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Mice lacking synaptophysin reproduce and form typical synaptic vesicles

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Abstract. Synaptophysin is one of the major integral membrane proteins of the small (30–50 nm diameter) electron-translucent transmitter-containing vesicles in neurons and of similar vesicles in neuroendocrine cells. Since its expression is tightly linked to the occurrence of these vesicle types, we mutated the X-chromosomally located synaptophysin gene in embryonic stem cells for the generation of synaptophysin-deficient mice in order to study the consequence of synaptophysin ablation for the formation and function of such vesicles in vivo. The behavior and appearance of mice lacking synaptophysin was indistinguishable from that of their litter mates and reproductive capacity was comparable to normal mice. Furthermore, no drastic compensatory changes were noted in the expression of several other neuronal polypeptides or in the mRNA levels of synaptophysin isoforms, the closely related neuronal synaptoporin/synaptophysinII, and the ubiquitous pantophysin. Immunofluorescence microscopy of several neuronal and neuroendocrine tissues showed that overall tissue architecture was maintained in the absence of synaptophysin, and that the distribution of other synaptic vesicle components was not visibly affected. In electron-microscopic preparations, large numbers of vesicles with a diameter of 39.9 nm and an electron-translucent interior were seen in synaptic regions of synaptophysin-deficient mice; these vesicles could be labeled by antibodies against synaptic vesicle proteins, such as synaptobrevin 2.

Key words: Synaptic vesicle – Synaptic vesicle protein – Synaptophysin – Neuroendocrine differentiation – Homologous recombination – Embryonal stem cell – Mouse (SV 129, C 57 BL/6, NMRI; cell clones, transgenic mice)

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Introduction

Synaptophysin was one of the first synaptic vesicle proteins for which specific antibodies were available (e.g., Jahn et al. 1985; Wiedenmann and Franke 1985) and whose complete cDNA was cloned (Leube et al. 1987; Südhof et al. 1987). These reagents have been valuable tools for the establishment of synaptophysin as a widely accepted marker for neuronal and neuroendocrine differentiation in tissue and tumor typing as it is an obligatory intrinsic membrane component of the abundant small (diameter between 30 and 50 nm) electron-translucent (SET) vesicles in these tissues (e.g., Jahn et al. 1985; Wiedenmann and Franke 1985; Wiedenmann et al. 1986; Navonne et al. 1986).

The function of synaptophysin is still subject to speculation. Since synaptophysin that has been reconstituted into planar lipid bilayers induces the formation of voltage-sensitive channels, it has been proposed that multimeric synaptophysin may form a fusion pore (Thomas et al. 1988). Furthermore, transmitter secretion can be inhibited either by synaptophysin antibodies or by synaptophysin antisense oligonucleotides (Alder et al. 1992a, b). The recently shown association of synaptophysin with synaptobrevin (Calakos and Scheller 1994, Edelmann et al. 1995; Washbourne et al. 1995), a component of the vesicle docking complex (Söllner et al. 1993), can be taken as another indication for the involvement of synaptophysin in regulated vesicle exocytosis. In addition, synaptophysin may be of importance for vesicle formation, since ectopic expression of synaptophysin in non-neuroendocrine cells of epithelial origin leads to the synthesis of distinct synaptophysin-rich vesicles (Leube et al. 1989, 1994).

In an attempt to learn more about the function(s) of synaptophysin, specifically with respect to vesicle trafficking and function we decided to disrupt the synaptophysin gene in embryonal stem (ES) cells by homologous recombination in order to generate synaptophysin-deficient mice. We show here that such mice are viable and fertile, and that normal-appearing synaptic vesicles are formed in these animals.

Materials and methods

Cloning of the murine synaptophysin gene and preparation of a targeting construct

The synaptophysin gene was isolated from a phage λ-Fix II library derived from SV129 mice (Stratagene, La Jolla, Calif.) by using the radioactively labeled *Bam*HI/*Hin*dIII insert of the rat synaptophysin-encoding cDNA clone pSR[5] (Leube et al. 1987).One of the isolated phage clones (λGSM1) was analyzed further by restriction enzyme mapping and nucleic acid sequencing and was shown to contain the 5′-end of the murine synaptophysin gene with its first three exons I, II and III, the two intervening introns 1 and 2, and part of intron 3, together with approximately 12.5 kb of the 5'-upstream region.

