

Cellular Localization of Synaptotagmin I, II, and III mRNAs in the Central Nervous System and Pituitary and Adrenal Glands of the Rat

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Three isoforms of synaptotagmin, a synaptic vesicle protein involved in neurotransmitter release, have been characterized in the rat, although functional differences between these isoforms have not been reported. *In situ* hybridization was used to define the localization of synaptotagmin I, II, and III transcripts in the rat CNS and pituitary and adrenal glands. Each of the three synaptotagmin genes has a unique expression pattern. The synaptotagmin III gene is expressed in most neurons, but transcripts are much less abundant than the products of the synaptotagmin I and II genes. A majority of neurons in the forebrain expressed both synaptotagmin I and III mRNAs while synaptotagmin II gene expression was confined to subsets of neurons in layers IV–VI of the cerebral cortex, in the dentate granule cell region, the hilus, and the CA1–CA3 areas of the hippocampus. In the cerebellum, all three transcripts were visualized in the granule cell layer. Furthermore, synaptotagmin I probes revealed striking differences between distinct populations of neurons, as in addition to moderate labeling of granule cells, much more prominent hybridization signals were detected on scattered cell bodies likely to be Golgi interneurons. In the most caudal part of the brain, synaptotagmin II transcripts were abundant and were coexpressed with synaptotagmin III mRNAs. This pattern was found in putative motoneurons of the spinal cord, suggesting that the two isoforms might be involved in exocytosis at the neuromuscular junction. Only synaptotagmin I mRNAs were detected in the anterior and intermediate pituitary and in adrenal medullary cells. These data reveal an unexpectedly subtle segregation of the expression of synaptotagmin genes and the existence of multiple combinations of synaptotagmin isoforms which may provide diversity in the regulation of neurosecretion.

[Key words: synaptotagmin, neurotransmitter release, mRNA distribution, CNS, pituitary gland, adrenal gland]

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Structural, biochemical, and physiological data are consistent with a crucial role of synaptotagmin in neurotransmitter release (reviewed by DeBello et al., 1993). It is one of the major membrane components of synaptic vesicles (Matthew et al., 1981; Fournier and Trifaro, 1988) and is exclusively expressed in neurons and neuroendocrine cells. Synaptotagmin binds Ca^{2+} at concentrations in the 10–100 μM range in a phospholipid-dependent manner (Brose et al., 1992) and has been postulated to be the Ca^{2+} sensor which induces exocytosis.

Synaptotagmin interacts *in vitro* with multimolecular complexes which include syntaxin/HPC-1 and the N-type Ca^{2+} channel (Bennett et al., 1992b; Lévêque et al., 1992, 1994; Yoshida et al., 1992), SNAP 25, synaptobrevin/VAMP (Söllner et al., 1993), the α -latrotoxin receptor, and other related proteins of the neurexin family (Petrenko et al., 1991; Hata et al., 1993; O'Connor et al., 1993). These complexes are thought to be involved in synaptic vesicle docking at presynaptic active zones in close vicinity to the site of Ca^{2+} entry.

Furthermore, synaptotagmin may prevent assembly of the constitutive core machinery, formed by the association of N-ethylmaleimide-sensitive fusion protein (NSF) with soluble NSF attachment proteins (SNAPs), and its putative receptor: the VAMP, syntaxin, and SNAP 25 complex (Söllner et al., 1993). Also, synaptotagmin appears to play a role in endocytosis through its interaction with the clathrin adaptor AP-2 (Zhang et al., 1994).

Drosophila mutants lacking synaptotagmin display a severe reduction of evoked neurotransmitter release and an increase in the frequency of spontaneous quantal events at the neuromuscular junction (Littleton et al., 1993; DiAntonio et al., 1994). In the giant synapse of the squid, injection of peptides from synaptotagmin into presynaptic terminals (Bommert et al., 1993) inhibits neurotransmission.

Synaptotagmin is expressed as variant isoforms encoded by different genes in the rat [synaptotagmin I (Perin et al., 1990); synaptotagmin II (Geppert et al., 1991); synaptotagmin III (Mizuta et al., 1994)]. The overall identity of amino acid sequence among them is relatively low; however, the region corresponding to the carboxyl terminal C_2 repeats is well conserved. Synaptotagmin I and II are more closely related to each other than to synaptotagmin III. No functional differences have been reported between these isoforms.

Variations in sequence between synaptotagmins could lead to differences in docking and fusion providing fine tuning of regulated vesicular trafficking required for development and neu-

Table 1. 5'-3' sequences of oligodeoxyribonucleotide probes used for *in situ* hybridization

Probes		Sequence
Synaptotagmin I		
TgIa	Complementary to bases 1800-1843	TACTGGCTAAAGAGCACTATGTGGGCAGA-TGCAGAAAGGCTTCG
TgIb	Complementary to bases 2527-2571	TGAAGCTATGCTAGATGCAGTGGTAGGAA-CGCATTGGCTCCTGTT
TgIs	Bases 1800-1843	CGAAGCCTTTTGCATCTGCCACATAGTG-CTCTTTAGCCAGTA
Synaptotagmin II		
TgIIa	Complementary to bases 1923-1966	TTTCGCAAGGACTATGAGAGCTTCTGGCCT-CTGACCACTTAAGC
TgIIb	Complementary to bases 2447-2491	AGTTGTGAGGAGCTCTGCAATGTCTAGCTT-GTCACTGTCCACCAA
TgIIs	Bases 1923-1966	GCTTAAGTGGTCAGAGGCCAGAAGCTCTC-ATAGTCCTTGCGAAA
Synaptotagmin III		
TgIIIa	Complementary to bases 1809-1853	TTCTCTGACAATCCTTTGCCGCCCTTGTA-AAGCTGCTTAGAGTC
TgIIIb	Complementary to bases 1853-1896	GTCCAATCCCAGGCCTAGACCAGACCCTC-ACTCTGAATTCTCTT
TgIIIs	Bases 1809-1853	GACTCTAAGCAGCTTTACCAAGGGCGGCA-AAGGATTGTCAGAGAA

rotransmission. In order to analyze the distribution of the different isoforms, we have carried out an *in situ* hybridization study of the distribution of synaptotagmin mRNAs in the rat CNS and in the adrenal and pituitary glands.

Materials and Methods

Oligonucleotide probes. Oligonucleotide probes [44-45 base pairs (bp)] of unique sequence were synthesized (CIML, France) and purified by ethanol precipitation. The probe sequences derived from the rat synaptotagmin I (Perin et al., 1990), synaptotagmin II (Geppert et al., 1991), and synaptotagmin III (Mizuta et al., 1994) cDNAs are compiled in Table 1. Sense probes TgIs, TgIIs, and TgIIIs exactly complementary to TgIa, TgIIa, and TgIIIa antisense probes, respectively, were used as controls.

Probes were 3'-end labeled with 5'-[α -³⁵S]-dATP (>1000 Ci/mmol; Amersham) to similar specific activities in the range of 2×10^8 - 1.4×10^9 dpm/ μ g using terminal deoxynucleotidyl transferase (Boehringer Mannheim) with a 30:1 molar ratio of dATP:oligonucleotide. Unincorporated nucleotides were removed by a spin column procedure using Sephadex G-25 (Pharmacia).

