PC12 cells (12). In that study, the presence of Ca^{2+}-evoked catecholamine release in the mutant cell line was interpreted as evidence that synaptotagmins were not essential for regulated release of catecholamines from dense-core vesicles. Although the phenotypic properties of clonal cell lines may vary greatly among laboratories, our results indicate that SYT3, and therefore synaptotagmin function, may have been present in these cells, thereby accounting for the observed Ca^{2+}-dependent release.

Recent genetic and electrophysiological evidence suggests that synaptotagmins are presynaptic Ca^{2+} sensors (13–15, 23). Therefore, the various isoforms might represent species that respond to different ranges of intracellular Ca^{2+}, thereby providing potential mechanisms for differential vesicle release. For example, each isoform could have unique Ca^{2+}-binding constants. Differences in the Ca^{2+}-binding properties of the synaptotagmins could be generated by physical interactions between synaptotagmin isoforms. It is known that Ca^{2+} binding to SYT1 depends highly upon the state of oligomerization of this synaptotagmin (9, 21). If it were possible for SYT1 to form mixed oligomers with other synaptotagmin isoforms, this might modify the Ca^{2+}-binding properties of the complex. Indeed, this type of situation would potentially provide for an enormous breadth in the Ca^{2+}-sensing range. It is also conceivable that particular synaptotagmins or combinations of synaptotagmins are present on distinct classes of vesicles. SYT1 has been demonstrated to exist on both synaptic vesicles and large dense-core vesicles (4). Therefore, rather than individual synaptotagmins being vesicle-specific, the particular combination of isoforms localized to a given vesicle may be characteristic. Thus, it will be important to establish not only the anatomical and subcellular distributions of all three synaptotagmin isoforms but also whether they are capable of heterodimerization.

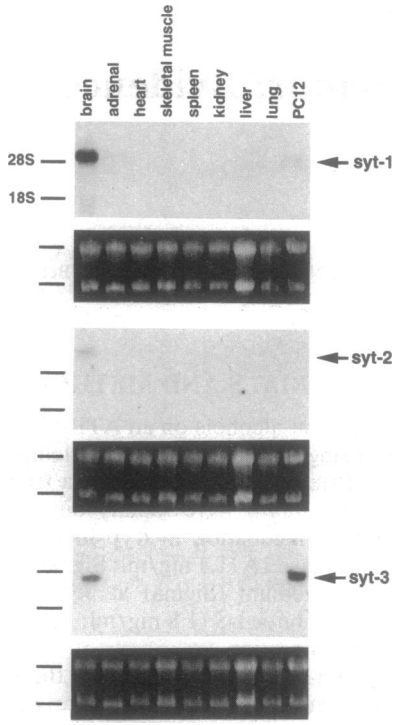

FIG. 3. Tissue distribution of synaptotagmin mRNA. Total RNA was prepared from various tissues and PC12 cells, as indicated. Samples (5 μg) were analyzed in triplicate on 0.8% agarose-formaldehyde gels, blotted to nitrocellulose, and hybridized with ^{32}P-labeled cDNA probes specific for *Syt1*, *-2*, and *-3* (see *Materials and Methods*). Blots were washed for 15 min at 55°C in 2× standard saline citrate (SSC), followed by 15 min at 60°C in 0.5× SSC. Positions of 28S and 18S ribosomal RNA are indicated at left, and the position of the single mRNA species obtained with each *Syt* cDNA probe is shown at right. A photograph of the ethidium bromide-stained gel is shown to indicate equal loading of RNA in each lane.

A final issue concerns the function of synaptotagmins in vesicular release. Several studies have demonstrated that SYT1 interacts with multiple presynaptic membrane proteins. These associations include the ability of SYT1 to bind *in vitro* to syntaxin (24), a proposed component of the vesicle docking and fusion apparatus (25), and with the cytoplasmic domains of neurexins (26, 27). In addition, SYT1 can be immunoprecipitated in a complex with Ca^{2+} channels (28). The physical association of this vesicle protein with components localized to release sites in the presynaptic membrane has led to the proposal that synaptotagmin plays a regulatory role in vesicle docking and fusion (5, 6, 23). The existence of additional synaptotagmin family members with unique protein–protein interactions or affinities to proteins in the fusion complex raises the possibility that multiple regulatory components are involved in initiating vesicle release in the presynaptic terminal. This hypothesis may account for the discrepancies between apparent loss of synaptotagmin function and the persistence of vesicular release in previous studies (12–15). Therefore, it will be important to determine the nature of the presynaptic components interacting with each synaptotagmin isoform and to further define the contributions that synaptotagmins make, not only singly but also combinatorially, to presynaptic function.

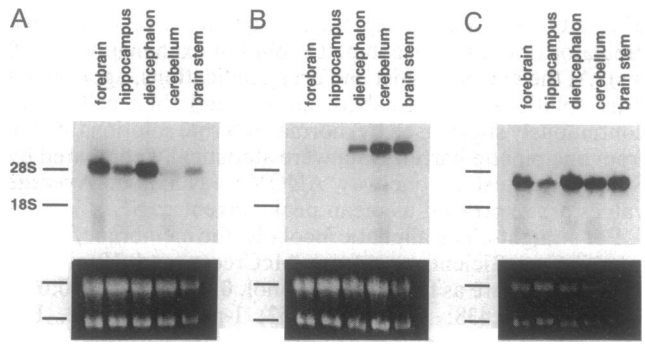

FIG. 4. Differential expression of *Syt* transcripts in the mouse CNS. RNA samples (5 μg) from various brain regions were run in triplicate, and *Syt* expression was analyzed by Northern blot using probes specific for *Syt1* (*A*), *Syt2* (*B*), and *Syt3* (*C*), as described in Fig. 3 legend. Positions of 28S and 18S ribosomal RNA are indicated at left.

Note Added in Proof. Recently, another synaptotagmin gene distinct from that reported here was published as Syt III (29). To avoid confusion, we refer to our sequence in the data base as *Syt4*.

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