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Differential expression of SNAP-25 protein isoforms during divergent vesicle fusion events of neural development

(neurotransmitter release/exocytosis/axon outgrowth)

I. CHRISTINA BARK*, KLAUS M. HAHN, ANDREY E. RYABININ, AND MICHAEL C. WILSON†

Department of Neuropharmacology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037

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ABSTRACT **The presynaptic plasma membrane protein SNAP-25 (synaptosome-associated protein of 25 kDa) has been implicated as one of several neural-specific components that direct constitutive fusion mechanisms to the regulated vesicle trafficking and exocytosis of neurotransmitter release. There exist two alternatively spliced isoforms of SNAP-25, a and b, which differ in a putative membrane-interacting domain. We show that these two isoforms have distinct quantitative and anatomical patterns of expression during brain development, in neurons, and in neuroendocrine cells and that the proteins localize differently in neurites of transfected PC12 pheochromocytoma cells. These findings indicate that alternative isoforms of SNAP-25 may play distinct roles in vesicular fusion events required for membrane addition during axonal outgrowth and for release of neuromodulatory peptides and neurotransmitters.**

In the nervous system, the regulated release of neurotransmitters and neuromodulatory peptides and the addition of proteins and other constituents required for neurite outgrowth proceed through intracellular trafficking and fusion of vesicles at the plasma membrane (for review, see refs. 1–4). This regulated fusion of donor vesicle and acceptor (target) membranes is likely mediated by the same ubiquitious ATP-dependent machinery, composed of soluble *N*-ethylmaleimide-sensitive factor (NSF) and NSF-associated proteins (SNAPs), that is utilized in constitutive pathways of all eukaryotic cells (5). To provide specificity for the fusion process, distinct sets of related proteins have been postulated to serve as membrane receptors, or SNAREs (6). These enable correct recognition between vesicle and target membranes at different intracellular compartments, and they engage the general fusion machinery. It is likely that specific SNAREs, together with additional cell-specific auxiliary proteins, are critical for the specialized sorting, targeting, and final recycling of vesicles which are required for the Ca^{2+}-triggered release of neurotransmitter and modulatory peptides unique to neurons and neuroendocrine cells (7–9). Although evidence suggests that membrane addition to the expanding plasmalemma of axonal growth cones is Ca^{2+} dependent (10), it is thought that there are independent vesicle-targeting and fusion mechanisms that chiefly support either axon outgrowth or neurotransmitter release. Recent studies do suggest, however, that at least one of the SNAREs, SNAP-25, is a key player in membrane fusion events of both developing and mature neurons.

The presynaptic nerve terminal protein SNAP-25 (synaptosome-associated protein of 25 kDa; ref. 11) has been identified as a plasma membrane protein that together with syntaxin and the synaptic vesicle proteins VAMP/synaptobrevin and synaptotagmin are thought to constitute an initial SNARE docking complex for regulated exocytosis (5, 12, 13). After docking, this complex can incorporate into a 20S fusion particle by associating with constitutive SNAPs and NSF, with displacement of synaptotagmin (14). During development, the expression of SNAP-25 correlates with synaptogenesis and neuronal maturation (15). In the adult nervous system, SNAP-25 appears localized to presynaptic terminals (11, 16), where it is conveyed by fast axonal transport (17, 18). The importance of SNAP-25 in synaptic transmission is demonstrated by its being a specific substrate for botulinum neurotoxins A and E, metalloproteases which effectively block neurotransmitter release (19, 20). However, prior to synapse formation SNAP-25 is detected in cell bodies and fibers of the neonatal rat brain which are virtually devoid of immunoreactive protein in the adult (21). Moreover, inhibition of SNAP-25 expression by antisense oligonucleotides significantly diminishes axonal extension in developing neurons (22). These observations suggest that the shift in subcellular distribution of SNAP-25 participates in determining the targeting of vesicle transport and fusion in neural cells, as well as possibly affecting the triggering properties that are necessary for further specialization of these events.

Recently, an isoform of SNAP-25, designated SNAP-25a, was identified that is generated by alternative splicing between two divergent versions of exon five (23, 24). SNAP-25a and SNAP-25b differ by nine amino acids in a central domain, two of which alter the relative positioning of clustered cysteine residues that are sites for posttranslational fatty acylation implicated in membrane anchoring (18). Incorporation of peptide sequence encoded by either exon 5a or exon 5b could alter the character of membrane association of the SNAP-25 protein. To explore whether SNAP-25 isoforms might function in the specialization of vesicular fusion, we examined their regulated expression during development and tested whether these proteins would differentially localize when coexpressed in cultured neural cells.

