Alternative splicing isoforms of synaptotagmin VII in the mouse, rat and human

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Synaptotagmin VII (Syt VII) has been proposed to regulate several different types of Ca^{2+}-dependent exocytosis, but its subcellular localization (lysosome or plasma membrane) and the number of alternative splicing isoforms of Syt VII (single or multiple forms) are matters of controversy. In the present study, we show by reverse transcriptase-PCR analysis that mouse Syt VII has one major isoform (Syt VIIα), the original Syt VII, and two minor isoforms (Syt VIIβ and Syt VIIγ), which contain unique insertions (of 44 and 116 amino acids respectively) in the spacer domain between the transmembrane and C2 domains of Syt VIIα. Similar results were obtained with respect to rat and human Syt VII mRNA expression. An antibody against the N-terminal domain of mouse Syt VII [anti-(Syt VII-N)], which specifically recognized recombinant Syt VII but not other Syt isoforms expressed in COS-7 cells, recognized two major, closely co-migrating bands (p58 and p60) and minor bands of approx. 65 kDa in mouse brain. Immunoaffinity purification of proteins that bind the anti-(Syt VII-N) antibody, and peptide sequence analysis revealed that: (i) the major p58 and p60 bands are identified as adenylate cyclase-associated protein 2; (ii) actin-binding protein is localized at the plasma membrane; and (iii) Syt VIIα (65 kDa) is the major Syt VII isoform, but with a much lower expression level than previously thought. It was also shown that FLAG-Syt VII–green-fluorescence-protein fusion protein stably expressed in PC12 cells is localized in the perinuclear region (co-localization with TGN38 protein, even after brefeldin A treatment) and in the tips of neurites (co-localization with Syt I), and not in the plasma membrane.

Key words: C2 domain, C-type tandem C2 protein, dense-core vesicle, exocytosis, membrane traffic.

INTRODUCTION

Synaptotagmin (Syt) is a family of C-terminal-type (C-type) tandem C2 proteins (known as the C2A domain and C2B domain) [1] with a single transmembrane domain at the N-terminus, and is thought to regulate membrane (or vesicular) trafficking, including synaptic vesicle exocytosis (reviewed in [2–5]). To date, 13 distinct *syt* genes have been reported in the mouse and human, and several genes have been reported in the fruit fly and nematode [6], with some of them (e.g. Syt VI and Syt VII) giving rise to several gene products by alternative splicing [7–9]. However, the presence of alternative splicing isoforms of Syt VII protein is somewhat controversial, because the isoforms reported by two groups were completely different. Craxton and Goedert [8] reported that Syt VII has several alternative splicing isoforms, produced mainly by transmembrane-domain-exon skipping, whereas Sugita et al. [9] reported that alternative splicing events occurred within the spacer domain (between the transmembrane domain and the C2A domain), and that multiple forms of Syt VII are expressed in the brain. The subcellular localization and functions of Syt VII are also a matter of debate [9–13]. Andrews and co-workers [10,11] showed that Syt VII protein is localized in the lysosomes of fibroblasts, and regulates Ca^{2+}-dependent lysosomal exocytosis to repair the plasma membrane, whereas Südhof and co-workers [9] reported that all the Syt VII splicing isoforms are present in the plasma membrane, and that they regulate Ca^{2+}-dependent secretory vesicle exocytosis by functioning as a plasma membrane Ca^{2+} sensor. Syt VII has also been suggested to be present in the insulin-containing vesicles in pancreatic β-cells and to regulate insulin secretion [12,13]. If multiple isoforms of Syt VII are present in mammals, it is quite important to determine the number of alternative splicing isoforms and to compare their expression levels. However, no quantitative analysis has ever been conducted.

In the present study, we have shown that mouse Syt VII has three alternative splicing isoforms, Syt VIIα, β and γ, and reverse transcriptase (RT)-PCR analysis and immunoblotting with specific antibodies revealed that Syt VIIα is the major splicing isoform in the brain. We have also shown that Syt VIIα protein stably expressed in PC12 cells is most likely to be present in the *trans*-Golgi network (TGN) and/or early endosomes and in dense-core vesicles, but not in the plasma membrane. On the basis of our findings, we discuss the reason for the conflicting results with regard to Syt VII alternative splicing events and subcellular localization.

MATERIALS AND METHODS

Materials

Ex-*Taq* and recombinant *Taq* DNA polymerases were obtained from Takara Shuzo Co., Ltd. (Shiga, Japan) and Toyobo Biochemicals (Tokyo, Japan) respectively. Horseradish peroxidase (HRP)-conjugated anti-T7 tag antibody was from Novagen (Madison, WI, U.S.A.). HRP-conjugated anti-FLAG tag (M2) and anti-synaptophysin antibodies were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Anti-TGN38 antibody was from

Abbreviations used: CAP, adenylate cyclase-associated protein; C-type, C-terminal-type; GFP, green fluorescent protein; GST, glutathione S-transferase; HRP, horseradish peroxidase; LC–MS/MS, liquid chromatography-tandem mass spectrometry; NGF, nerve growth factor; RT, reverse transcriptase; Syt(s), synaptotagmin(s); TGN, *trans*-Golgi network.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AB075900-1.

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Transduction Laboratories (Lexington, KY, U.S.A.). Anti-actin and anti-(secretogranin II) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Anti-(Syt I) (SYA148) antibody was from StreeGen Biotechnologies Corp. (Victoria, BC, Canada). Anti-(Syt II) and anti-(Syt IX) antibodies was prepared as described previously [14,15]. All other chemicals were commercial products of reagent grade. Solutions were made up in deionized water prepared with an Elix10 Water Purification System and Milli-Q Biocel A10 System (Millipore Corp., Bedford, MA, U.S.A.).