In situ hybridization. Nonperfused rat brains were removed and frozen on dry ice. Cryostat sections (12 μ m) were cut, mounted onto poly-L-lysine-coated slides, and dried at room temperature. Sections were fixed in 4% paraformaldehyde, rinsed in phosphate-buffered saline (PBS), and dehydrated into 95% ethanol for storage at 4°C, or dried and stored at -80°C until required. Radiolabeled probe was dissolved in hybridization buffer [50% (v/v) formamide, 4 \times saline sodium citrate (SSC: 0.15 M NaCl, 0.015 M Na-citrate), and 10% (w/v) dextran sulfate] in final concentrations of 0.14-0.5 nM and applied to sections. Hybridization was performed at 42°C under a Parafilm cover. Sections were washed to a final stringency of 1 \times SSC at 55°C and exposed to Kodak X-Omat film at room temperature, or dipped in Ilford K5 emulsion. To examine the specificity of the signals observed, parallel sections were hybridized with two independent antisense oligonucleotides complementary to different regions of each transcript. Furthermore, control experiments were done with the sense oligonucleotide probes TgIs, TgIIs, and TgIIIs as detailed above. Some sections were also hybridized with the antisense probes in the presence of a large excess (50-fold) of the unlabeled oligonucleotide. The anatomy of sections and autoradiographs was determined using the atlas of Paxinos and Watson (1986).

Results

Specificity of probes

Probes to each of the three rat synaptotagmins produced unique patterns of hybridization in the rat brain (Figs. 1-5), spinal cord (Figs. 6, 7), and adrenal and pituitary glands (Fig. 8). Two probes, complementary to different regions of the same cDNA, were tested for each synaptotagmin. In each case, both members of the pair produced identical patterns of hybridization. No signal could be detected with the sense probes TgIs, TgIIs, and TgIIIs when used in the same conditions at equivalent specific activity (data not shown). Also, no labeling was observed on sections hybridized with radiolabeled TgIa and TgIIa in the presence of an excess of the unlabeled oligonucleotide (data not shown).

In situ hybridization

Macroscopic transcript distributions on sagittal and coronal sections of brain (Fig. 1), spinal cord (Fig. 6), and adrenal and pituitary glands (Fig. 8) are shown. Each probe produced regionally distinct hybridization signals. Regions of white matter such as the corpus callosum were devoid of synaptotagmin mRNAs. Cellular localizations of synaptotagmin transcripts were analyzed in different areas of the brain. While synaptotagmin I transcripts were confined to the most rostral part of the CNS, synaptotagmin II mRNAs were strongly expressed in spinal cord and brainstem. Synaptotagmin III mRNAs were weakly expressed throughout the nervous system. In the pituitary and adrenal glands synaptotagmin I transcripts were the major form.

The results are semiquantitatively summarized in Table 2. These data were compiled by evaluating both x-ray films (Fig. 1; see also Figs. 6, 8) and emulsion-coated slides (Figs. 2-5, 7).

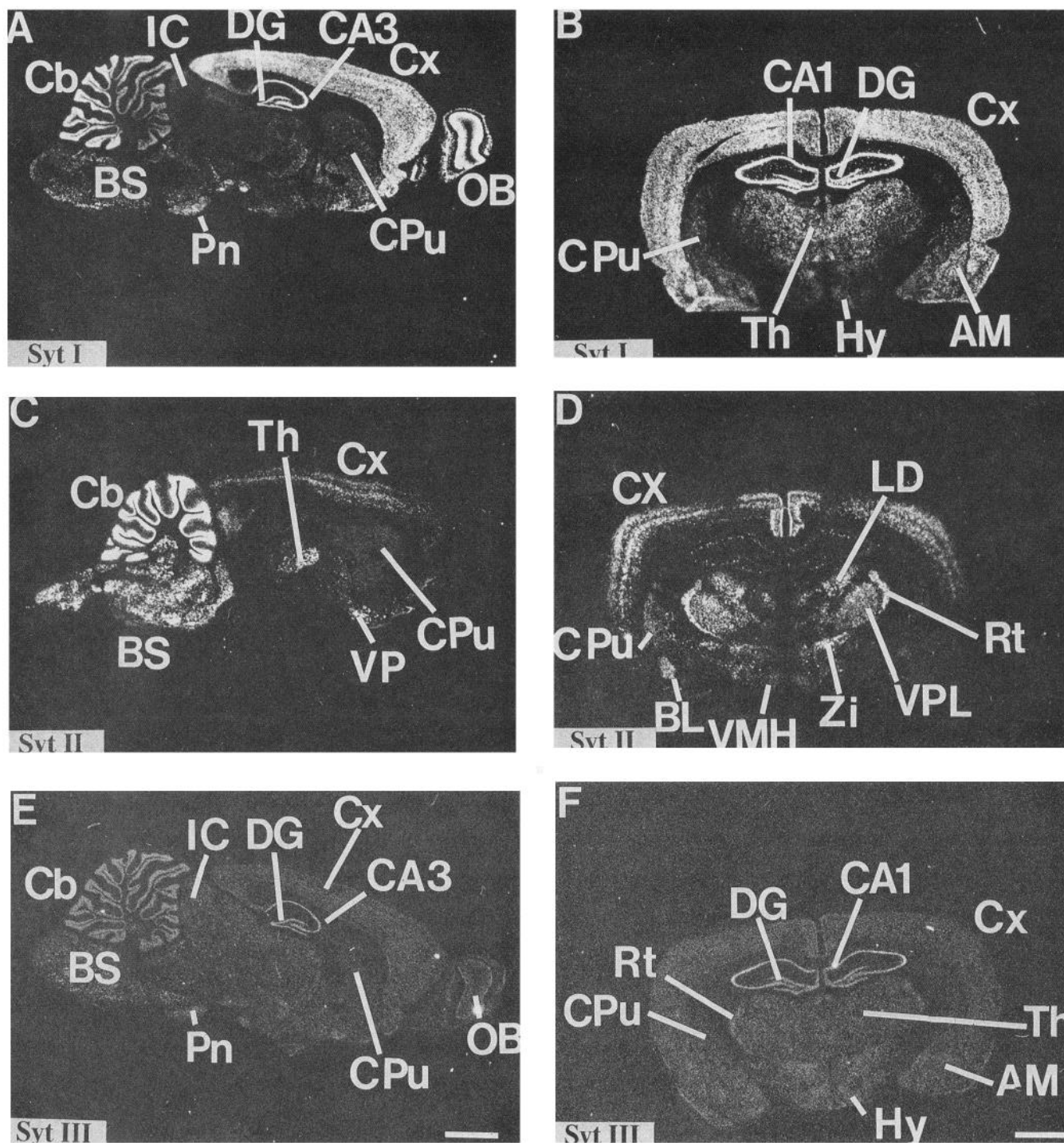


Figure 1. *In situ* hybridization of synaptotagmin I (A, B; *syt I*), II (C, D; *syt II*), and III (E, F; *syt III*) mRNAs in sagittal (A, C, E) and coronal (B, D, F) sections of rat brain. AM, amygdala; BL, basolateral amygdaloid nu.; BS, brainstem; Cb, cerebellum; Cpu, caudate putamen; Cx, cortex; DG, dentate gyrus; Hy, hypothalamus; IC, inferior colliculus; LD, laterodorsal thalamic nu.; OB, olfactory bulb; Pn, pontine nu.; Rt, reticular thalamic nu.; Th, thalamus; VMH, ventromedial hypothalamic nu.; VP, ventral pallidum; VPL, ventral posterolateral thalamic nu.; Zi, zona incerta. Exposure was on Kodak X-Omat film for 10 weeks. Scale bars: A, C, E, 3.3 mm; B, D, F, 2.4 mm.