MATERIALS AND METHODS

RNase Protection Assay. Total RNA was prepared, by the guanidinium thiocyanate procedure (25), from brains, dissected free of brainstem and later developing cerebellum, of BALB/c mice from embryonic day 15 (E15) to 16 weeks of age. RNA was similarly isolated from tissues dissected from adult male Wistar rats (Charles River Breeding Laboratories) and from PC12 cells harvested after culturing in either the absence or the presence of nerve growth factor (NGF; Boehringer Mannheim) at 50 ng/ml. The SNAP-25 probe was synthesized by using an *Ava* II-linearized subclone from a SNAP-25b cDNA, 8.52 (11), truncated at the *Sma* I site in exon 7 (see

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Abbreviations: NSF, *N*-ethylmaleimide-sensitive factor; NGF, nerve growth factor; HA, hemagglutinin; E*n*, embryonic day *n*; P*n*, postnatal day *n*.

*Present address: Department of Developmental Neuroscience, Uppsala University, Biomedical Center, Box 587, S-751 23 Uppsala, Sweden.

†To whom reprint requests should be addressed.

Fig. 1); for internal control probes, templates of an 18S ribosomal RNA cDNA (Ambion, Austin, TX) or ribosomal protein L32 were used. The *in vitro* transcribed SNAP-25b, 18S, and RPL32 RNA probes (247, 109, and 78 nt in length) were hybridized in excess to 10–20 μg of total RNA. Hybrid protected fragments were assayed according to the RPAII kit (Ambion) protocol, using a mixture of RNase A and T_1 and separated on an 8% (wt/vol) polyacrylamide/7 M urea gel. As controls, mouse liver RNA and yeast RNA were included. For quantification, the radioactivity of protected fragments was measured directly from the dried gel by using an AMBIS Systems Radioanalytic Imaging Detector and corrected for the number of incorporated radioactive nucleotides, and the radioactivities of the SNAP-25a and -b fragments, 184 and 159 nt in length, respectively, were compared with those of L32 or 18S RNAs.

***In Situ* Hybridization.** *In situ* hybridization was performed on cryostat sections of paraformaldehyde-perfused brains as described previously (26), but with hybridization at 42°C overnight followed by washes in 2× SSC at room temperature and 0.5× SSC at 56°C and 60°C (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). The sections were air dried, exposed first to x-ray film (DuPont), and dipped in NBT2 photographic emulsion (Kodak) for the dark-field images. The oligodeoxynucleotide probes, each 34 nt in length and comparable in G+C content, were labeled with terminal deoxynucleotidyltransferase and deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate by using standard procedures (27). The oligodeoxynucleotide probes were derived from cloned mouse cDNA sequence (I.C.B. and M.C.W., unpublished work): exon 5a antisense, 5′-TTGGTTGATATGGTTCATGCCTTCTTC-GACACGA-3′; exon 5b antisense, 5′-CTTATTGATTTGTC-CATCCCTTCCTCAATGCGT-3′; exon 6 antisense, 5′-TCCTGATTATTGCCCCAGGCTTTTTTGTAAGCAT-3′; and exon 6 sense, 5′-ATGCTTACAAAAAAGCCT-GGGGCAATAATCAGGA-3′.

Culture and Transfection of PC12 Cells. PC12 rat adrenal pheochromocytoma cells, generously provided by G. Guroff (National Institutes of Health, Bethesda, MD) were cultured as described (28). For transfection, the cells were preprimed with NGF at 25 ng/ml for 5–6 days and then replated on collagen-coated (4–6 μg/cm^2) dishes in the presence of NGF. Cotransfections of isoforms bearing different tags were performed by using Lipofectamine (GIBCO/BRL) according to the supplier's protocol. Expression constructs were generated from the previously described SNAP-25b cDNA clone p8.52 (11) and a SNAP-25a cDNA generated by PCR from 7-day-old BALB/c mouse brain RNA characterized by DNA sequencing (I.C.B. and M.C.W., unpublished results). Each SNAP-25a and -b cDNA was tagged with the human c-Myc epitope (29) or the hemagglutinin (HA) epitope from influenza virus (30) one or two amino acids from the carboxyl terminus by using patch-PCR (31) and inserted into the pCMX expression vector (ref. 32; provided by R. Evans, Salk Institute, La Jolla, CA).

Immunocytochemistry and Image Analysis. Forty-eight hours after transfection cells were fixed in 4% paraformaldehyde in phosphate-buffered 0.15 M saline, pH 7.4 (PBS). The cells were permeabilized in PBS containing 0.4% saponin and 10% goat serum for 10 min and incubated in blocking solution (PBS and 10% goat serum) for 30 min. Primary antibodies (c-Myc, monoclonal antibody 9E10, Santa Cruz Biotechnology; HA, monoclonal antibody 12CA5, a generous gift from I. A. Wilson, The Scripps Research Institute) were diluted in blocking solution and incubated with the cells for 30 min at room temperature, followed by Texas red- and fluorescein-conjugated isotype-specific secondary antibodies (Southern Biotechnology Associates). Microscope images were captured and analyzed by using a Photometrics cooled charged-coupled device camera and BDS Image software (33). Images were registered, background was subtracted, and images were masked through inspection prior to calculating ratios.