RT-PCR analysis

Mouse first-strand cDNAs (Mouse MTC Panel I) were prepared from various tissues and at developmental stages were obtained from Clontech Laboratories, Inc. (Palo Alto, CA, U.S.A.). Rat and human first-strand cDNAs prepared from various tissues (Rat and Human MTC Panel I) were also obtained from Clontech Laboratories, Inc. PCR was performed in the presence of Perfect Match PCR Enhancer (Stratagene, La Jolla, CA, U.S.A.) for 35 (or 30) cycles, with each round of PCR consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 2 min. Species-specific primers corresponding to the 5′- and 3′-ends of the spacer domain of Syt VII used for amplification were as follows: 5′-CGCAAACTGGGCAAACGCTA-3′ (common Syt VII spacer 5′ primer); 5′-GCACTAGTCAGCAGCCCTCGTGGGCCTC-3′ (mouse Syt VII spacer 3′ primer); 5′-GCAGCCCTCATGGGCCTC-3′ (rat Syt VII spacer 3′ primer); and 5′-GCAACCCTCGTGGGCCTC-3′ (human Syt VII spacer 3′ primer). The PCR products were analysed by 1.2% (w/v) agarose gel electrophoresis and ethidium bromide staining [16]. The authenticity of the products was verified by subcloning into a pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) and DNA sequencing with a Hitachi SQ-5500 DNA sequencer, as described previously [17].

Molecular cloning of mouse adenylate cyclase-associated protein 1 (CAP1) and CAP2

cDNA encoding a full open reading frame of CAP1 and CAP2 was amplified by RT-PCR using the following primers with restriction-enzyme sites (underlined) that were designed on the basis of the mouse sequence as described previously [18,19]: 5′-GGATCCATGGCTGACATGCAAAATCTT-3′ (CAP1 Met primer; sense; amino acid residues 1–7; Genbank® accession number L12367) and 5′-TTATCCAGCGATTTCTGTCA-3′ (CAP1 stop primer; antisense; amino acid residues 469–474); 5′-GGATCCATGACAGACATGGCGGGACTG-3′ (CAP2 Met primer; sense; amino acid residues 1–7; GenBank® accession number AK013331) and 5′-TCAGGCCATGATCTCTGCAG-3′ (CAP2 stop primer; antisense; amino acid residues 471–476). Reactions were performed in the presence of a Perfect Match PCR enhancer for 37 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 50 °C for 2 min, and extension at 72 °C for 2 min. The PCR products were purified from agarose gel with a Microspin column (Amersham Biosciences, Little Chalfont, Bucks., U.K.), as described previously [17], and directly inserted into the pGEM-T Easy vector. Addition of the FLAG tag to the N-terminus of CAP1 and CAP2, and construction of the expression vectors (pEF-FLAG-CAP1 and -CAP2), were performed as described previously [20–22].

Expression constructs

Full-length mouse Syt VIIβ and Syt VIIγ (referred to as pGEM-T-Syt VIIβ and γ respectively) were constructed by replacement of the *Eco*RI–*Hin*cII insert between Syt VIIα (original Syt VII) [17] and Syt VIIβ (or γ). pEF-T7-Syt VIIβ and -Syt VIIγ were constructed by PCR essentially as described previously [20–22]. pEF-FLAG-Syt VIIα–GFP (green fluorescence protein) was constructed by two-step PCR techniques, as described previously [23]. Briefly, Syt VIIα cDNA without a stop codon (left halves) was amplified by PCR using the following oligonucleotides (where the restriction sites are underlined): 5′-CGGGATCCATGTACCGGGACCCGGAGGCGG-3′ (Met primer; sense) and 5′-CAATTGGGCTTTCAGCTGGTGCCACT-3′ (C primer; antisense). GFP cDNA (right halves) was also amplified by PCR using pEGFP-N1 (Clontech Laboratories, Inc.) as a template: 5′-CGCAATTGCCGGGAATTTCC**GGTGGTGGTGGTGGT**ATGGTGAGCAAGGGCGAGGAGCTGT-3′ (GFP-glycine 5′ primer; sense strand, where the glycine linker in shown in bold), and GFP-C sequencing primer (antisense; Clontech Laboratories, Inc.). The two purified PCR products were digested with *Mun*I (restriction site shown underlined above), ligated to each other, and re-amplified by Met primer and GFP-C1 primer. The resulting Syt VII–Gly-GFP fragments were subcloned into the modified pEF-FLAG mammalian expression vector (pEF-FLAG-Syt VIIα–GFP), as described previously [22,24]. pShooter vector (Invitrogen Corp., Carlsbad, CA, U.S.A.) was digested with *Pml*I–*Xba*I (removal of the myc epitope tag), blunted with the Blunting kit (Takara Shuzo Co.), and ligated to a *Not*I linker. FLAG-Syt VIIα–GFP was inserted into the *Not*I site of the modified pShooter vector (referred to as pShooter-FLAG-Syt VIIα–GFP). pShooter-FLAG–GFP was similarly constructed by PCR, as described above. Other expression constructs (pEF-FLAG-Syts I–XI) were prepared as described previously [22,24]. Plasmid DNA was prepared with Wizard Mini preps (Promega) or the Qiagen Maxi prep kit (Chatsworth, CA, U.S.A.).

Antibody production

Rabbit antibody specific for the mouse Syt VII N-terminus was generated against synthetic peptides with a C-terminal artificial cysteine residue (MYRDPEAASPGAPTRDVC; amino acids shown in the single-letter code), and affinity-purified on immobilized peptides on 2-fluoro-1-methylpyridinium toluene-4-sulphonate-activated cellulofine beads (Seikagaku Co.), as described previously [14]. Construction of the pGEX-4T-3-Syt VIIβ spacer and -Syt VIIγ spacer domains, and preparation of glutathione S-transferase (GST) fusion proteins, were performed as described previously [20,25]. Rabbit antibodies specific for mouse Syt VIIβ or Syt VIIγ were raised against the GST–Syt VIIβ spacer domain (amino acids 86–129 of mouse Syt VIIβ) and the GST–Syt VIIγ spacer domain (amino acids 72–187 of mouse Syt VIIγ), and affinity-purified on antigen-immobilized Affi-Gel 10 beads (Bio-Rad Laboratories, Hercules, CA, U.S.A.), as described previously [7]. Antibody specificity was confirmed by immunoblotting with extracts of COS-7 cells transfected with T7-tagged Syts I–XIII ([16,17,26–28]; see Figure 3). The anti-(Syt VII-N) antibody was used for immunoisolation of Syt VII; the immunoisolated proteins were immunoblotted with the anti-(Syt VII-C2A) antibody, which recognized an epitope different from the first antibody (see below and Figure 4A). Since the first antibody recognized CAP2 and Syt VII (but not the other Syt isoforms; see Figure 4B), and the second antibody recognized specific Syt isoforms (I, II and VII), but not other C-type tandem C2 proteins (results not shown), the 65 kDa protein detected and shown in Figure 4(A) should be Syt VII. The protein concentrations were determined with the Bio-Rad protein assay kit, using BSA as a reference.