To prepare a targeting construct for homologous recombination, the 5.2-kb *Eco*RI-fragment containing the 5'-end of the murine synaptophysin gene was excised from phage λGSM1 and inserted into the Bluescript vector (plasmid pGSM10). A 500-bp *Spe*I/*Kpn*I segment comprising exon II and flanking intron sequences was further subcloned from pGSM10 into Bluescript (plasmid pGSM4). The synaptophysin-coding gene sequence was interrupted by the insertion of a 1.8-kb blunt-ended *Eco*RI/*Hin*dIII-casette, with the neomycin-resistance gene being under the control of the phosphoglycerate kinase promoter [from plasmid pGEM7(Neo), kindly provided by Dr. Thomas Magin, University of Bonn, Bonn, Germany] into the *Pst*I site located in exon II contained in pGSM4, thereby generating plasmid pGSM11. In parallel, plasmid clone pGSM18 was constructed by inserting the 3.2-kb *Eco*RI/*Sph*I fragment from pGSM10 into vector pSP72 (Promega, Madison, Wis.). The 2.5-kb *Spe*I/*Kpn*I insert of pGSM11 was then exchanged with the 500-bp *Spe*I/*Kpn*I segment of clone pGSM18, thus generating plasmid pGSM19. Subsequently, the 5'-located 5.25-kb *Eco*RI fragment was excised from phage λGSM1 and introduced with the correct orientation into pGSM19, resulting in plasmid pGSM20. The 11-kb *Cla*I/*Hin*dIII insert was then transferred into pBT/SP-TK(Xba#1), which contained the thymidine kinase gene under the control of the PYF41 enhancer and TK promoter (also kindly provided by Dr. Thomas Magin) to produce the final targeting construct pGSM21 as depicted in Fig. 1. This vector could be linearized with *Cla*I.

Establishment of synaptophysin-deficient ES cell clones and transgenic mice

ES cells (line R1, passage 11) were obtained from Dr. Andras Nagy (Mount Sinai Hospital, Toronto, Canada) and grown on embryonal feeder cells derived from fibroblasts (embryonal day 15) of either normal mice or neomycin-resistant transgenic mice (kindly provided by the animal facility of the EMBL, Heidelberg, Germany) under standard conditions (Nagy et al. 1993; Torres and Mansouri 1994) in the presence of 1000 U/ml leukemia inhibitory factor (ESGRO, Gibco BRL, Gaithersburg, Md.). Linearized plasmid DNA was transfected into ES cells (passage 13 or 14) by electroporation. In a typical experiment, 40 μg DNA were incubated with 10^7 trypsinized cells in 0.8 ml phosphate-buffered saline (PBS) in an electroporation cuvette, and electroporation was carried out with a Gene Pulser (Bio-Rad Laboratories, Hercules, Calif.) with one pulse of 500 μF and 24 V. After 24 h, G418 (Gibco BRL) was added at 300 μg/ml. Between days 6 and 9 after electroporation, gancyclovir (Cymoven; Syntex, Aachen, Germany) was added to a final concentration of 2 μM. Clones were selected with a drawn-out Pasteur pipette and seeded directly into 24-well dishes. Of the resulting confluent cultures, 75% were frozen, and DNA was prepared from the remaining cells after amplification of the cells as described (Torres and Mansouri 1994). For Southern blot analysis, the radioactively labeled 1.8-kb *Eco*RI/*Sph*I fragment of pGSM10 was used; this fragment reacted either with the wild type 5-kb or mutated 7-kb *Eco*RI fragment (see Fig. 1).

Clones containing a disrupted synaptophysin gene were thawed, and between 10 and 15 cells that were taken from an early passage were injected into blastocysts of C57BL/6 mice. These blastocysts were transplanted into NMRI pseudopregnant foster mothers following established procedures (e.g., Joyner 1993; Hogan et al. 1994). Chimeras were then bred with C57BL/6 mice to homozygosity under special pathogen-free conditions.

RNA preparation and Northern blotting

Brains were dissected and immediately disintegrated in 4 M guanidinium isothiocyanate buffer, in 0.1 M TRIS-HCl, pH 7.5, 10 mM dithiothreitole (DTT), 5 mM EDTA, with the help of a polytron. RNA was precipitated with half the volume of cold ethanol for 6 h at −20° C. After centrifugation (10000 *g*; 10 min), the pellet was resuspended in 7 M guanidinium-hydrochloride buffer (in 0.1 M sodium acetate, pH 5.5), 10 mM DTT, and extensively homogenized; nucleic acids were precipitated with ethanol. This cycle was repeated once more, and the final pellet was washed with 70% ethanol, dried, then dissolved in 10 mM TRIS-HCl (pH 7.0) containing 0.5% SDS, 10 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, 1 mg/ml proteinase K (Boehringer, Mannheim, Germany), and incubated for 2 h at 45° C. Residual proteins and proteinase K were removed by three cycles of phenol/chloroform extraction. Poly-A+-RNA was prepared by affinity chromatography using oligo(dT)-cellulose (Pharmacia, Uppsala, Sweden). Purified RNA was separated electrophoretically in formaldehyde-containing agarose gels and blotted onto filter membranes as described (Leube 1994, 1995). As the probe for synaptophysin, the *Eco*RI insert of the rat synaptophysin cDNA clone pSR[5] was radioactively labeled by the random primer labeling method using a kit (Amersham, Buckinghamshire, UK) and was hybridized in hybridization solution comprising 50% formamide, 5×SSC=150 mM NaCl/15 mM sodium citrate, pH 7.0), 5× Denhardt's solution (1× Denhardt's solution=0.02% bovine serum albumin/0.02% polyvinylpyrrolidone/0.02% Ficoll), 1% SDS, 100 μg/ml yeast tRNA, at 42° C. The most stringent wash was at 50° C in a buffer containing 0.1% SDS/0.1×SSC. To generate a probe for synaptoporin, a cDNA was first amplified from rat brain by reverse transcription and the polymerase chain rection (RT-PCR) with amplimers taken from the published cDNA sequence (Knaus et al. 1990). By using the resulting radioactively labeled fragment as a probe, a mouse synaptoporin cDNA was isolated from a λ-ZAP II cDNA library from mouse brain (Stratagene) by standard procedures (cf. Leube 1994). The resulting clone (pPO2) was shown by nucleic-acid sequencing to contain the entire coding sequence of synaptoporin and an additional 1.6 kb of the 3'-non-coding region. Following linearization with *Hin*dIII, a radioactively labeled, antisense transcript was produced with T3-RNA polymerase (cf. Leube 1994) and hybridized with the filter-bound RNA at 61° C in hybridization buffer overnight. Afterwards filters were washed extensively in 0.1% SDS/0.1×SSC at 72° C. For production of a pantophysin probe, the redundant primers SY-92502 and SY-92501 were used in an RT-PCR with RNA extracted from murine 3T3L1 fibroblasts as described recently (Leube 1994), and the nucleic-acid sequence of the cloned amplified product (clone pPhM1) was determined. Plasmid clone pPhM1 was linearized with *Hin*dIII prior to transcription with T7-RNA polymerase in the presence of ^{32}P-containing ribonucleotides. Hybridization and washing conditions were the same as for the detection of synaptoporin mRNA.