RESULTS

Developmental Expression of SNAP-25 Isoforms. To examine whether the two isoforms of SNAP-25 are regulated during mouse brain development, RNase protection assays were performed. As shown in Fig. 1, the expression patterns of SNAP-25a and SNAP-25b mRNAs differed from E15 to 16 weeks of age. Despite its relatively low mRNA level, the SNAP-25a isoform was the predominant species during embryonic and early postnatal development. In contrast, SNAP-25b transcripts, which were initially of very low abundance, reached similar levels to those of the SNAP-25a mRNA 7 days after birth. After the first postnatal week, SNAP-25b isoform transcripts became the predominant species, and they increased approximately 40-fold from their lowest level to become the majority of SNAP-25 mRNA in adult brain. Interestingly, the most dramatic increase in SNAP-25b expression was observed

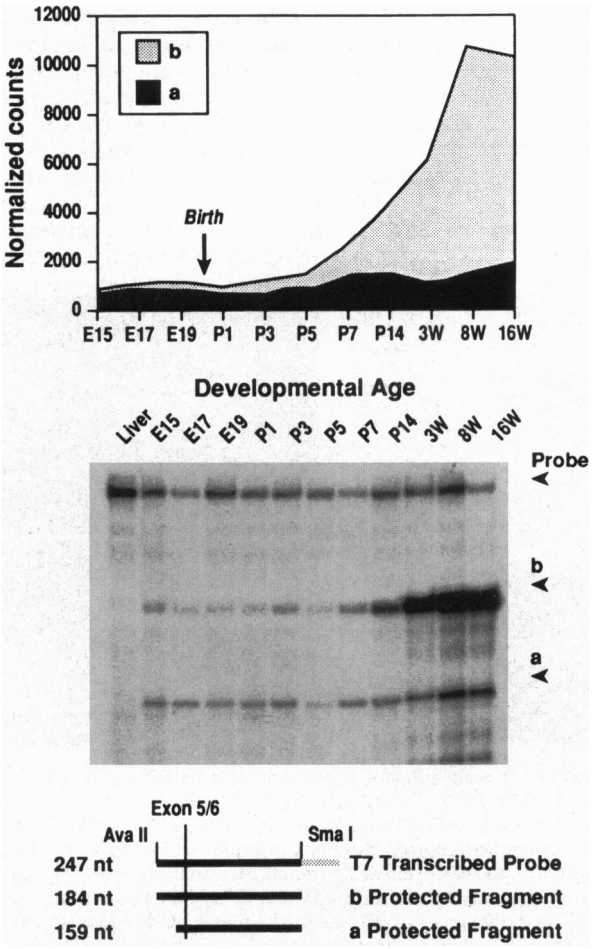

FIG. 1. Developmentally regulated expression of SNAP-25a and SNAP-25b mRNAs in developing mouse brain. (*Top*) Relative levels of SNAP-25 transcripts, a and b, contributing to total SNAP-25 mRNA at different stages of development, determined by RNase protection hybridization assay; P, postnatal day; W, weeks. The values correspond to the radioactivity of the protected fragments, normalized to 18S RNA (not shown) and the number of radioactive ribonucleotides in each fragment. (*Middle*) Autoradiograph of the assay, indicating the position on the gel of undigested probe and protected fragments corresponding to SNAP-25a and -b mRNAs (a, b). (*Bottom*) Diagram depicting the SNAP-25b cDNA probe bounded by *Ava* II and *Sma* I sites in exons 5b and 7, respectively, and expected sizes of fragments protected by the two isoform mRNAs.

between 3 and 8 weeks after birth, a period when many final cortical synapses attain their mature morphology (34).