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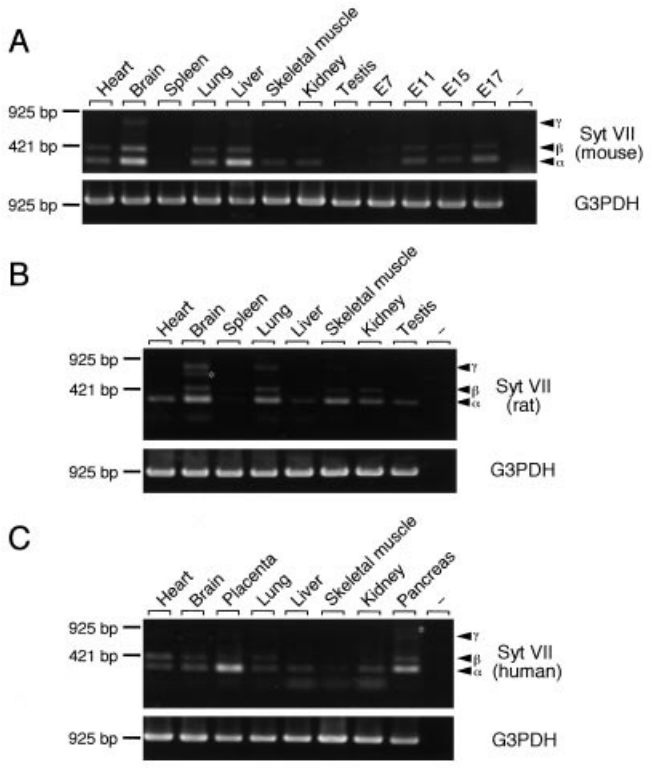

Figure 1 RT-PCR analysis of alternative splicing isoforms of Syt VII in the mouse, rat and human

(**A**) RT-PCR analysis of alternatively spliced isoforms of mouse Syt VII in various tissues (heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis) and on embryonic days 7, 11, 15 and 17 (E7, E11, E15 and E17 respectively). (**B**) RT-PCR analysis of alternative splicing isoforms of rat Syt VII in various tissues (heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis). (**C**) RT-PCR analysis of alternatively spliced isoforms of human Syt VII in various tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas). RT-PCR analysis of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) expression was also performed (bottom panels in **A–C**) to ensure that equal amounts of first-strand cDNA had been used for RT-PCR analysis. The lane marked with a minus sign contained no templates, and served as a negative control. The sizes of the molecular-mass markers (λ/*Sty*I) are shown to the left of each panel. Arrowheads indicate the position of three alternatively spliced variants of Syt VII, Syt VIIα, β and γ. Additional PCR bands were found in the rat and human (shown by white asterisks in **B** and **C**), but sequence analysis showed that they were not derived from Syt VII (results not shown). The results shown are representative of at least two or three independent experiments.

Purification of proteins that react with anti-(Syt VII-N) antibody

Purified anti-(Syt VII-N) antibody (170 μg) was immobilized with Affi-Gel 10 beads (250 μl, wet volume) according to the manufacturer's instructions. Adult mouse cerebellum (approx. 4 g) was homogenized in 10 ml of 0.32 M sucrose, 1 mM EDTA, 0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A, 1 mM 2-mercaptoethanol and 50 mM Hepes/KOH, pH 7.2, in a Teflon/glass Potter homogenizer with ten strokes at 900–1000 rev./min, and the homogenate was centrifuged at 1000 ***g*** for 10 min at 4 °C. The supernatant was centrifuged further at 100000 ***g*** for 1 h at 4 °C to precipitate membrane fractions. The membrane fractions were re-suspended in 50 mM Hepes/KOH, pH 7.2/1 mM 2-mercaptoethanol/1 mM EDTA containing proteinase inhibitors, and were solubilized with 1 % (v/v) Triton X-100. After removal of insoluble materials by centrifugation at 100000 ***g*** for 1 h at 4 °C, protein concentrations were adjusted to 5 mg/ml.

Approx. 8 ml of the solubilized membrane fraction was applied to the anti-(Syt VII-N) antibody-immobilized Affi-Gel 10 beads, and incubated for 2 h at 4 °C. After washing sequentially with 10 ml of Hepes/KOH, pH 7.2/1 M NaCl and 1 ml of Hepes/KOH, pH 7.2, 500 μl of 1 mM antigenic peptides (Syt VII-N) was applied to the column and gently agitated for 1 h at 4 °C to compete with the brain Syt VII. The eluate was recovered and analysed by SDS/PAGE [7.5 % (w/v) gels], followed by immunoblotting with anti-Syt VII antibodies [17] or staining with Coomassie Brilliant Blue R-250. Four major proteins (p50, p58, p60 and IgG heavy chain) were obtained from the affinity column (see Figure 4A). After lysyl-endopeptidase digestion, the peptides were analysed by liquid chromatography–tandem mass spectrometry (LC–MS/MS).

Isolation of PC12 cells stably expressing FLAG-Syt VIIα–GFP protein

Transfection of pShooter-FLAG-Syt VIIα–GFP into PC12 cells (2×10^6 cells/10-cm dish the day before transfection) was performed with LIPOFECTAMINE™ Plus reagent, according to the manufacturer's instructions (Life Technologies, Rockville, MD, U.S.A.) [7,27]. Three independent cell lines stably expressing FLAG-Syt VIIα–GFP and FLAG–GFP (as a control) were selected with 400 μg/ml Geneticin (Life Technologies) and GFP fluorescence. Brefeldin A (Sigma Chemical Co.) and nerve growth factor (NGF; Merck KGaA, Darmstadt, Germany) treatment was performed as described previously [27,28]. After fixing and permeabilizing the cells, they were incubated with anti-TGN38 antibody (1:250 dilution) or anti-(Syt I) antibody (1:250 dilution) and visualized with a second antibody (anti-mouse Alexa 568 antibody, 1:5000 dilution; Molecular Probes, Eugene, OR, U.S.A.), as described previously [7,28]. The cells were then examined with a confocal fluorescence microscope (Fluoview; Olympus, Tokyo, Japan).