Telencephalon

Olfactory bulb. Only synaptotagmin I and III mRNAs were detected in olfactory bulb tissue (Fig. 2). Strong labeling was obtained with the synaptotagmin I probe while synaptotagmin III hybridization was weak. In mitral cells synaptotagmin I-positive cells were heavily labeled (Fig. 2A). Transcripts specific for syn-

aptotagmin I and III were observed in the internal granule cells, in tufted cells of the external plexiform layer, and in the glomerular cell layer (Fig. 2B,F).

Cerebral cortex. Autoradiographs (Fig. 1) showed that all three mRNAs are expressed in the cortex but display different distributions. Synaptotagmin I (Figs. 1A,B; 3A) and III (Figs.

Table 2. Relative distribution of synaptotagmin I (Syt I), II (Syt II), and III (Syt III) mRNAs in different regions of the CNS and pituitary and adrenal gland of the rat

Area	Syt I	Syt II	Syt III
Olfactory bulb			
Periglomerular	+++	ND	+
External plexiform layer	+++	ND	+
Mitral cell layer	++++	ND	++
Internal granule cell layer	+++	ND	+
Neocortex			
Layer II/III	+++	ND	+
Layer IV	+++	++	+
Layer V/VI	+++	++	+
Hippocampus			
CA1 pyramidal cell layer	+++	some cells ++	++
CA3 pyramidal cell layer	+++	some cells ++	++
DG granule cells	++	some cells ++	++
Basal nuclei			
Caudate putamen	+	+	+
Globus pallidus	+	++	+
Amygdala			
Basolateral amygdaloid nu	++	++	+
Central amygdaloid nu., lateral	++	ND	+
Medial amygdaloid nu.	++	ND	+
Medial habenula	++	ND	+
Thalamus			
Central medial nu.	++	ND	+
Laterodorsal nu.	++	++	+
Ventral posterolateral nu.	+	++	+
Ventromedial nu.	++	ND	+
Reticular nu.	ND	++	+
Zona incerta	+	+	+
Hypothalamus	+	+	+
Colliculi			
Inferior	++	++	+
Superior	+	+	+
Cerebellum			
Molecular cell layer	+	+++	++
Purkinje cell layer	ND	+++	ND
Granule cell layer	+++	+++	++
	some cells +++++		
Pons/medulla	+	++	+
Pontine nu.	++	++	+
Spinal cord			
Dorsal horn	++	+++	++
Ventral horn	ND	+++	++
Pituitary gland			
Anterior lobe	++	ND	ND
Intermediate lobe	+++	ND	ND
Posterior lobe	ND	ND	ND
Adrenal gland	++	ND	ND
	some cells +++		

In situ hybridization signals obtained with ³⁵S-labeled oligonucleotide probes on sections were assessed as intense (++++), strongly positive (+++), positive (++), weakly positive (+), or not detectable (ND).

1E,F; 3C) transcripts were present in all areas and laminae explored of the cerebral cortex. Synaptotagmin I transcripts were the most abundant. Sparsely distributed cells displaying horizontally oriented perikarya situated in deep layer VI were strongly labeled with synaptotagmin I probes (data not shown). A restricted expression of synaptotagmin II mRNAs was found.

Synaptotagmin II transcripts were not detected in regions of the frontal (Fig. 1C) and perirhinal (Fig. 1D) cortex. On emulsion-dipped slides, labeling was confined to a few cells of layer IV, pyramidal cells of layer V (Fig. 3B), and some neurons of layer VI.

Hippocampus. In the hippocampus, distinct patterns of hy-

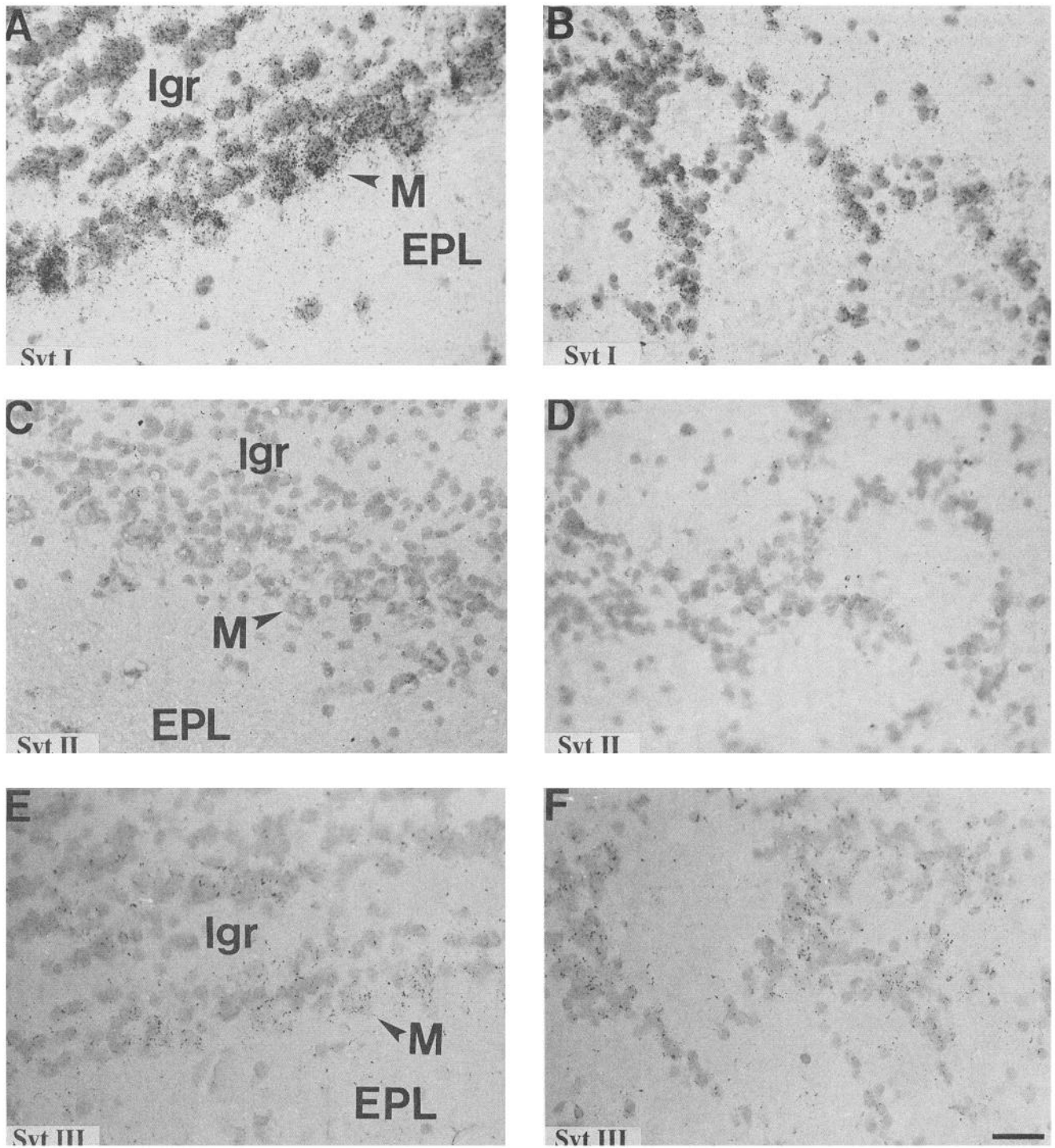


Figure 2. High-power bright-field photomicrographs of the olfactory bulb through the internal granular and mitral cell layers (*A, C, E*) and glomerular cell layer (*B, D, F*) showing the distribution of synaptotagmin I (*A, B*), synaptotagmin II (*C, D*), and synaptotagmin III (*E, F*) mRNAs. *Igr*, internal granular layer; *EPL*, external plexiform layer; *M*, mitral cell layer. Exposure times of the emulsion-coated slides were 7 weeks (*A–D*) and 10 weeks (*E, F*). Scale bars, 30 μ m.