The neuroanatomical pattern of expression in mouse brain was correlated with the temporal regulation of the two isoforms by *in situ* hybridization with deoxyoligonucleotide probes specific to the alternative exon 5 sequences (Fig. 2). At early neonatal stages—i.e., P1—the SNAP-25a transcript was dominant and found throughout cortex, subiculum, and hippocampus. During the next 2 weeks of development, while the expression of SNAP-25b increased and the level of the SNAP-25a isoform remained relatively constant, a complementary pattern emerged (Fig. 2). At P14 SNAP-25a mRNA was found preferentially in cells corresponding to layer IV of cortex, whereas the SNAP-25b isoform was present in the outer and inner cortical layers. In hippocampus, pyramidal CA3 neurons were found to express both SNAP-25a and -b at P14; however, SNAP-25a expression was decreased in adults and SNAP-25b isoform transcripts accounted for the characteristic high levels of SNAP-25 previously observed (11). In general, the expression of the two forms appeared to correspond to a switch in splicing pattern from the 5a to the 5b exon by individual neurons, and in the hippocampal formation this occurred during the period when the subcellular localization of the SNAP-25 protein shifted to nerve terminals (21). Low but detectable levels of SNAP-25a mRNA were still present in deep layers of cortex and selected thalamic structures in adult brain (Fig. 2, compare *g* and *h*, *k* and *l*, and *m* and *n*).

Expression of SNAP-25 in Neuroendocrine Cells. The sensitivity of Ca^{2+}-dependent catecholamine release from adrenal chromaffin cells to botulinum neurotoxin A (35, 36) and the identification of SNAP-25 in vesicle docking complexes in these cells (37) suggest that SNAP-25 also mediates regulated exocytosis in neuroendocrine cells. The levels of the SNAP-25 transcripts were therefore examined in rat adrenal and pituitary glands and selected regions of the central and peripheral nervous systems. As shown in Fig. 3, the relative abundance of SNAP-25 mRNA in these neuroendocrine tissues was significantly lower than in neuronal tissue but was clearly evident compared with the nonneural tissues liver and heart. While the abundance in adrenal gland appears lower than in pituitary, the expression of SNAP-25 in neuroendocrine medullary cells is likely underestimated due to nonneural cortical cells in the total adrenal tissue. More importantly, the relative expression of SNAP-25a and -b mRNAs was markedly different compared with regions of the adult nervous system. In RNA preparations of both pituitary and adrenal glands, the SNAP-25a transcript was predominant and made up 70–80% of the SNAP-25 mRNA. In contrast, as in adult mouse brain, the SNAP-25b isoform RNA was by far the major species in adult rat central regions, spinal cord, and cortex, as well as in the peripheral dorsal root ganglia.

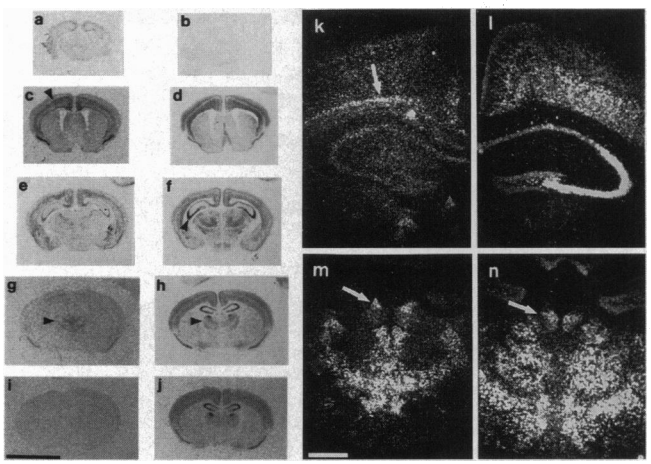

FIG. 2. Localization of SNAP-25a and -b RNAs in developing mouse brain. (*a–j*) X-ray film images of *in situ* hybridizations using probes to distinguish SNAP-25a and -b, and total SNAP-25 transcripts on representative coronal sections of brains to illustrate the distinct localization in neocortex at P1 (*a* and *b*), in parietal cortex (*c* and *d*; layer IV indicated by arrowhead), and in hippocampus (CA3; arrowhead) and thalamus at P14 (*e* and *f*) and at 8 weeks (*g* and *h*; arrowheads). Sections on the left (*a*, *c*, *e*, and *g*) hybridized to a SNAP-25a oligodeoxynucleotide probe are compared with nearly adjacent sections on the right (*b*, *d*, *f*, and *h*) hybridized with the SNAP-25b probe. (*i* and *j*) Controls using exon 6 sense- and antisense-derived oligodeoxynucleotide probes for background (*i*) and total SNAP-25 mRNA (*j*) hybridization. (*k–n*) Dark-field photomicrographs of an 8-week brain, illustrating low levels of exon 5a hybridization in hippocampus (*k*) but high levels in complementary subsets of thalamic nuclei (*m*; arrow to medial habenular nuclei), compared with exon 5b hybridization to these structures in adjacent sections (*l* and *n*; paraventricular nuclei indicated with arrow). Note the prominent hybridization of the 5a antisense oligodeoxynucleotide probe to the cortical subplate (*k*; arrow). (Scale bar in *a–j* indicates 5 mm, and the bar in *k–m* indicates 500 μm.)