Subcellular fractionation

The stable PC12 cell lines (5×10^6 cells/10-cm dish) were cultured with NGF for 3 days. Cells from two confluent plates were harvested and homogenized in 1 ml of 0.3 M sucrose/5 mM EDTA/5 mM Hepes/KOH, pH 7.3, with ten strokes at 1000 rev./min in a Teflon/glass Potter homogenizer. The homogenate was centrifuged at 10000 ***g*** for 10 min at 4 °C to remove debris. The supernatant was loaded on a linear sucrose gradient (0.6–1.8 M) and spun at 100000 ***g*** for 6 h in an SW41TI rotor (Beckman Instruments, Fullerton, CA, U.S.A.). A total of 14 fractions of 0.75 ml each were collected from the top of the gradient and analysed by immunoblotting. The following antibodies were used as organelle markers: anti-(secretogranin II), anti-synaptophysin and anti-(Syt IX) [15].

Miscellaneous procedures

Transfection of pEF-T7-Syt or FLAG-Syt into COS-7 cells (7.5×10^5 cells the day before transfection/10-cm dish) was performed by using the LIPOFECTAMINE™ Plus reagent according to the manufacturer's instructions (Life Technologies) [17,26]. SDS/PAGE and immunoblotting with HRP-conjugated anti-T7 (or FLAG)-tag antibody were performed as described previously [17,26,29].

RESULTS

Identification of three alternative splicing isoforms of Syt VII (α, β and γ) in the mouse, rat and human

In our previous study, we searched for alternative splicing isoforms of the mouse Syt family in the brain by RT-PCR using

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two sets of primers (amplified from the N-terminus to the end of the C2A domain, and from the C2A domain to the C-terminus) [7]. We found that, except for Syt VI and Syt VII, alternative splicing events in the Syt family were quite rare in the brain. The majority of the Syt VI (Syt VIΔTM) in the brain lacks a transmembrane domain, and as a result it is present in various membrane fractions, as well as the soluble fraction [7,27,30]. In contrast, no transmembrane exon-skipping type of Syt VII was detected in mouse brain [8], but a novel isoform that contains a 44-amino-acid insertion was found in the spacer domain of original Syt VII [17,31].

To characterize further the alternative splicing events in the spacer domain of Syt VII, we amplified the spacer domains of Syt VII by RT-PCR in various mouse tissues and developmental stages. As shown in Figure 1(A), three PCR products were detected in mouse brain (one major band of 280 bp, and two minor bands of 410 bp and 630 bp). Sequence analysis revealed that the 280 bp PCR fragments corresponded to the original Syt VII (referred to as Syt VIIα below), and the 410 and 630 bp PCR products were 44- and 116-amino-acid insertions (referred to as Syt VIIβ and γ respectively) in the spacer domain of Syt VIIα (see Figure 2). Quantitative analysis indicated that the proportion of the three Syt VII mRNAs in mouse brain is approximately $\alpha:\beta:\gamma = 77:20:3$. However, we discovered that the amount of Syt VIIγ mRNA was overestimated, because contamination by non-Syt clones or Syt VIIα or β PCR products was often found when the 630 bp PCR fragments were subcloned into pGEM-T Easy vector (results not shown). Mouse Syt VIIα mRNA was most abundant in brain, but found in all tissues tested, although its expression levels differed with the tissue. Syt VIIα expression also increased from the embryonic day 7 (E7) to the adult stage. A similar expression profile was observed for the mouse Syt VIIβ mRNA, although Syt VIIγ mRNA was difficult to detect, with the exception of the brain.

Similar results for Syt VII by RT-PCR analysis were obtained in the rat and human (Figures 1B and 1C). Syt VIIα is the dominant isoform, Syt VIIβ is the second most abundant isoform, and Syt VIIγ is very rare. Syt VIIα mRNA expression levels were higher in the human placenta and pancreas than in the brain. We therefore concluded that alternative splicing events in the Syt VII spacer domain are common in the mouse, rat and human.

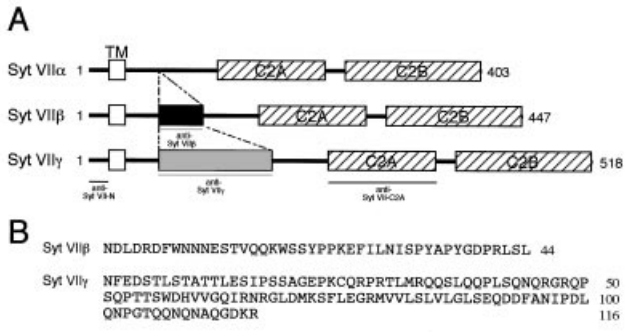

Figure 2 Three alternatively spliced isoforms of Syt VII

(**A**) Schematic representation of mouse Syt VIIα, β and γ. The transmembrane domain (TM) and two C2 domains are represented by open boxes and hatched boxes respectively. Insertions of 44 (black box) and 116 (grey box) amino acids were found in the spacer domain of Syt VIIβ and γ respectively. Amino acid numbers are indicated on the left and right of the boxed regions. Bars indicate the region used for antibody production (also see Figure 3B). (**B**) Amino acid sequence that is unique to mouse Syt VIIβ and Syt VIIγ in the spacer domain.