bridization were again observed for the different transcripts. A strong hybridization signal was produced by synaptotagmin I probes in the CA1–CA4 areas and in the dentate gyrus (Fig. 1*A,B*). Localization of transcripts at the cellular level is shown in Figure 4. Synaptotagmin I-positive CA3 cells (Fig. 4*B*) could be seen in addition to labeled granule cells in the dentate gyrus (Fig. 4*A*). The same pattern of expression was seen for synap-

totagmin III, but transcripts were present in much lesser amounts (Fig. 4*E,F*). Sections hybridized with synaptotagmin II probes exhibited a confined pattern of labeling. Very few large neurons in the granule cell layer (Fig. 4*C*) and in the hilar region expressed the synaptotagmin II gene. Furthermore, a number of scattered cells in the CA4–CA1 pyramidal cell layer were also labeled (Fig. 4*D*). The frequency of cells expressing synaptotag-

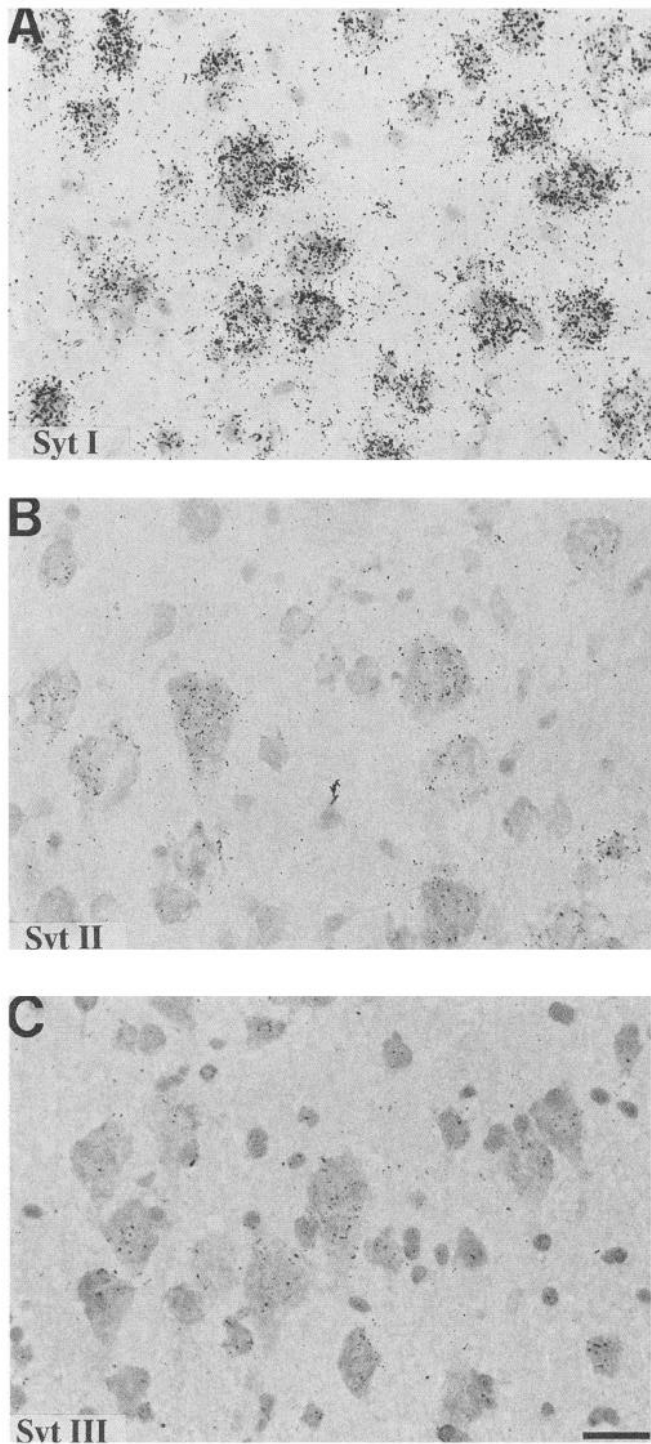


Figure 3. High-power bright-field photomicrographs of the cerebral cortex cell layer V, showing the distribution of synaptotagmin I (A), II (B), and III (C) transcripts. Exposure times were 7 weeks (A, B) and 8 weeks (C). Scale bars, 27 μ m.

min II gene was low throughout hippocampus: around 10–25 cells per 12 μ m sagittal section cut in the same plane as in Figure 1C.

Amygdala. Synaptotagmin I- and III-positive cell bodies (Fig. 1B,F, respectively) were found in all nuclei of the amygdaloid complex. Synaptotagmin I transcripts were again the major form.

Hybridization to synaptotagmin II mRNAs was restricted to the basolateral amygdaloid nucleus (Fig. 1D).

Striatum. Cells of the caudate putamen contained low levels of synaptotagmin mRNAs. In the globus pallidus (Fig. 1D) and ventral pallidum (Fig. 1C) synaptotagmin II transcripts were present at a moderate level.

Diencephalon

Thalamus–hypothalamus. Characteristic distributions for each of the synaptotagmin transcripts were seen in the thalamus. Synaptotagmin I transcripts were abundant in most nuclei of the thalamus observed, but were not seen in the reticular thalamic nucleus (Fig. 1A,B). Low levels of synaptotagmin III mRNAs were also present throughout the whole thalamus (Fig. 1E,F). Synaptotagmin II transcripts were restricted to certain nuclei such as the ventral posterolateral nucleus and the zona incerta (Fig. 1D). The reticular thalamic nucleus exhibited strong signals. This area appeared to contain only one type of neuron. These cells are thus likely to express both synaptotagmin II and III mRNAs. The rest of the diencephalon did not show a strong labeling. Synaptotagmin I (Fig. 1B) and III (Fig. 1F) transcripts were ubiquitously expressed in the hypothalamus although at a low level. The three mRNAs were present in the ventromedial hypothalamic nucleus (Fig. 1B,D,F).

Metencephalon

Cerebellum. Autoradiographs indicated that all of the transcripts were expressed in granule cells (Fig. 1A,C,E). Examination of sections exposed to photographic emulsion confirmed this observation (Fig. 5B,D,F). Furthermore, with synaptotagmin I probes, a subpopulation of neurons in the granule cell layer was more strongly labeled. These cells were scattered homogeneously in all granule cell layers in each lobule of the cerebellar cortex (Fig. 5B). It was not possible to determine the morphology of the positive cells but their frequency suggested that they were Golgi II cells (Shivers et al., 1986). Synaptotagmin I and III transcripts were not detectable in Purkinje cells (Fig. 5A,E, respectively). They were present most probably in the stellate/basket cells of the molecular cell layer (Fig. 5A,E). Synaptotagmin II was widely expressed through all cell layers (Fig. 5C,D).