Subcellular Localization of SNAP-25 Isoforms. The correspondence between the regulated expression of the SNAP-25 mRNAs and the difference in subcellular distribution of the protein during development (21) suggested that the two isoforms might localize differently when expressed within the same cell. To test this possibility, each isoform was tagged with either influenza HA or human c-Myc sequences and coexpressed in PC12 cells to compare their distribution by immunofluorescence using epitope-specific antibodies. PC12 cells endogenously express SNAP-25 that is translocated into the developing processes promoted by NGF (38). RNA analysis of PC12 cells either untreated or cultured with NGF showed that, although both isoforms are expressed, as in developing neurons and mature neuroendocrine cells, the SNAP-25a isoform transcript was the predominant species (>90%; Fig. 4*C*).

After transient expression, dual immunofluorescent detection of tagged SNAP-25 isoforms demonstrated that both proteins were translocated into NGF-induced processes of PC12 cells (Fig. 4). The SNAP-25b isoform, however, accumulated to a higher extent in the terminals and varicosities, whereas the SNAP-25a protein usually showed a more diffuse distribution extending throughout the processes. The localization of tagged SNAP-25a was similar to the diffuse distribution of endogenous SNAP-25 in PC12 cells after NGF treatment (38), consistent with the predominance of SNAP-25a mRNA in these cells. The difference in the distribution of transiently expressed SNAP-25 protein isoforms was verified by calculating the ratio of SNAP-25b to SNAP-25a immunofluorescence intensity throughout the length of the extended processes (Fig. 5). Control experiments in which the epitope tags were switched between the SNAP-25a and SNAP-25b cDNAs confirmed that the differential distribution was isoform specific and not due to addition of the tag epitope.

DISCUSSION

The differential expression of SNAP-25 isoforms during development and between neurons and neuroendocrine cells suggests

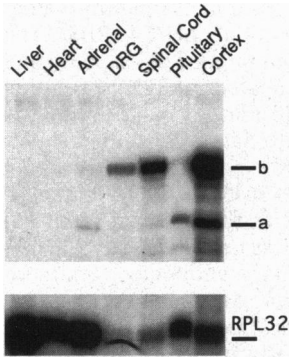

FIG. 3. Expression of SNAP-25 isoforms in neuroendocrine and neuronal tissues. Total RNA was evaluated as described for Fig. 1. As internal control for RNA input, the RNAs were also hybridized with a probe to the ribosomal protein L32 (RPL32). To provide comparable signal intensities the following amounts of total RNA from each tissue were assayed: liver, 20 μg; heart, 20 μg; adrenal, 20 μg; dorsal root ganglia (DRG), 8 μg; spinal cord, 10 μg; pituitary, 10 μg; and cortex, 10 μg.

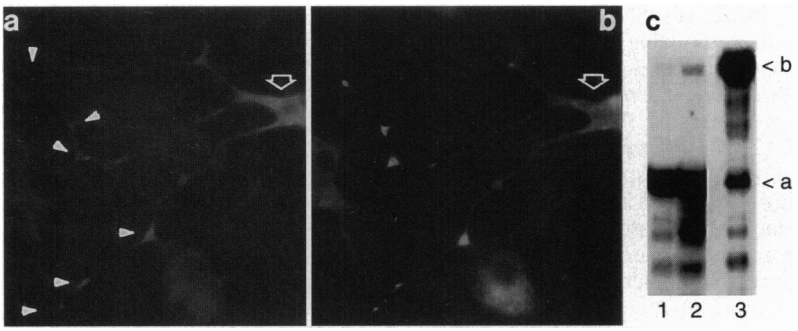

FIG. 4. Dual immunofluorescence of transfected epitope-tagged SNAP-25 isoforms and expression of endogenous SNAP-25 in NGF-treated PC12 cells. (*a* and *b*) Individual immunofluorescent staining in the same cell for HA-tagged SNAP-25a and c-Myc-tagged SNAP-25b, respectively, visualized with fluorescein- and Texas red-labeled isotype-specific secondary antisera. (×200.) The open arrow indicates the cell body; the arrowheads in *a* point to branchpoints and terminals of processes poorly stained for SNAP-25a that show prominent accumulation of SNAP-25b in *b*. (*c*) RNase protection analysis of SNAP-25 mRNAs in untransfected PC12 cells and adult mouse brain. Lane 1, uninduced PC12 cells; lane 2, PC12 cells treated with NGF (50 ng/ml) for 3 days; lane 3, 8-week-old mouse brain.