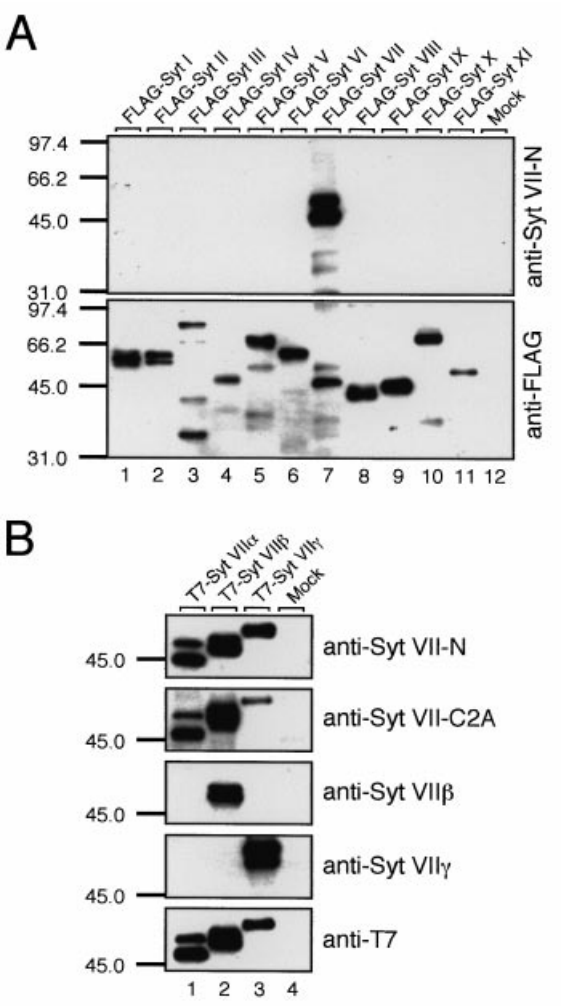

Figure 3 Production of isoform-specific antibody

(**A**) Specific recognition by the anti-(Syt VII-N) antibody of Syt VII, but not other Syt isoforms (Syts I–VI, VIII–XI) (lane 7). (**B**) Specificity of the anti-(Syt VII-N), anti-(Syt VII-C2A), anti-(Syt VIIβ) and anti-(Syt VIIγ) antibodies. Recombinant FLAG-tagged Syts I–XI and T7-tagged Syt VIIα–γ, prepared as described previously [17], were subjected to SDS/PAGE (10% gels), and transferred to a PVDF membrane. The blots were first probed with the anti-(Syt VII-N), anti-(Syt VII-C2A), anti-(Syt VIIβ) or anti-(Syt VIIγ) antibodies (all 1 μg/ml; the first, second, third and fourth panels from the top in **B** respectively). The same blots were stripped [48], and re-probed with HRP-conjugated anti-FLAG tag or anti-T7 tag antibody (1:10000 dilution; bottom panels in **A** and **B** respectively) to ensure loading of the same amounts of Syt proteins. Note that, whereas the anti-(Syt VII-N) and anti-(Syt VII-C2A) antibodies both recognized Syt VIIα–γ, the anti-(Syt VIIβ) and anti-(Syt VIIγ) antibodies specifically recognized Syt VIIβ and Syt VIIγ respectively. The positions of the M_r markers ($\times 10^{-3}$) are shown on the left. 'Mock' indicates transfection of a vector control (pEF-BOS).

Syt VIIα is the major isoform of Syt VII in the mouse, rat and human

Since the protein expression pattern of the Syt family was often different from its mRNA expression pattern [27,30–32], we next investigated whether the mRNA of the three Syt VII isoforms was indeed translated into protein in mouse tissues. To do so, we produced two anti-pan-Syt VII antibodies [anti-(Syt VII-N) and anti-(Syt VII-C2A)] and Syt VIIβ- and Syt VIIγ-specific antibodies (Figures 2A and 3). The specificity of each antibody was checked by probing with recombinant Syts I–XIII, Syt VIIβ and Syt VIIγ expressed in COS-7 cells [28] (Figure 3). Since the anti-

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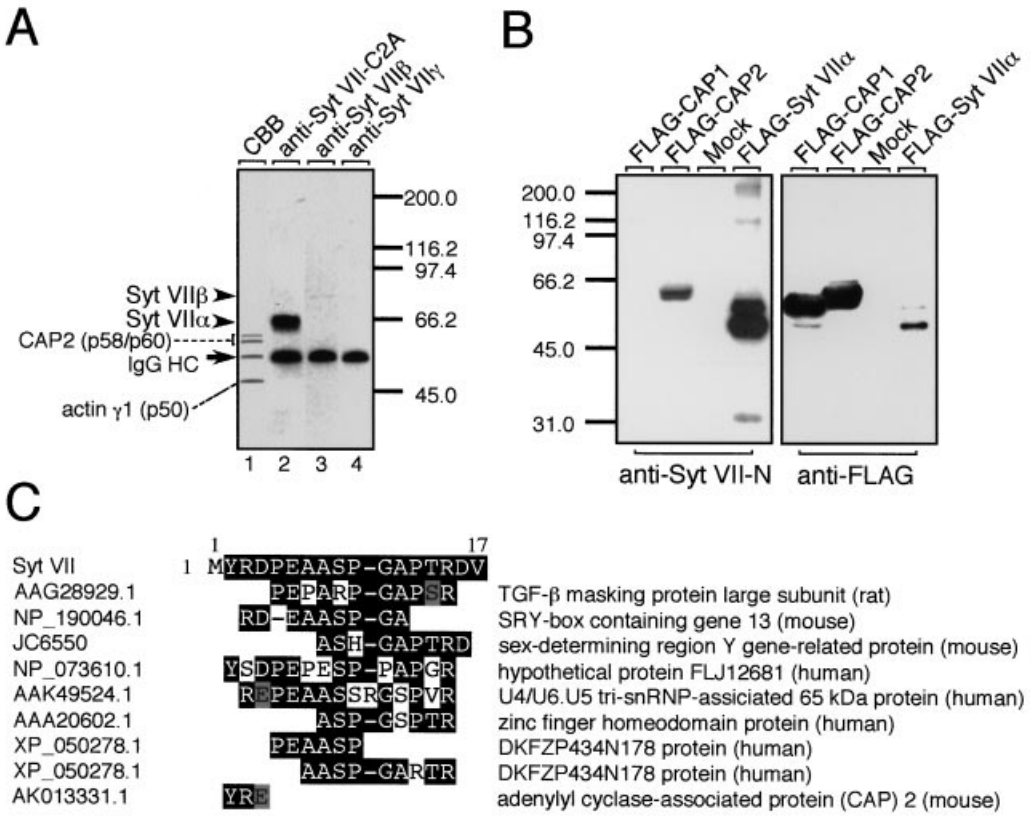

Figure 4 Characterization of the anti-(Syt VII-N) antibody-immunoreactive bands in mouse brain