Immunoblotting and synaptosome preparation

Mouse brains were directly minced in protein solubilization buffer (2% SDS, 100 mM DTT, 60 mM TRIS-HCl, pH 6.8, 0.001% bromophenol blue, 10% glycerol) by 30 up and down strokes with a dounce homogenizer; large DNA fragments were destroyed by the

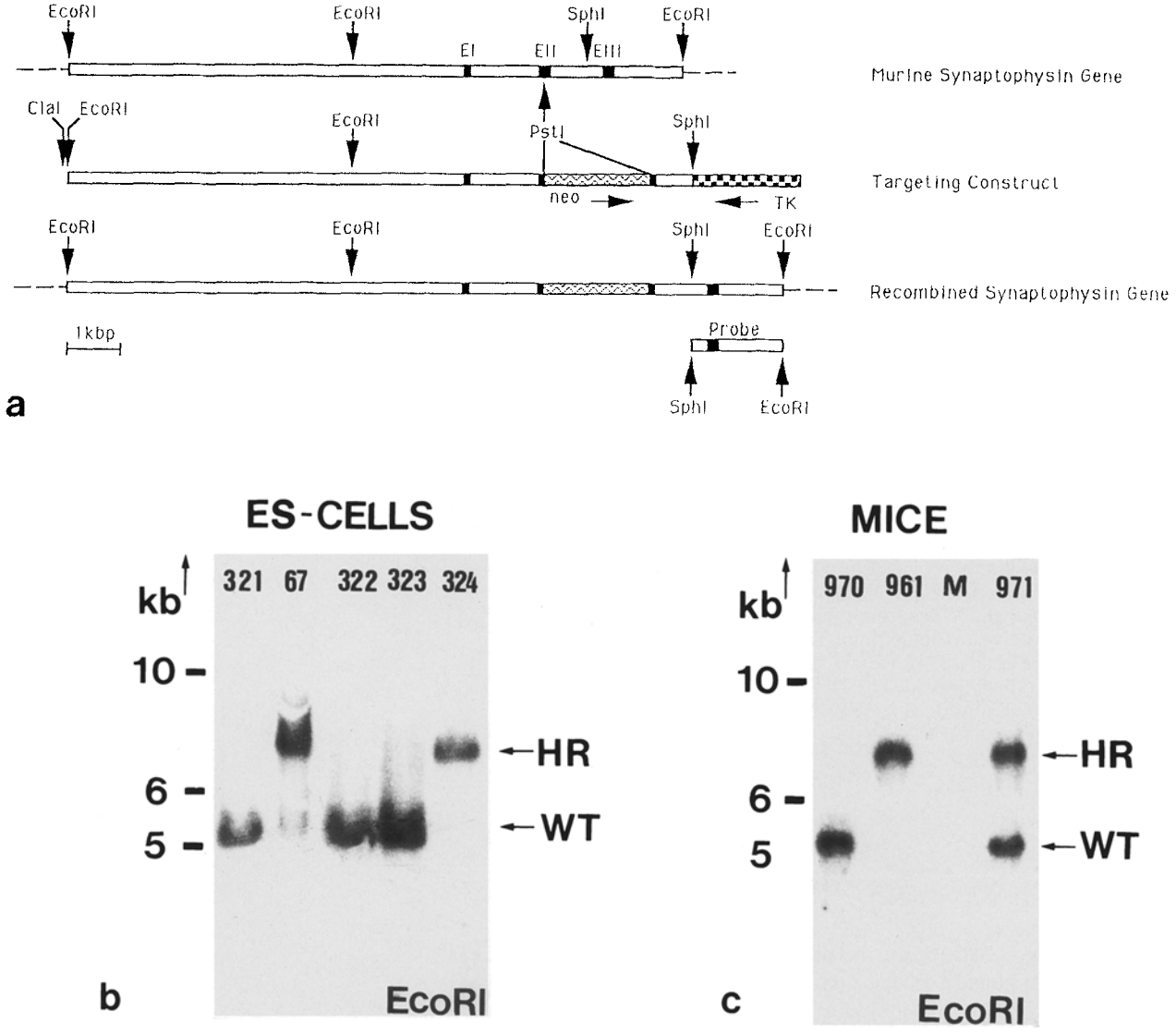

Fig. 1a–c. Targeting strategy for the disruption of the murine synaptophysin gene. **a** Map of the murine synaptophysin gene with its 5'-end encompassing the first three exons *EI*, *EII* and *EIII* (*black boxes*), the targeting construct, and the altered gene locus after homologous recombination. The interrupting sequence carrying the neomycin resistance gene is marked by *wavy lines*, the thymidine kinase gene (*TK*) used for negative selection is shown *right* of the targeting construct (direction of transcription is indicated by an *arrow* in each case). Positions of important restriction sites are marked. The probe used for Southern blot analysis located outside the targeting construct is also shown. *Interrupted lines* denote neighboring gene regions. **b** Southern blot analysis of embryonal stem cells (*ES-CELLS*) after transfection with the *Cla*I-linearized targeting construct and selection. The *Eco*RI fragment hybridizing to the probe shown in **a** is approximately 5 kb (*WT*) in wild-type cells (clones *321*, *322* and *323*), whereas it is about 7 kb (*HR*) in cells containing the mutated synaptophysin gene (clone *324*; clone *67* contained some contaminating normal cells resulting in a weak signal at 5 kb and had to be recloned prior to use). Positions and sizes (in kb) of co-electrophoresed size markers are given *left*. **c** Autoradiograph of a Southern blot with *Eco*RI-digested DNA obtained from mice that contain either a normal synaptophysin gene (male mouse *970*), an interrupted synaptophysin gene (male mouse *961*), or both (female mouse *971*) after hybridization with the probe shown in **a**. The size marker was loaded in *lane M* of the agarose gel with positions and size (in kb) of fragments denoted *left*

addition of benzonase (Merck, Darmstadt, Germany). The resulting solution was boiled for 10 min, following which the polypeptides were separated by SDS-polyacrylamide gel electrophoresis, blotted, and incubated with antibodies as described; immunoreactions were detected using an enhanced chemiluminescence system (cf. Leube 1994, 1995).