Spinal cord

We processed sections of the spinal cord at cervical (Fig. 6) and thoracic (data not shown) levels. Synaptotagmin I transcripts were restricted to the dorsal horn of the spinal cord, possibly in layers I and II (Fig. 6A). Some scattered positive cells were found in layers V–VII. Labeling in the intermediolateral nucleus was visible on thoracic sections. The cellular localization with emulsion-coated sections confirmed these results. Putative motoneurons were devoid of labeling (Fig. 7A), while some positive neurons were seen in the dorsal horn (Fig. 7B). Synaptotagmin II mRNAs were abundant throughout the entire spinal cord section (Fig. 6B), showing strong expression in large cells in the ventral horn, probably motoneurons (Fig. 7C), and in all cells of the dorsal horn (Fig. 7D). Synaptotagmin III mRNA distribution followed the same pattern (Fig. 6C), but the level of hybridization was lower (Fig. 7E,F).

Endocrine cells

Pituitary gland. The autoradiograph in Figure 8A showed that synaptotagmin I transcripts were distributed throughout the anterior and intermediate lobe of the pituitary. Labeling was ho-

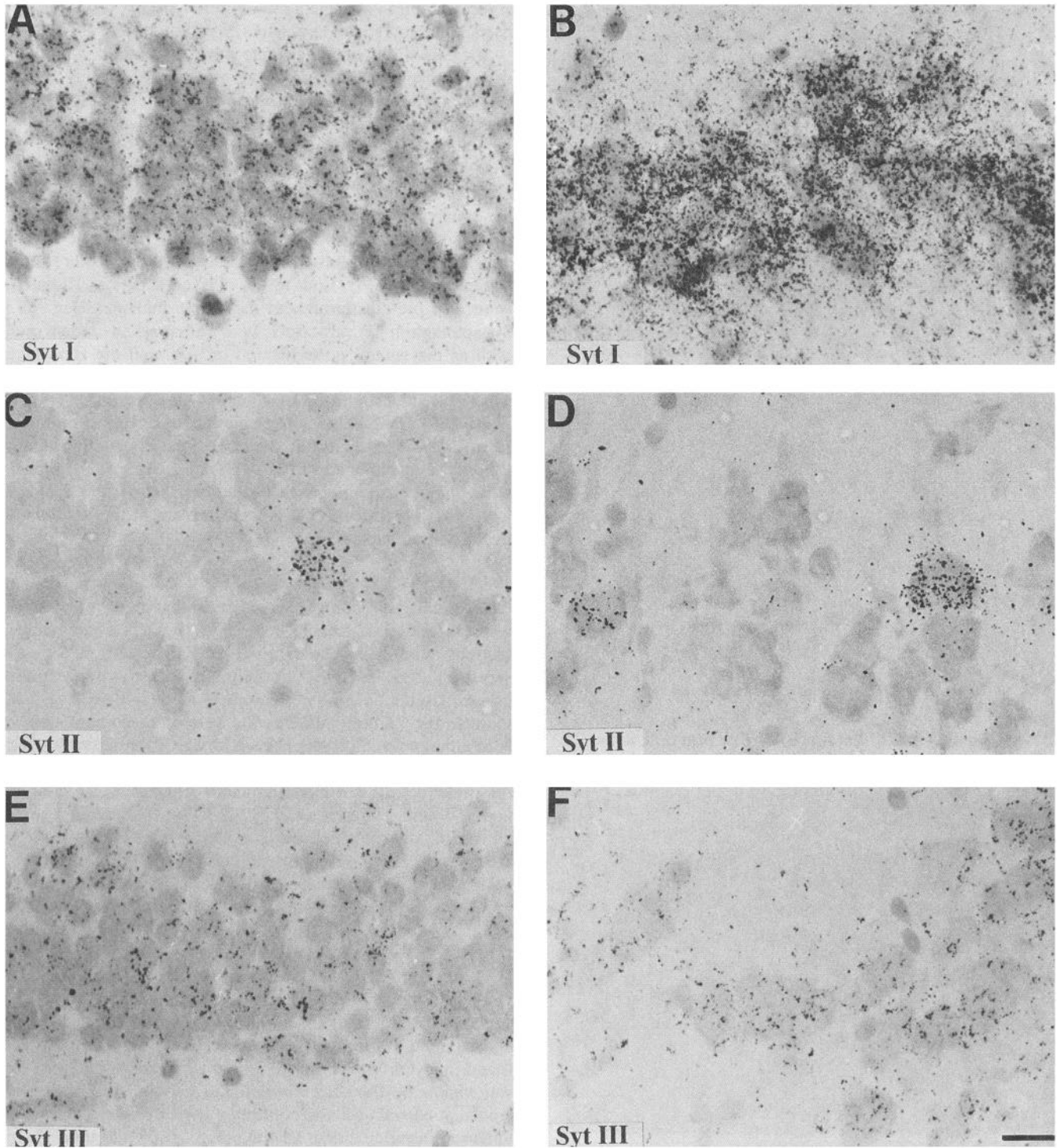


Figure 4. High-power bright-field photomicrographs of the hippocampus through the dentate gyrus (*A, C, E*) and the CA3 cell layer (*B, D, F*) illustrating the distribution of synaptotagmin I (*A, B*), II (*C, D*), and III (*E, F*) transcripts. Exposure times were 7 weeks (*A–D*) and 10 weeks (*E, F*). Scale bars, 15 μm .

mogeneous on all cells. No specific signals were detected with synaptotagmin II and III probes (data not shown).

Adrenal gland. Synaptotagmin I-positive cells were confined to the medulla (Fig. 8*B*). Some scattered cells were very strongly labeled. Two to four clusters of 2–6 cells were observed in each medullary section. Their size suggests that they are sympathetic ganglion cells described in the rat adrenal medulla (Dellmann

and Brown, 1981). No cells were specifically labeled with synaptotagmin II and III probes (data not shown).

Discussion

These *in situ* hybridization studies indicate a number of interesting features of the expression of synaptotagmin transcripts in the CNS and in endocrine cells.