(**A**) Identification of p58 and p60 as mouse CAP2 by LC–MS/MS. The eluate from the anti-(Syt VII-N) antibody-conjugated column was subjected to SDS/PAGE (7.5% gels) and stained with Coomassie Brilliant Blue R-250 (lane 1) or immunoblotted with anti-(Syt VII C2A) (lane 2), anti-(Syt VIIβ) (lane 3) or anti-(Syt VIIγ) antibodies (lane 4). The p50, p58 and p60 bands were excised from the gels, digested with lysyl-endopeptidase and analysed by LC–MS/MS. Note that the anti-(Syt VII-C2A) antibody recognized a single band of approx. 65 kDa (arrowhead, Syt VIIα), but not p50 (actin γ1), p58 or p60 (CAP2), and that the anti-(Syt VIIβ) antibody recognized a faint band of approx. 70 kDa (arrowhead, Syt VIIβ). The immunoreactive p65 kDa band was higher than the molecular mass of the recombinant Syt VII in Figure 3(A), but this difference may be attributable to the difference in post-translational modification (e.g. fatty-acylation or O-glycosylation). The arrow indicates the heavy chain of IgG. (**B**) The anti-(Syt VII-N) antibody recognized recombinant FLAG-tagged CAP2, but not CAP1, a closely related isoform of CAP2 (left panel) [33–36]. After stripping [48], the blots were re-probed with HRP-conjugated anti-FLAG tag antibody (1/10000 dilution) to ensure loading of the same amounts of CAP proteins (right panel). (**C**) Proteins that contain a sequence similar to the N-terminal 17 amino acids of Syt VII [immunogenic peptides of the anti-(Syt VII-N) antibody] were searched with the standard Protein BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/). Conserved and similar residues are shown against a black- or a grey-shaded background respectively.

(Syt VII-C2A) antibody cross-reacted with Syts I and II (results not shown), we initially used the anti-(Syt VII-N) antibody to detect endogenous Syt VII in various mouse tissues.

The anti-(Syt VII-N) antibody specifically recognized two closely co-migrating bands (a 58 kDa band and a 60 kDa band), referred to as p58 and p60 respectively, in all brain regions tested, but essentially not in non-neuronal tissues (see Figure 4A; also results not shown). The p58 and p60 bands completely disappeared in the presence of immunogenic peptides (results not shown), indicating specific recognition of p58 and p60 by the anti-(Syt VII-N) antibody. The p58/p60 ratio varied with the region of the brain under scrutiny, and expression of the p58 and p60 proteins gradually increased towards adulthood in parallel with synaptogenesis, consistent with a previous report [9] (results not shown). To investigate whether the 58 kDa- and 60 kDa-immunoreactive bands are indeed Syt VII, these were immunoisolated using the antibody-immobilized affinity column (see the Materials and methods section for details). As shown in Figure 4(A), we succeeded in purifying p58, p60 and additional p50 proteins from the affinity column. MS analysis and expressed sequence tag ('EST') database searching revealed the p58 and p60 proteins to be mouse CAP2 [18,33–35] and p50 to be actin γ1 [36], but surprisingly we were unable to obtain any peptide sequence related to Syt VII (Figure 4A). Since CAP2 is known to bind actin [37,38] and the anti-(Syt VII-N) antibody did not recognize p50, we concluded that actin γ1 (p50) was co-purified with CAP2 (p58 and p60). Consistent with this finding, the anti-(Syt VII-N) antibody recognized recombinant CAP2 (two closely co-migrating bands), but not its close homologue CAP1, indicating that the immunoreactive bands (p58 and p60) observed in mouse brain were derived from CAP2 (Figure 4B, left panel). Unexpectedly, however, CAP2 did not contain any sequence that was highly similar to the Syt VII N-terminus, and only a short sequence (YREFP) that was related to Syt VII, which was not found in CAP1, was identified (Figure 4C). Alternatively, the anti-(Syt VII-N) antibody might recognize the secondary or tertiary structure of CAP2, rather than its primary sequence.

To rule out the possibility that the anti-(Syt VII-N) antibody is unable to recognize endogenous Syt VII in brain, the eluate from the anti-(Syt VII-N) antibody-affinity column was immunoblotted with anti-(Syt VII-C2A), anti-(Syt VIIβ), anti-(Syt VIIγ) and anti-(Syt I) antibodies. As shown in Figure 4(A), the anti-(Syt VII-C2A) antibody recognized a single immunoreactive band (approx. 65 kDa), and the anti-(Syt VIIβ) antibody recog-

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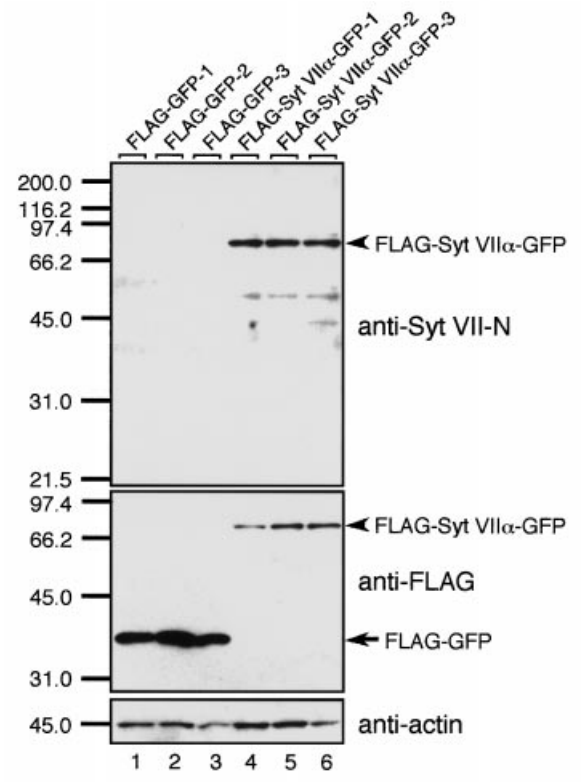

Figure 5 Expression of Syt VII in PC12 cells

Three independent PC12 cell lines stably expressing FLAG-Syt VIIα–GFP (lanes 4–6) or FLAG–GFP alone (lanes 1–3) were homogenized in 1% (w/v) SDS with a 27-gauge syringe. After addition of SDS sample buffer (+2-mercaptoethanol), the solubilized proteins were boiled for 3 min, subjected to SDS/PAGE (10% gels), and immunoblotted with anti-(Syt VII-N) antibody (top panel), HRP-conjugated anti-FLAG tag antibody (1:10000 dilution; middle panel), or anti-actin antibody (1:200 dilution; bottom panel). As described previously [15], we were unable to detect endogenous Syt VII proteins in control PC12 cells, but easily detected recombinant FLAG-Syt VIIα–GFP (arrowheads in lanes 4–6). The positions of the M_r markers ($\times 10^{-3}$) are shown on the left.

nized only a faint signal (approx. 70 kDa) that was not detected by the anti-(Syt VII-C2A) antibody. In contrast, no immunoreactive bands were detected with anti-(Syt VIIγ) or anti-(Syt I) under our experimental conditions, although Syt I itself was abundant in the brain lysate (results not shown). Since the mRNA expression level of Syt VIIα was much higher than that of Syt VIIβ (Figure 1), and the Syt family undergoes post-translational modifications (e.g. palmitoylation and O-glycosylation) [14,22,26,39–41] (M. Fukuda, unpublished work), we concluded that the anti-(Syt VII-N) antibody recognizes brain Syt VII and that the 65 kDa immunoreactive band corresponds to Syt VIIα, the major isoform in brain, with Syt VIIβ being only a very minor isoform.