For synaptosomal preparations, brains were homogenized with a loose glass-teflon homogenizer (ten times up and down; 9000 rpm) in homogenization buffer (320 mM sucrose, 4 mM Hepes adjusted with NaOH to pH 7.3, 0.2 mM phenylmethylsulfonylfluoride, 1 μM pepstatin) and centrifuged at 1000 *g* for 10 min. The resulting supernatant was recentrifuged at 12000*g* for 15 min, and the pellet fraction was then resuspended in homogenization buffer. The crude synaptosomal pellet obtained after another centrifugation step (12000 *g*, 15 min) was used for further analyses.

Immunohistology and electron microscopy

Immunofluorescence-microscopic procedures were as described recently (Leube et al. 1994, Leube 1995). Cryosections (5 μm

thick) were fixed with a freshly prepared 2% formaldehyde solution in PBS for 20 min, treated subsequently with 0.1% saponin for 10 min, and incubated for another 10 min in PBS supplemented with 1% goat serum and 50 mM ammonium chloride. Alternatively, the cryosections were treated with acetone at −20° C for 10 min prior to antibody incubation. Secondary antibodies were coupled either to Texas Red or to cyanine Cy2 (Dianova, Hamburg, Germany; Biotrend, Köln, Germany). Electron microscopy and immuno-electron microscopy were performed as described (Wiedenmann and Franke 1985; Leube et al. 1994; Rose et al. 1995).

The following primary antibodies were used for immunodetection: murine monoclonal antibodies against synaptophysin [SY38 (Wiedenmann and Franke 1985; for epitope mapping, see Knaus and Betz 1990), SVP-38 (Sigma, St. Louis, Mo.)], SV2 (kindly provided by Dr. Kathleen Buckley, Harvard Medical School, Boston, Mass.; Buckley and Kelly 1985), 68-kDa neurofilament NF-L (clone NR 4; Boehringer, Mannheim, Germany), 160-kDa neurofilament NF-M (clone BF10; Boehringer), syntaxin (clone HPC-1; Sigma), SNAP-25 (clone SMI 81; Sternberger Monoclonals, Baltimore, Md.), secretory carrier membrane proteins (SCAMPs; antibody SG7C12; kindly provided by Dr. David Castle, University of Virginia, Charlottesville, Va.; cf. Brand et al. 1991), and synaptobrevin 2 (clone 69.1; kindly provided by Dr. Reinhard Jahn, Yale University, New Haven, Conn.; Edelmann et al. 1995), and affinity purified polyclonal antibodies from rabbit against the cytoplasmic carboxyterminus of synaptophysin (DAKO, Hamburg, Germany; see also Leube et al. 1994), the cytoplasmic aminoterminus of synaptophysin (kindly provided by Dr. Bertram Wiedenmann, Free University of Berlin, Berlin, Germany), and synapsin 1(a+b) (Biogenesis, Sandown, N.H.).