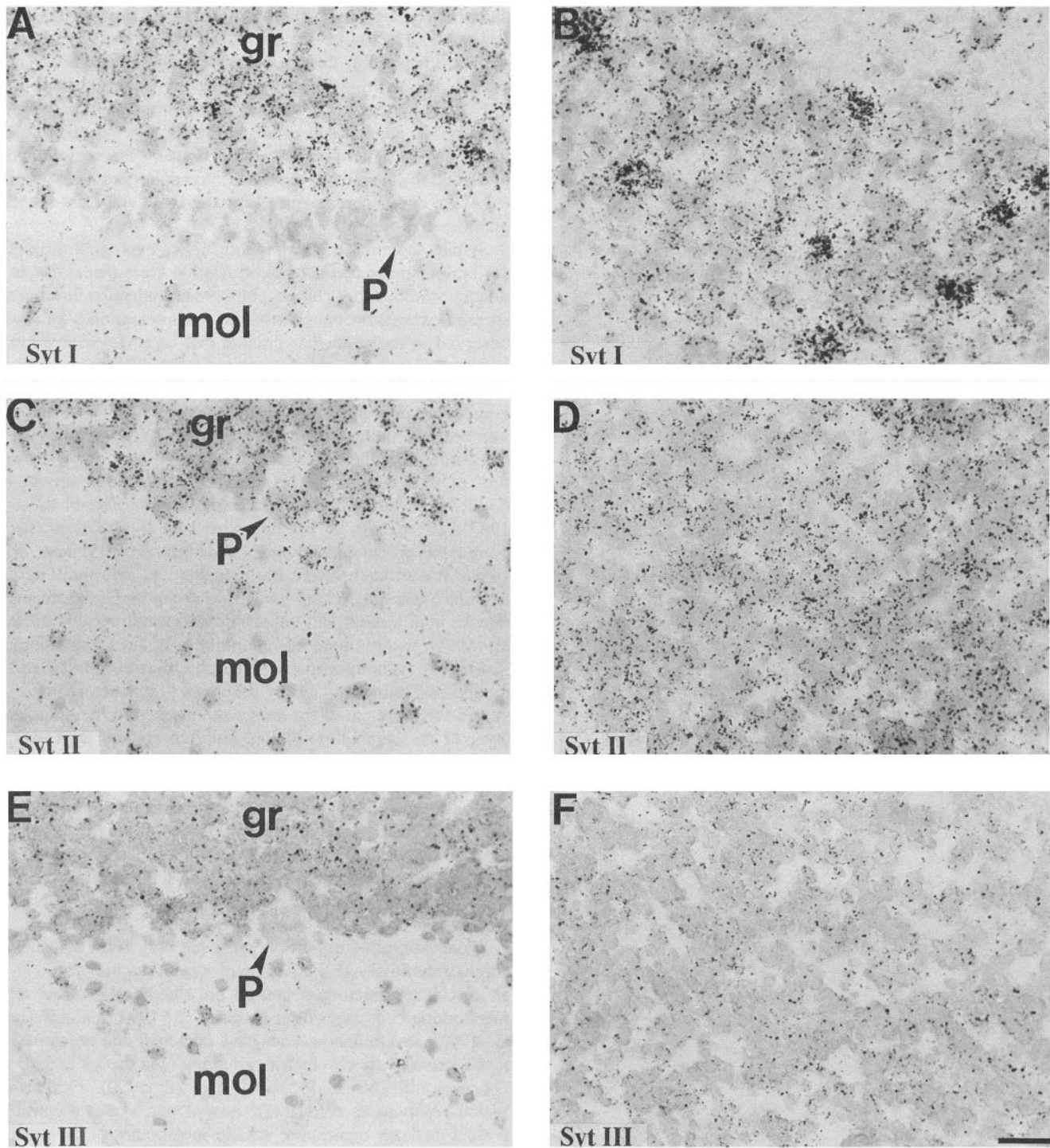


Figure 5. High-power bright-field photomicrographs of the cerebellum through the molecular and Purkinje cell layers (*A, C, E*) and granular cell layer (*B, D, F*), displaying the distribution of synaptotagmin I (*A, B*), II (*C, D*), and III (*E, F*) transcripts. *gr*, granular cell layer; *mol*, molecular layer; *P*, Purkinje cells (*arrows*). Exposure times were 7 weeks (*A–D*) and 8 weeks (*E, F*). Scale bars, 27 μ m.

Three variants of synaptotagmin have been characterized in rat brain, with a relatively low overall identity of amino acid sequence. However, the sequence corresponding to the C-terminal part of the protein thought to contain, in the case of synaptotagmin I, a Ca^{2+} /phospholipid binding site (Davletov et al., 1993), high-affinity binding sites for the neurexins (Perin, 1994), and the clathrin adaptor AP-2 (Zhang et al., 1994) is well con-

served. Synaptotagmin III is more distantly related to the two other variants. It shares the highest homology with o-p65C, one of the three isoforms characterized in the marine ray (Wendland et al., 1991). No differences in functional and biochemical properties between the synaptotagmins have been reported.

In situ hybridization demonstrated that each of the three synaptotagmin genes has a unique expression pattern. The synap-

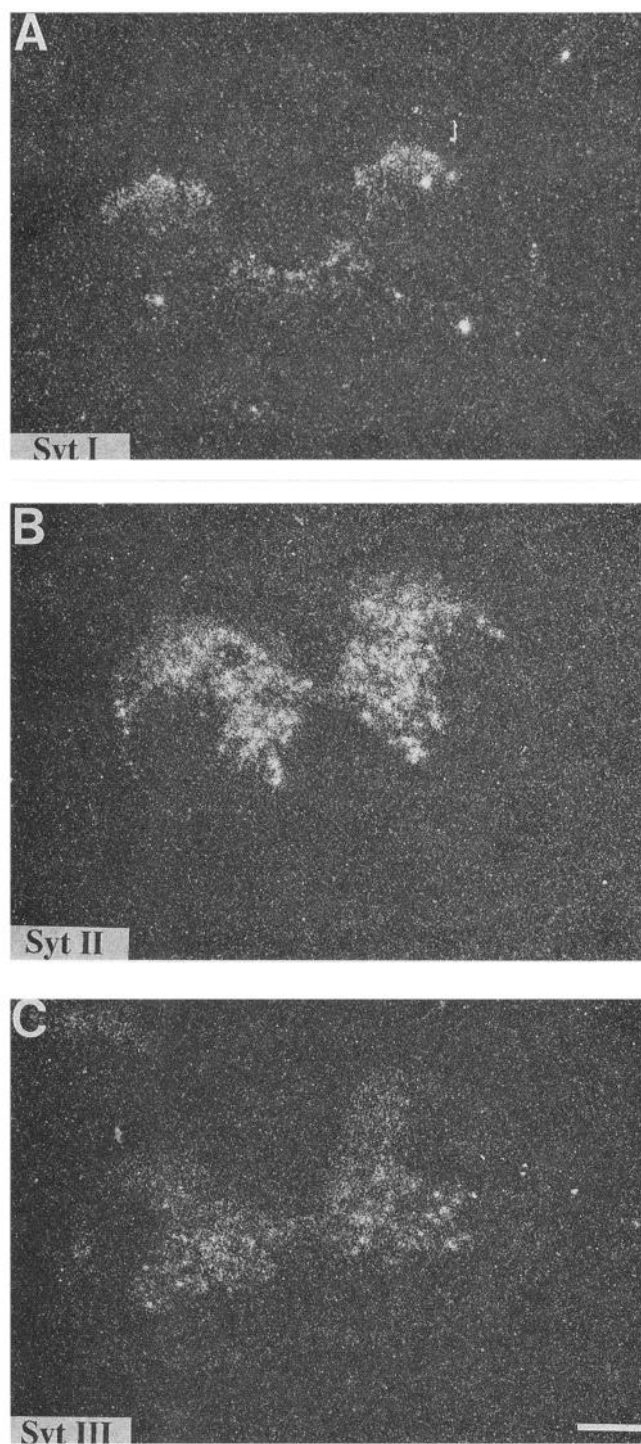


Figure 6. *In situ* hybridization of synaptotagmin I (A), II (B), and III (C) mRNAs in spinal cord. Sections are oriented with the dorsal horn towards the top of the figure. Exposures were on Kodak X-Omat film for 10 weeks. Scale bars, 0.5 mm.

tagmin III gene is the one expressed in most neurons, but transcripts are much less abundant than the products of the synaptotagmin I and II genes. Among the cells examined, only Purkinje cells were not labeled with synaptotagmin III probes. In the several endocrine cells explored no hybridization signal was visualized. However, occasionally on pituitary sections very weak specific hybridization was detected (data not shown). Al-

though Northern blots (Mizuta et al., 1994) suggest that synaptotagmin III mRNAs are expressed in adrenal and pituitary glands, it is possible that the oligonucleotide hybridization method was not sensitive enough to detect very low transcript levels. This study indicates that synaptotagmin I transcripts are the most abundant in adrenal and pituitary glands. It would therefore be interesting to determine if in these tissues synaptotagmin III is preferentially localized on small synaptic-like microvesicles (SLMV), and synaptotagmin I on the more abundant secretory granules.