that the isoforms may be involved in differentially targeting the subcellular localization of regulated vesicular fusion at the plasma membrane. During neurite outgrowth, membrane fusion for plasmalemma expansion at the growth cone (10), as well as possibly throughout the extending neurite (39), appears generally targeted to the plasma membrane. Similarly, the regulated secretion of neuromodulatory peptides from secretory granules in neuroendocrine cells and from large dense cored vesicles of axon terminals of neurons also appears diffusely located along the plasma membrane (2, 40). This contrasts markedly with the organized, tight clustering of more highly abundant synaptic vesicles at the active zone of presynaptic terminals of most neurons. Thus, the presence of SNAP-25a in neuroendocrine cells and in developing axons and processes of PC12 cells (which largely do not make fully differentiated synaptic connections typical of mature neurons) is consistent with a broader repertoire for this isoform. In addition to serving as a plasma membrane receptor or SNARE for secretion of neuropeptides and for catecholamine-con-

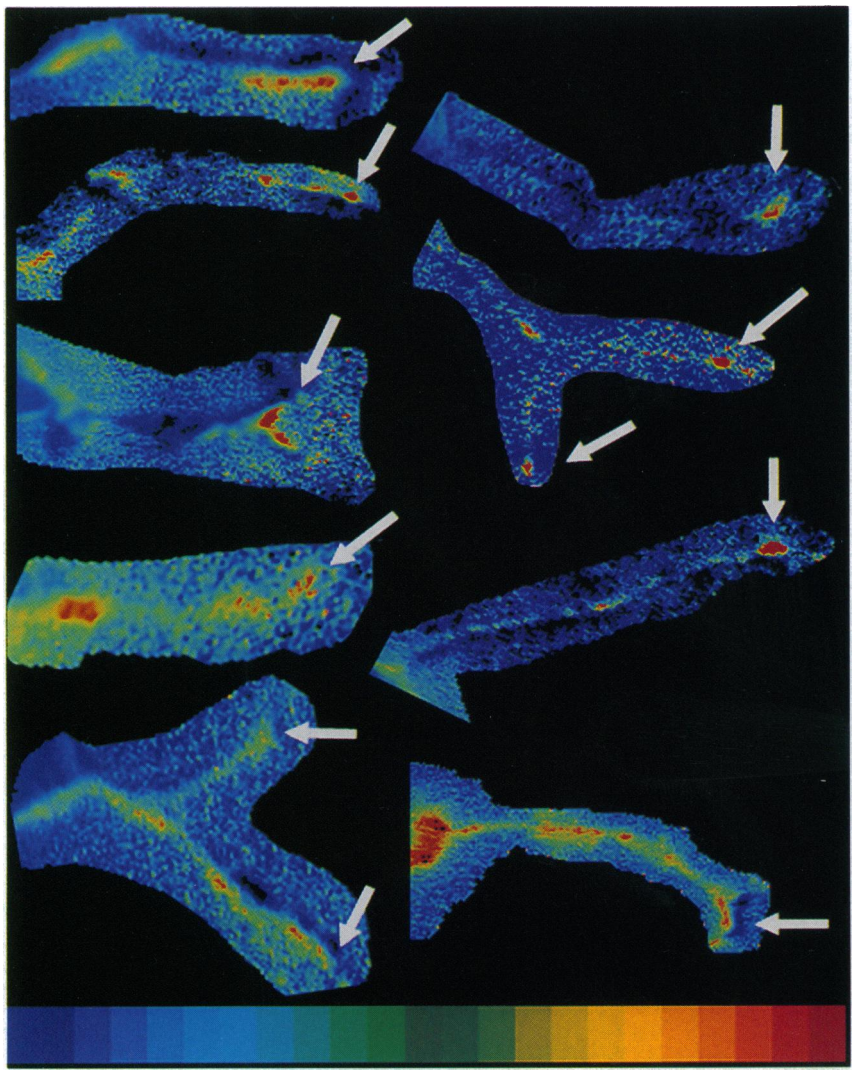

FIG. 5. Image analysis of the relative subcellular distribution of SNAP-25 isoforms in processes of PC12 cells. A gallery of quantitative ratio images is presented of processes taken from representative cells expressing detectable levels of both isoforms in the same cell 48 hr after cotransfection. Each ratio image was produced by dividing a SNAP-25b image by a SNAP-25a image on a pixel-by-pixel basis. Warmer colors correspond to higher ratios, indicating more pronounced accumulation of SNAP-25b isoform in varicosities, branchpoints, and termini of processes (arrows indicate termini). The upper seven processes are images of dual immunofluorescence of Texas red- and fluorescein-labeled antibodies to HA-tagged SNAP-25a and c-Myc-tagged SNAP-25b, respectively. The lower two processes depict the localized accumulation of SNAP-25b in control studies in which the HA and c-Myc tags on the two isoforms were switched. The color scale of the ratio values is shown. For each image (clockwise from upper left), the lowest value (blue) = 1, highest values (red) are 4.4, 4.0, 5.0, 5.2, 1.9, 3.6, 4.7, 9.0, and 5.6. (×500–1200.)