Subcellular localization of Syt VIIα stably expressed in PC12 cells

Since most Syt family mRNAs were known to be expressed in PC12 cells [5] (M. Fukuda, unpublished work), and several Syt proteins (Syt I and Syt IX in the dense-core vesicles, and Syt IV in the Golgi and at the tips of neurites) had been detected at the protein level [15,28,42,43], we selected PC12 cells to investigate the subcellular localization of endogenous Syt VIIα. However, we were unable to detect Syt VII-related molecules by either immunocytochemistry or immunoblotting with our antibodies, probably because of their low expression levels in PC12 cells (Figure 5, lanes 1–3; also results not shown). Since the Syt family lacks a signal peptide, and the Syt protein itself should have correct type I membrane topology as well as specific membrane targeting [14,22,39], we next attempted to determine the subcellular localization of recombinant Syt VIIα by plasmid transfection. To do so, we established three independent lines of PC12 cells stably expressing Syt VIIα–GFP (Figure 5, lanes 4–6; indicated by arrowheads), because transient overexpression of Syt molecules sometimes causes mislocalization of proteins, probably due to saturation of normal transport pathway (M. Fukuda, unpublished work). To minimize the effect of C-terminal fusion of GFP on proper folding of the C2B domain [44], Syt VIIα and GFP were separated by a glycine linker. In all three cell lines, Syt VIIα–GFP protein was localized in the perinuclear region, in addition to many 'dots' being seen in the cell body (Figure 6). Our findings were in complete conflict with a previous report of transient overexpression of Syt VII–GFP localized in the plasma membrane [9]. The perinuclear staining of Syt VIIα–GFP was co-localized with that of TGN38, a marker for the TGN [Figures 6A (Syt VII in green), 6B (TGN38 in red) and 6C (overlay of Figures 6A and 6B)]. This immunostaining pattern of Syt VIIα was similar to that of recombinant Syt IV, Syt XI, Syt XIII and B/K protein in PC12 cells [16,27,39]. When cells were treated with brefeldin A, which caused redistribution of the Golgi markers to the endoplasmic reticulum and collapse of the TGN markers and early endosomal markers into the microtubule-organizing centre [27,28,39], Syt VIIα signals were still co-localized with TGN38 [Figures 6D–6F, shown by arrows (Figure 6D; Syt VII in green, 6E, TGN38 in red; and 6F, overlay of Figures 6D and 6E)]. When cells were treated with NGF, strong Syt VIIα signals were also detected at the tips of neurites, where dense-core vesicles are abundant, and were co-localized with Syt I [Figures 6G–6I, shown by arrowheads (Figure 6G, Syt VII in green; 6H, Syt I in red; and 6I, overlay of Figures 6G and 6H)]. Localization of Syt VIIα in dense-core vesicles was confirmed further by a subcellular fractionation study, as shown in Figure 7. The Syt VIIα protein was co-distributed with secretogranin II (a marker for the dense-core vesicles) and Syt IX [15] (peaks in fractions 5–7), but not with synaptophysin (a marker for synaptic-like microvesicles; peaks in fractions 3 and 4). These results strongly suggest that Syt VIIα protein is localized in the TGN and/or early endosomes of PC12 cells, as well as in the dense-core vesicles.

DISCUSSION

Syt VII has recently been proposed to be the third most abundant Syt isoform in the brain [9] and to regulate different types of Ca^{2+}-dependent vesicular trafficking [9–13]. However, the precise localization of Syt VII and the heterogeneity of Syt VII molecules produced by alternative splicing is a matter of controversy. In the present study, RT-PCR analysis revealed that mouse Syt VII has three alternative splicing isoforms, Syt VIIα, β and γ, in the unique spacer domain between the transmembrane and C2A domains (Figures 1 and 2), and that this splicing pattern is conserved in the mouse, rat and human. The original Syt VII (Syt VIIα) mRNA predominated in all tissues tested and at different developmental stages (more than 75% of total Syt VII mRNA in the mouse brain), and others were minor components. Similar results were obtained at the protein expression level when the anti-Syt VII antibodies were used (Figure 4A): Syt VIIα is a major isoform, Syt VIIβ is a very minor isoform, and no Syt VIIγ expression was detected. Therefore, although the heterogeneous Syt VII molecules pro-

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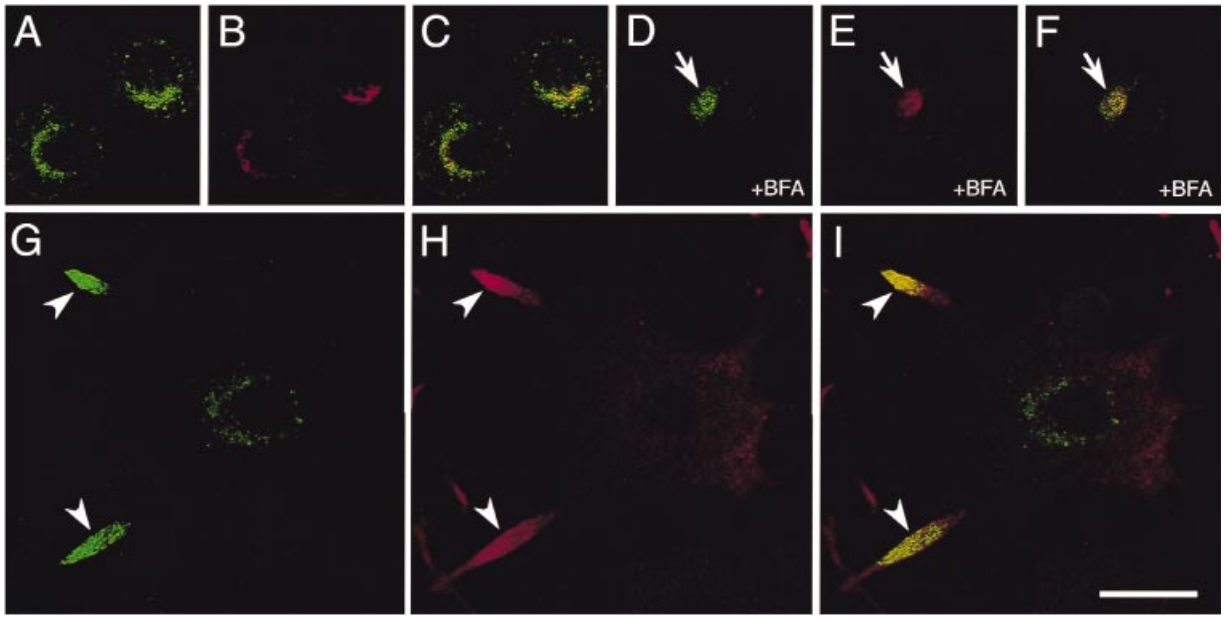