Synaptotagmin I transcripts are highly expressed throughout the brain. Strong levels of hybridization were detectable in the olfactory bulb, cortex, hippocampus, and thalamus. Striking cell-specific expression patterns of the synaptotagmin I gene were observed in the cerebellar granule cell layer. In addition to the labeling of granule cells, prominent hybridization signals on scattered cell bodies were detected. These patches of silver grains probably correspond to the large perikarya of the Golgi interneurons that synapse on dendrites of numerous granule cells, inhibiting their activation by mossy fibers. A high proportion of Golgi cells are believed to use γ -aminobutyric acid (GABA) as an inhibitory neurotransmitter (Schulman et al., 1983). The pattern of labeling seen here is similar to that reported for proenkephalin mRNA distributions (Shivers et al., 1986). It should therefore be interesting to determine if Golgi neurons expressing a high number of synaptotagmin I transcripts release both GABA and enkephalins. Interestingly, Matthew et al. (1981), using a monoclonal antibody against a nonidentified isoform of synaptotagmin, and Takahashi et al. (1991), using a monoclonal antibody which recognizes synaptotagmin I, revealed the presence of the antigen in patches in the granule cell layer of the cerebellum which could correspond to Golgi cell synapses on granule cell dendrites.

In the most caudal part of the CNS, weak hybridization signals with synaptotagmin I probes were observed in the brainstem and spinal cord. In this area, only neurons of the dorsal horn are labeled. Our results are in good agreement with Northern blotting data of Geppert et al. (1991), who reported that the distribution of synaptotagmin I mRNA follows a rostrocaudal gradient.

Synaptotagmin I transcripts were the only form detectable in adrenal medullary cells. Although synaptotagmin's role in the exocytosis of secretory vesicles is not clearly established in neuroendocrine cells (see Shoji-Kasai et al., 1992, and Elferink et al., 1993), several laboratories have demonstrated synaptotagmin to be present on chromaffin granules (Matthew et al., 1981; Fournier and Trifaro, 1988; Perin et al., 1990). Furthermore, Walch-Solimena et al. (1993) showed that synaptotagmin I is present on large dense core vesicle membranes of the posterior pituitary. We observed the presence of synaptotagmin I mRNAs in the pituitary gland in agreement with Northern blotting data of Mizuta et al. (1994). They are homogeneously expressed among the anterior and intermediate pituitary cells. Thus synaptotagmin I may be involved in the release of the several peptide hormones from secretory granules. The hybridization level found in the intermediate pituitary was particularly strong. These cells are known to release peptides derived from the pro-opiomelanocortin precursor (POMC). A population of cells that synthesize POMC also occurs in the anterior pituitary, and peptide synthesis in the anterior and intermediate lobes is submitted to differential regulations (Eipper and Mains, 1980). Double-labeling experiments would allow a comparison of the regulation

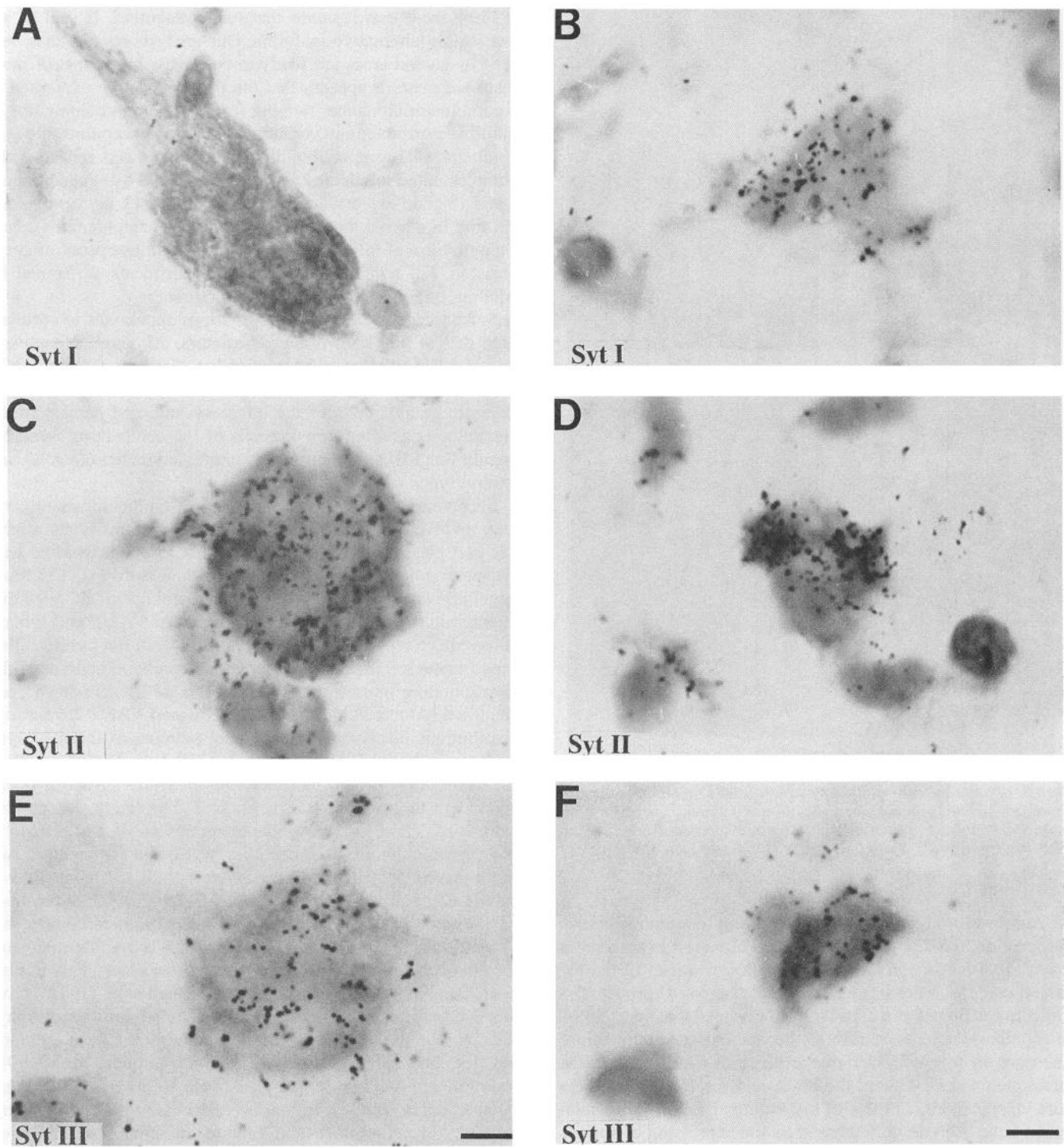


Figure 7. High-power bright-field photomicrographs of the ventral (*A, C, E*) and dorsal horn (*B, D, F*) cells of the spinal cord, showing the distribution of synaptotagmin I (*A, B*), II (*C, D*), and III (*E, F*) transcripts. Exposure times were 8 weeks (*A–D*) and 10 weeks (*E, F*). Scale bars: *A, C, E*, 7 μm ; *B, D, F*, 5 μm .

of synaptotagmin I and POMC gene expression in these two populations of cells.