taining dense core vesicles of PC12 cells, SNAP-25a is likely to participate in axonal outgrowth by directing regulated fusion of "construction" vesicles that supply general membrane components needed for elongation throughout the neurite, as well as for plasmalemma expansion at growth cones. The suppression of neurite extension both in PC12 cells *in vitro* and in embryonic neurons *in vivo* by blocking of SNAP-25 expression (22) is consistent with the important role of SNAP-25a in membrane cycling required for outgrowth of neuronal processes, including axons.

In the adult nervous system, the predominance of SNAP-25b transcripts in central and peripheral neurons indicates that this isoform is likely most important for release of classical neurotransmitters from synaptic vesicles. Although SNAP-25a constitutes the major species in neuroendocrine cells, we cannot rule out that the SNAP-25b isoform is entirely responsible for neuropeptide secretion. However, the sequence previously reported for SNAP-25 in bovine brain membrane fractions which associates with NSF/SNARE fusion complexes corresponds to SNAP-25a (5), suggesting that this isoform can effectively participate in the vesicle fusion complex to mediate exocytotic release. As in neuroendocrine cells, the low but persistent expression of SNAP-25a in brain, therefore, may reflect the need for differential targeting of neuropeptide release from dense core vesicles at nerve terminals. Nevertheless, it is also tempting to consider that expression of SNAP-25a reflects the requirement of certain neurons, such as those in cortex layer IV which relay incoming thalamic information (as well as other thalamic nuclei), for neurite outgrowth and plasticity in response to learning and sensory input.

Alternative *Snap* gene exon 5 sequences have been found in mammals (24), chicken (23), and zebrafish (D. Larhammar, personal communication) but thus far not in *Drosophila* (41). Incorporation of these alternative exon-encoded sequences results not only in an altered arrangement of the "cysteine quartet" that constitutes the fatty acylation and putative membrane binding domain (11, 18) but also in an increased net positive charge in the SNAP-25a isoform. Such changes might also lead to altered patterns of palmitoylation and thereby affect membrane association and hence distribution.

The demonstration of selective interactions between synaptic vesicle-associated VAMPs (synaptobrevins), cell-specific members of the syntaxin family, and SNAP-25 at the target membrane provides evidence for a combinatorial role of SNAREs and associated proteins in specifying the intracellular traffic of vesicles for neurotransmitter release (42, 43). The expression of two functionally distinct isoforms of the vesicle docking protein SNAP-25 with altered membrane-interacting domains supports the idea that modifications in the components participating in vesicular targeting and fusion also contribute to the diversity of these mechanisms (3). Furthermore, the regulation of SNAP-25 isoforms through alternative splicing suggests posttranscriptional processing could help mediate dynamic cellular alterations in directed vesicular fusion, in response to developmental events and changing environmental stimuli. The control of vesicular fusion performed by differently expressed auxiliary proteins might be an important regulatory mechanism for coordinating neurite extension, neurotransmitter release, and synaptic remodeling in complex neuronal systems.