Figure 6 Subcellular localization of Syt VII–GFP stably transfected into PC12 cells

Subcellular localization of Syt VIIα–GFP in undifferentiated (**A–F**) and NGF-differentiated PC12 cells (**G–I**). PC12 cells stably expressing FLAG-Syt VIIα–GFP were fixed and permeabilized, and then visualized with GFP fluorescence (green coloration in **A**, **C**, **D**, **F**, **G** and **I**) and anti-TGN38 antibody (1:250 dilution; red coloration in **B**, **C**, **E** and **F**) or anti-(Syt I) antibody (1:250 dilution; red coloration in **H** and **I**). **C**, **F** and **I** are superimpositions of **A** and **B**, **D** and **E** and **G** and **H** respectively. Immunoreactivity was assessed with a confocal fluorescence microscope, as described in the Materials and methods section. Images were pseudo-coloured and superimposed with Adobe Photoshop software (Version 5.0). Note that, in undifferentiated PC12 cells, Syt VIIα–GFP was localized at the perinuclear region, which overlapped well with TGN38 even after brefeldin A (arrowheads; +BFA in **D–F**) treatment, and that in NGF-differentiated PC12 cells Syt VIIα–GFP was co-localized with Syt I at the tips of the neurites (arrowheads in **G–I**). The scale bar represents 25 μm.

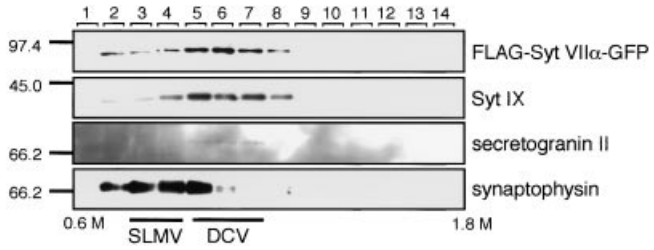

Figure 7 Subcellular distribution of Syt VII–GFP determined by sucrose-gradient fractionation of PC12 cells

PC12 cells stably expressing FLAG-Syt VIIα–GFP were fractionated on a 0.6–1.8 M sucrose gradient. The fractions were analysed by immunoblotting with anti-FLAG (1:10000 dilution; top panel), anti-(Syt IX) (1 μg/ml; second panel), anti-(secretogranin II) [1:200 dilution, third panel: a marker for dense-core vesicles (DCV)], and anti-synaptophysin antibody [1:4000 dilution, bottom panel: a marker for synaptic-like microvesicles (SLMV)]. Note that Syt VII–GFP migrated predominantly to the fractions corresponding to the DCV-enriched fractions (fractions 5–7), the same as secretogranin II and Syt IX, not with the SLMV-enriched fractions (fractions 3–4). The positions of the M_r markers are shown on the left (1×10^{-3}).

duced by alternative splicing are very attractive with regard to functional diversity, the multiple alternative splicing events reported previously [8,9] occur very rarely, if at all, or alternatively, they may be derived from retained introns.

Since the anti-(Syt VII-N) antibody recognized CAP2 (p58 and p60) in the brain rather than Syt VIIα (Figure 4), and the antibody showed much higher affinity for recombinant Syt VII than for recombinant CAP2 (Figure 4B), endogenous expression levels of Syt VIIα must be lower than previously thought [9]. Consistent with this, Syt VIIα is not a third abundant Syt isoform in the brain, according to the results of our preliminary experiments (M. Fukuda, unpublished work). It should be noted that previous attempts at subcellular localization of Syt VII by electron microscopy used a similar antibody generated against the same N-terminal peptide (MYRDPEAASPGAPTRDVC) [9] or a slightly shorter peptide (MYRDPEAASPGAC) [10]. Although both reports showed that incubation with antigenic peptides resulted in disappearance of signals, and such experiments were often used to check the antibody specificity for other Syt isoforms [15,45–47], characterization of our Syt VII-N antibody strongly indicated that absorption by immunogenic peptides alone is insufficient to indicate recognition of endogenous Syt VII protein. More importantly, several proteins containing a sequence similar to that of Syt VII N-terminal peptide are present in the databases (Figure 4C). Accordingly, the tissue distribution and abundance of Syt VII molecules should be re-evaluated by using a highly specific antibody.

Since both our own antibodies and those from studies published before are inappropriate to determine the subcellular localization of Syt VII, we stably expressed Syt VII–GFP in PC12 cells and showed that Syt VIIα protein is localized in the perinuclear region (TGN and/or early endosomes), as well as in the tips of neurites (dense-core vesicles) of NGF-differentiated PC12 cells (Figures 6 and 7). Our observation strongly supports the notion that Syt VIIα is present in the insulin-containing vesicles of pancreatic β-cells [12,13], but is completely different from previous reports that Syt VIIα is localized in the lysosomes [10,11] or plasma membrane [9]. These discrepancies may be attributable to the difference in plasmid transfection methods (transient overexpression in previous work [9,10] compared with stable expression in the present study), cross-reactivity of the anti-(Syt VII-N) antibody with non-Syt proteins, or differences in cell types (fibroblasts compared with neuroendocrine cells).

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In summary, we have identified the conserved alternative splicing events in the spacer domain of Syt VII in the mouse, rat and human, but only Syt VIIα protein was expressed predominantly in mouse brain. We have also shown that Syt VIIα–GFP is mainly localized in the perinuclear region and tips of neurites (dense-core vesicles), and not in the plasma membrane.

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