Synaptotagmin II displays a very different pattern of expression. It is the major form in the caudal brain: cerebellum, brainstem, and spinal cord. Similar results were observed by Geppert et al. (1991), who described an inverse gradient of expression for synaptotagmin I and II genes. Synaptotagmin II mRNAs were strongly expressed throughout the entire cerebellum. It was

the only form detected in Purkinje cells. An unanticipated finding was that synaptotagmin II mRNAs were found in only subsets of forebrain neurons. In the posterior thalamus, synaptotagmin II transcripts were restricted to the ventral posteromedial, ventral posterolateral, and laterodorsal nuclei. Central regions were not labeled. Synaptotagmin II transcripts were abundant in the reticular thalamic nuclei while synaptotagmin I mRNAs were not detectable. This area appears to contain only one type of

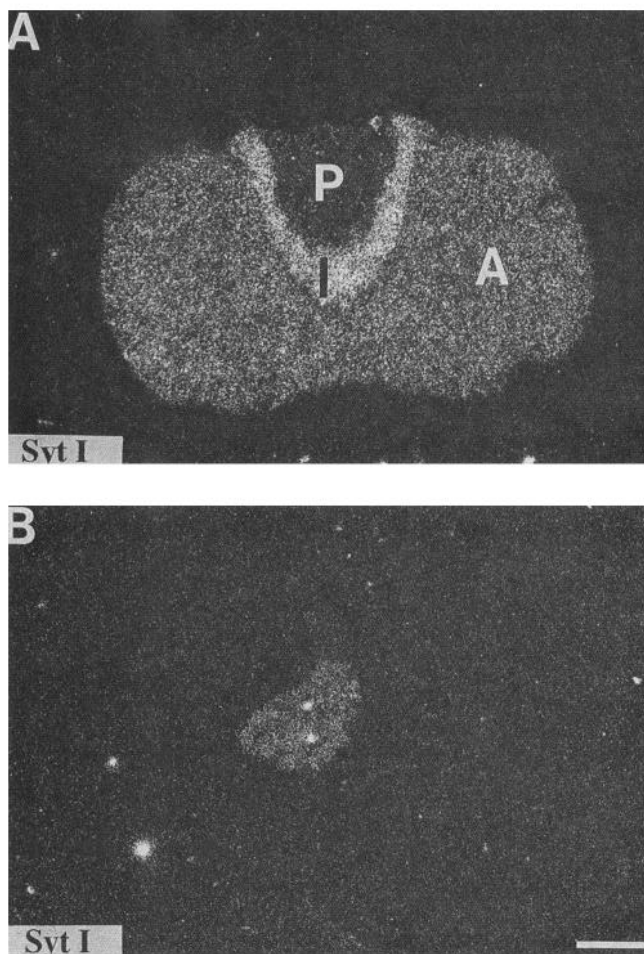


Figure 8. *In situ* hybridization of synaptotagmin I on sections through the pituitary (A) and adrenal gland (B). Pituitary gland: A, anterior lobe; I, intermediate lobe; P, posterior lobe. Exposure were on Kodak X-Omat film for 8 weeks (A, B) and 10 weeks (C). Scale bars, 0.5 mm.

GABAergic neuron (Jones, 1983) projecting to thalamic nuclei. Neuropeptide Y mRNAs have also been observed in these cells (Morris, 1989). Also, in amygdala only a few nuclei of the basolateral complex were labeled by synaptotagmin II probes. Experiments performed at a cellular level revealed that synaptotagmin II probes distinguish strikingly between discrete populations of neurons in layers IV–VI of the cerebral cortex and in the dentate granule cell region, the hilus, and the CA1–CA3 areas of the hippocampus. Subsets of hippocampal and cortical interneurons have been described based on the expression of various neurotransmitters, neuropeptides, and receptors. More recently, Weiser et al. (1994) labeled scattered neurons throughout the hippocampus with a probe for a specific isoform of a Shaw-related K^+ channel. Furthermore, transcripts coding for the NR2D subunit of the NMDA receptor show a very similar distribution (Monyer et al., 1994).

In spinal cord high levels of expression are seen in putative motoneurons, demonstrating that synaptotagmin II together with synaptotagmin III may be involved in neurotransmitter release at the neuromuscular junction. No hybridization signals were detectable in the endocrine cells explored. Identical results have been found in the adrenal gland by Northern blots (Geppert et al., 1991) and in PC12 cells (Shoji-Kasai et al., 1992).

From these data it seems that synaptotagmin I, II, and III are not simply alternative isoforms. Our analysis reveals that neurons in several areas are likely to coexpress transcripts of more than one gene. It appears that the three genes are expressed in a combinatorial manner ranging from expression of only one to multiple synaptotagmin variants. With a few exceptions, the synaptotagmin III gene is ubiquitously expressed and appears to be complemented in selected neurons of the CNS by synaptotagmin I and II gene expression; but in Purkinje cells (synaptotagmin II), and in adrenal and pituitary glands (synaptotagmin I), only a single form of mRNAs was detected. Definitive proof of coexpression will require colocalization experiments performed by labeling oligonucleotides with distinct tracers.

Synaptotagmin transcript distributions appear not to correlate with that of any known neuromediators. Although synaptotagmin II mRNAs seem to be confined in discrete, possibly inhibitory interneurons containing GABA, they are absent in GABAergic granule cells of the olfactory bulb and present in the glutamate-containing granule cells of the cerebellum. Synaptotagmin I and III genes are widely expressed in neurons releasing several types of neurotransmitter.

Synaptotagmin appears to be part of a multicomponent complex involved in neurotransmitter release. Several synaptic vesicle and plasma membrane proteins that associate *in vitro* with synaptotagmin are also present in multiple isoforms. The functional relevance of these variants is also unknown, although differences in interactions between isoforms of VAMP and syntaxin have been reported (Calakos et al., 1994). *In situ* hybridization experiments have revealed differential expression patterns of the corresponding transcripts. Two isoforms of VAMPs have been identified (Archer et al., 1990). VAMP 1 and VAMP 2 transcript distributions display similar regional patterns to those of synaptotagmin II and I, respectively. VAMP 1 transcripts are present in the most caudal part of the brain while VAMP 2 is expressed in the most rostral areas. It is thus likely that combinations of VAMP 1 with synaptotagmins II–III and VAMP 2 with synaptotagmins I–III mRNAs will occur in neurons. Another protein which associates *in vitro* with synaptotagmin is SNAP 25. A limited regional SNAP 25 mRNA distribution which has been reported by Oyler et al. (1989) resembles that of synaptotagmin I and III. Synaptophysin is another major integral membrane protein of small synaptic vesicles likely to play a role in neurosecretion (Betz, 1990). Bennett et al. (1992a) have found that synaptotagmin/p65 was associated with synaptophysin/p38 in a large protein complex solubilized from synaptic vesicles. Data from *in situ* hybridization (Marqu ze et al., 1991) and immunohistochemistry (Fykse et al., 1993) have shown that synaptophysin and synaptoporin genes are differentially expressed, resulting in several unique combinations. However, none of these patterns fully match the distribution of synaptotagmin mRNA.

Thus, our results on synaptotagmin transcript segregation will be particularly useful in attempts to identify native multicomponent complexes used by neurons and endocrine cells to perform secretion. It is likely that specific sets of synaptic and plasma membrane protein variants will confer different properties to the synapse. Further studies on colocalization of these various isoforms will be necessary in order to sort out the potential combinations that may be used by individual neurons. Cellular expression of multiple isoforms composing the secretory machinery will require complex genetic control during neuronal development and plasticity that it will be essential to understand.

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