Results

Viability and fertility of synaptophysin-deficient mice

To prepare a targeting construct for the disruption of the murine synaptophysin gene, λ phage clones were isolated from a genomic phage library. One of the clones (λGSM1) was shown by nucleic acid sequencing to contain the first three exons of the synaptophysin gene together with approximately 12.5 kb of the 5'-flanking region. The positions of the intron/exon junctions were identical to those determined for the rat and human synaptophysin genes (Özcelik et al. 1990; Bargou and Leube 1991). From this clone, a targeting vector was constructed that comprised 7.5 kb of homology at the 5'-end and only 700 bp at the 3'-end. A 1.8-kb cassette with the neomycin gene under the control of the phosphoglycerate kinase promoter was inserted into the *Pst*I site of exon II for positive selection and the thymidine kinase gene with the PYF45 enhancer was added at the 3'-end to allow for negative selection (Fig. 1a). The 600 selected ES cell clones obtained in two independent electro-

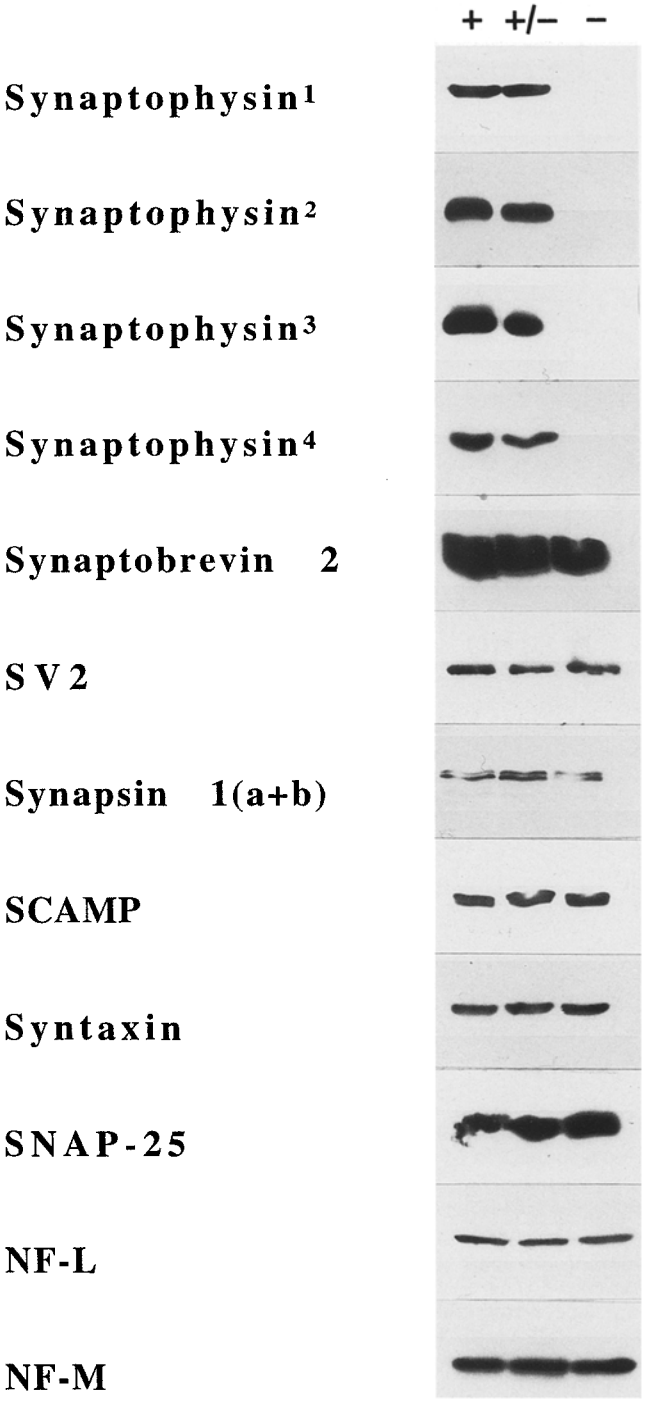

Fig. 2. Immunoblot analysis of total brain homogenates from normal male mice (+), heterozygous female mice (+/−) or from male mice with a mutated synaptophysin gene (−). Equal amounts of solubilized polypeptides were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. For detection of antibody binding, secondary antibodies coupled to horseradish peroxidase were used in combination with an enhanced chemiluminescence system. For identification of synaptophysin, the reactions of four different antibodies are shown: *1* Murine monoclonal antibody SY38 recognizing the cytoplasmic carboxyterminus; *2* affinity-purified antibodies from rabbit against a peptide located in the cytoplasmic carboxyterminus; *3* murine monoclonal antibody SVP38 from Sigma; *4* affinity-purified antibodies from rabbit against a peptide located in the cytoplasmic aminoterminus. In addition, antibodies were used for detection of the synaptic vesicle proteins synaptobrevin 2, SV2, synapsin 1(a+b), secretory carrier membrane proteins (SCAMP), the target membrane receptors syntaxin and SNAP-25, and the neurofilaments NF-L and NF-M