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1. De Camilli, P. & Jahn, R. (1990) *Annu. Rev. Physiol.* **52,** 625–645.
2. Hökfelt, T. (1991) *Neuron* **7,** 867–879.
3. Bark, I. C. & Wilson, M. C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4621–4624.
4. Catsicas, S., Grenningloh, G. & Pich, E. M. (1994) *Trends Neurosci.* **17,** 368–372.
5. Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. & Rothman, J. E. (1993) *Nature (London)* **362,** 318–323.
6. Rothman, J. E. & Warren, G. (1994) *Curr. Biol.* **4,** 220–233.
7. Bennett, M. K. & Scheller, R. H. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2559–2563.
8. Südhof, T. C., De Camilli, P., Niemann, H. & Jahn, R. (1993) *Cell* **75,** 1–4.
9. Ferro-Novick, S. & Jahn, R. (1994) *Nature (London)* **370,** 191–193.
10. Locherbie, R. O., Miller, V. E. & Pfenninger, K. H. (1991) *J. Cell Biol.* **112,** 1215–1227.
11. Oyler, G. A., Higgins, G. A., Hart, R. A., Battenberg, E., Billingsley, M., Bloom, F. E. & Wilson, M. C. (1989) *J. Cell Biol.* **109,** 3039–3052.
12. O'Connor, V. M., Shamotienko, O., Grichin, E. & Betz, H. (1993) *FEBS Lett.* **326,** 255–260.
13. Horikawa, H. P. H., Saisu, H., Ishizuka, T., Sekine, Y., Tsugita, A., Odani, S. & Abe, T. (1993) *FEBS Lett.* **330,** 236–240.
14. Söllner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H. & Rothman, J. E. (1993) *Cell* **75,** 409–418.
15. Catsicas, S., Larhammar, D., Blomqvist, A., Sanna, P. P., Milner, R. J. & Wilson, M. C. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 785–789.
16. Geddes, J. W., Hess, E. J., Hart, R. A., Kesslak, J. P., Cotman, C. W. & Wilson, M. C. (1990) *Neuroscience* **38,** 515–525.
17. Lowey, A., Lui, W.-S., Baitinger, C. & Willard, M. B. (1991) *J. Neurosci.* **11,** 3412–3421.
18. Hess, D. T., Slater, T. M., Wilson, M. C. & Skene, J. H. P. (1992) *J. Neurosci.* **12,** 4634–4641.
19. Blasi, J., Chapman, E. R., Link, E., Binz, T., Yamasaki, S., De Camilli, P., Südhof, T. C., Niemann, H. & Jahn, R. (1993) *Nature (London)* **365,** 160–163.
20. Schaivo, G., Rossetto, O., Catsicas, S., Polverino De Laureto, P., DasGupta, B. R., Benfenati, F. & Montecucco, C. (1993) *J. Biol. Chem.* **268,** 23784–23787.
21. Oyler, G. A., Polli, J. W., Wilson, M. C. & Billingsley, M. L. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5247–5251.
22. Osen-Sand, A., Catsicas, M., Staple, J. K., Jones, K. A., Ayala, G., Knowles, J., Grenningloh, G. & Catsicas, C. (1993) *Nature (London)* **364,** 445–448.
23. Bark, I. C. (1993) *J. Mol. Biol.* **233,** 67–76.
24. Bark, I. C. & Wilson, M. C. (1993) *Gene* **139,** 291–292.
25. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
26. Wilson, M. C. & Higgins, G. A. (1989) in *Neuromethods 16: Molecular Neurobiological Techniques*, eds. Bouton, A. A., Baker, G. B. & Campagnoni, A. T. (Humana, Clifton, NJ), pp. 239–284.
27. Sambrook, J., Fritsch, E. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
28. Greene, L. A. & Tischler, A. S. (1982) *Adv. Cell. Neurobiol.* **3,** 373–414.
29. Evan, G. I., Lewis, G. K., Ramsay, G. & Bishop, J. M. (1985) *Mol. Cell. Biol.* **5,** 3610–3616.
30. Field, J., Nikawa, J.-I., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A. & Wigler, M. (1988) *Mol. Cell. Biol.* **8,** 2159–2165.
31. Squinto, S. P., Aldrich, T. H., Lindsay, R. M., Morrissey, D. M., Panayotas, N., Bianco, S. M., Furth, M. E. & Yancopoulos, G. D. (1990) *Neuron* **5,** 757–766.
32. Umesono, K., Murakami, K. K., Thompson, C. C. & Evans, R. M. (1991) *Cell* **65,** 1255–1266.
33. Bright, G. R., Fisher, G. W., Rogowska, J. & Taylor, D. L. (1989) *Methods Cell Biol.* **30,** 157–192.
34. Aghajanian, G. K. & Bloom, F. E. (1967) *Brain Res.* **6,** 716–727.
35. Penner, R., Neher, E. & Dreyer, F. (1986) *Nature (London)* **324,** 76–78.
36. Strecher, B., Gratzl, M. & Ahnert-Hilger, G. (1989) *FEBS Lett.* **248,** 23–27.
37. Roth, D. & Burgoyne, R. D. (1994) *FEBS Lett.* **351,** 207–210.
38. Sanna, P. P., Bloom, F. E. & Wilson, M. C. (1991) *Dev. Brain Res.* **59,** 104–108.
39. Popov, S., Brown, A. & Poo, M.-M. (1993) *Science* **259,** 244–246.
40. Thureson-Klein, A. K. & Klein, R. L. (1990) *Int. Rev. Cytol.* **121,** 67–126.
41. Risinger, C., Blomqvist, A. G., Lundell, I., Lambertsson, A., Nässel, D., Pieribone, V. A., Brodin, L. & Larhammar, D. (1993) *J. Biol. Chem.* **268,** 24408–24414.
42. Calakos, N., Bennett, M. K., Peterson, K. E. & Scheller, R. H. (1994) *Science* **263,** 1146–1149.
43. Pevsner, J., Hsu, S.-C., Braun, E. A. E., Calakos, N., Ting, A. E., Bennett, M. K. & Scheller, R. H. (1994) *Neuron* **13,** 353–361.