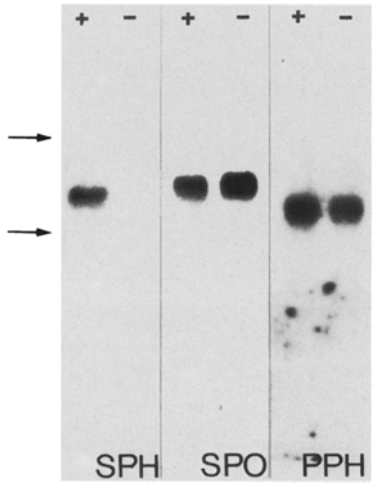

Fig. 3. Autoradiographs of Northern blots containing RNA extracted either from normal mouse brain (+) or from the brain of a male mouse with a disrupted synaptophysin gene (–); hybridization either to a random primer labeled probe for synaptophysin (*SPH*), a specific riboprobe for synaptoporin (*SPO*), or a cRNA probe for pantophysin (*PPH*). *Lanes 1, 2* 20 μg total RNA; *lanes 3, 4* 25 μg total RNA; *lanes 5, 6* 2 μg poly-A$^+$-RNA. Note the absence of any reactivity for synaptophysin in *lane 2* in the RNA taken from the mutant mouse. The positions of the 28S and 18S ribosomal RNAs are marked by *arrows* on the *left*

poration experiments using the *Cla*I-linearized targeting construct were examined by Southern blot hybridization of their *Eco*RI-digested DNA. Ten clones were shown to contain a recombined synaptophysin gene (see, for example, clone 324 in Fig. 1b). Since synaptophysin is located on the X chromosome (Öczelik et al. 1990), a single targeting event was sufficient to disrupt the gene in the male mouse blastocyst-derived R1 cells (Nagy et al. 1993). Three clones (including clone 324; see Fig. 1b) were selected for injection of blastocysts from C57BL/6 mice. Many chimeras with a high contribution of ES cells were obtained for each of these ES cell clones. The male chimeras were crossed with C57BL/6 females. *Agouti* female progeny (P generation) were heterozygous for the mutant synaptophysin allele and were bred with C57BL/6 males. In the following F1 generation, Southern blot hybridization experiments were performed to identify animals that were either homozygous males (e.g., mouse 961 in Fig. 1c) or heterozygous females (e.g., mouse 971 in Fig. 1c). Next, either homozygous males from the F1 generation or male chimeras were crossed with heterozygous females. From the resulting F2 generation, homozygous females were bred with homozygous males.

None of the mice lacking either one or both synaptophysin genes showed any visible abnormalities. Weight development was normal and overall behavior and mobility were apparently not affected. Furthermore, synaptophysin-deficient mice were able to reproduce and had normal litter sizes. No increased mortality was observed after birth and the oldest mouse is now 4.5 months old and healthy.

Expression of synaptic polypeptides in synaptophysin-deficient mice

To exclude that either a trace amount of synaptophysin or a mutant form of synaptophysin was still expressed in mice containing the mutated synaptophysin gene(s), extensive immunoblotting was performed using a panel of different antibodies directed against various epitopes, including the amino- and carboxyterminus of the synaptophysin molecule. None of these reagents detected synaptophysin or any other reactive polypeptide of different apparent mobility in total brain homogenates of homozygous mutant animals (Fig. 2). Furthermore, no significant synaptophysin mRNA levels or relevant RNAs of other sizes were seen in Northern blot hybridization experiments (Fig. 3). Taken together, these observations excluded that a partial or altered synaptophysin molecule resulted in an incomplete knockout.

In contrast to synaptophysin, expression of other synaptic vesicle proteins, including synaptobrevins, SV2, or synapsins, was clearly demonstrable in mutant mice (Fig. 2). Similarly, no significant differences in the expression levels were noted for SCAMPs, the target membrane receptors syntaxin or SNAP-25, or the neurofilament proteins NF-L and NF-M (Fig. 2).

Since no immunological reagents were available for the other known members of the synaptophysin gene family, synaptoporin (also referred to as synaptophysin II) and pantophysin, Northern blot hybridization experiments were performed with appropriate probes. No major differences in mRNA levels were detected in comparisons of normal with synaptophysin-deficient mice, although a slightly increased signal was noted for synaptoporin that was, however, still at least an order of magnitude weaker than that seen for synaptophysin (Fig. 3).

Synapses and synaptic vesicles in synaptophysin-deficient mice

No abnormalities in tissue architecture were noted in routine formalin sections of various brain regions that were subjected to a variety of histological staining procedures (not shown). In further analyses, the distribution of synaptic vesicle proteins was compared, by immunofluorescence microscopy, in tissue sections taken from normal and mutant animals. As an example, the typical staining patterns obtained in the cerebellum by using antibodies against synaptophysin, synaptobrevin 2, and SV2 are shown in Fig. 4. Multipunctate labeling of glomeruli in the granule cell layer and dense "dotted" staining of the molecular cell layer was seen in all instances (for a comparison, see also Leclerc et al. 1989; Marquèze-Pouey et al. 1991; Fykse et al. 1993). The spatial organization of neurons was particularly evident in the retina, and double immunofluorescence microscopy demonstrated that synaptobrevin 2 immunoreactivity in synaptophysin-deficient mice remained restricted to the inner and outer plexiform layers, i.e., the regions in which synaptic contacts are formed (Fig. 5; for a com-

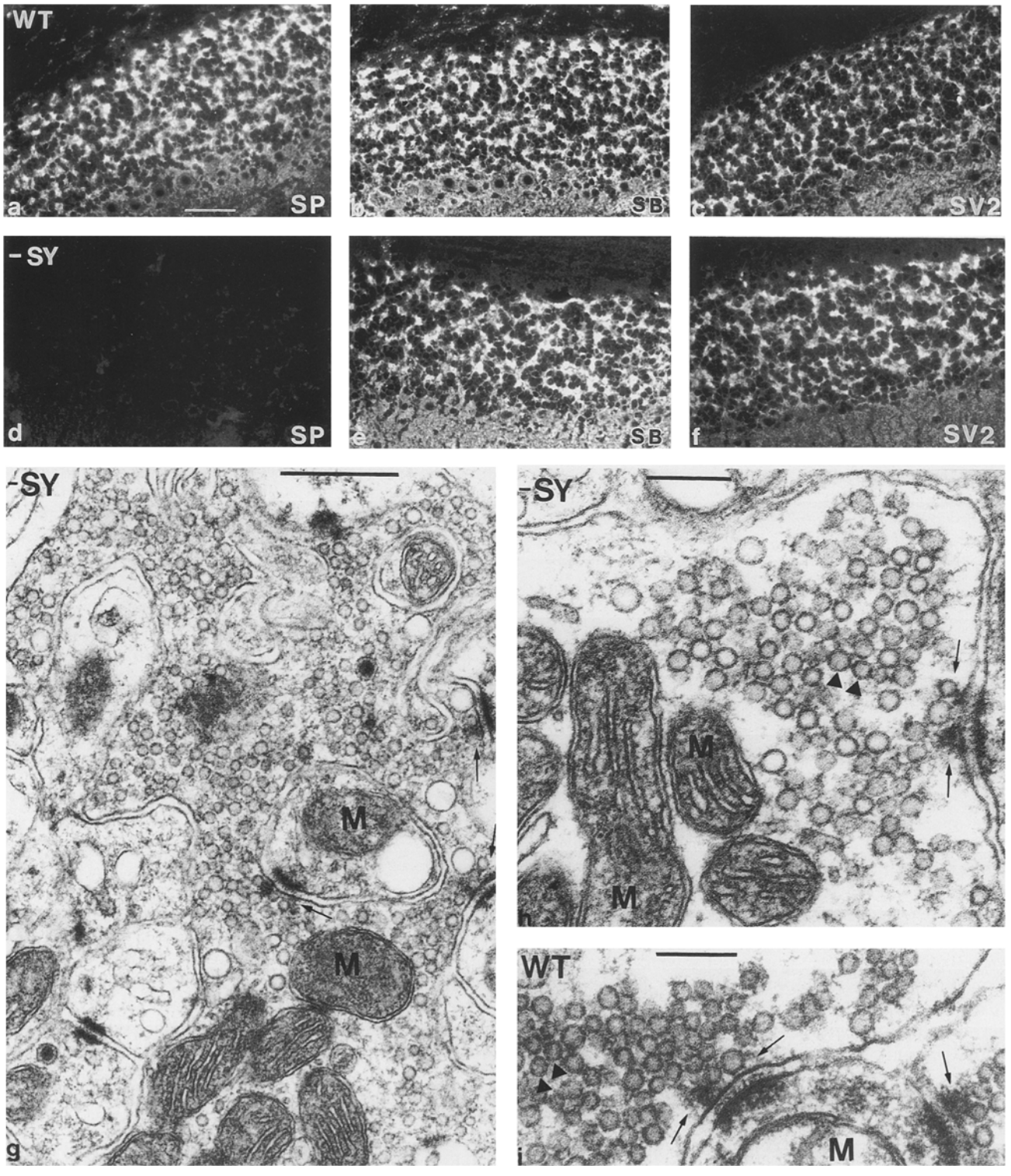

Fig. 4a–i. Immunohistology and electron microscopy of mouse cerebellar cortex. **a–f** Immunofluorescence microscopy of 5-µm-thick acetone-fixed cryosections showing the distribution of synaptophysin (*SP*), synaptobrevin 2 (*SB*) and *SV2* in normal (*WT*; **a–c**) and synaptophysin-deficient (*-SY*; **d–f**) mice. Note the labeling of the molecular (*lower part* of each micrograph) and granule cell layers in **a–c** and **e**, **f** in contrast to the lack of fluorescence using the monoclonal synaptophysin antibody SY38 in the mutant mouse (**d**). *Bar*: 50 µm in **a**; same magnification in **b–f**. **g–i** Ultrastructure of regions in the cerebellar glomeruli of a mutant mouse (*-SY*) in comparison with a similar region of a wild-type mouse (*WT*). The survey micrograph in **g** shows the abundance of similarly, sized small electron-translucent (SET) vesicles in a cerebellar glomerulus. **h**, **i** Higher magnifications to depict ultrastructural details. Note the almost identical size of the vesicles, the close apposition of individual vesicles (*arrowheads* in **h**, **i**) and the normal appearance of the active zones with docked vesicles (*arrows* in **g–i**) in the mutant and normal mouse. *M* Mitochondrion. *Bars:* 500 nm in **g**; 100 nm in **h**, **i**

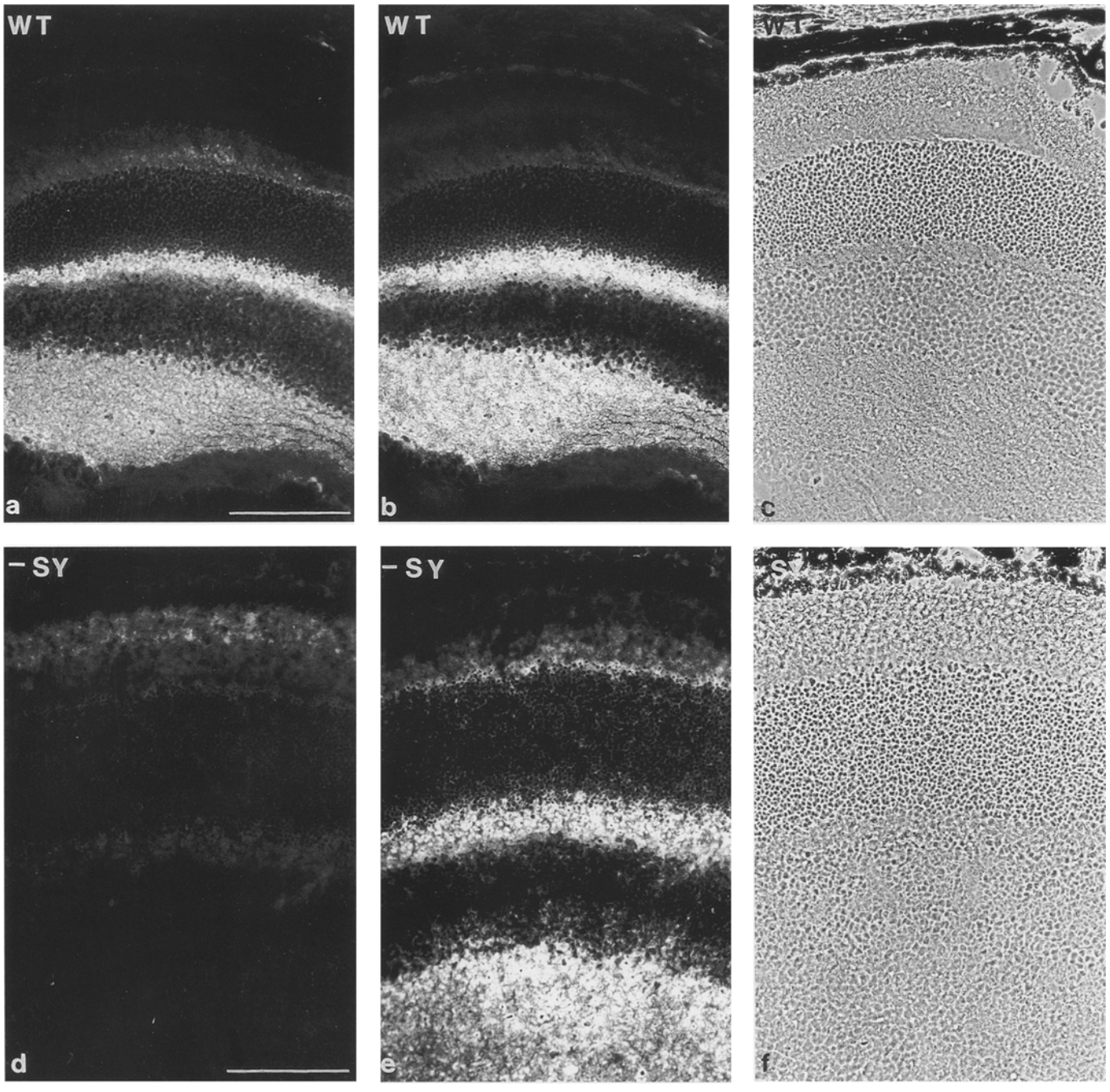

Fig. 5a–f. Double-label immunofluorescence microscopy of formaldehyde-fixed tissue sections from retina of a normal (*WT*) and synaptophysin-deficient (*-SY*) mouse with antibodies against synaptophysin (**a**, **d** rabbit antibodies against the cytoplasmic carboxyterminus) and synaptobrevin 2 (**b**, **e**). The corresponding phase-contrast images are shown in (**c**) and (**f**), respectively. Note the strong labeling of the inner (*bottom*) and outer (*center*) plexiform layers in **a**, **b**, **e** and the lack of specific fluorescence in **d**. Weak autofluorescence can be observed in the pigment epithelium (*top* in **a**, **b**, **d**, **e**). *Bars:* 50 μm

parison, see also Wiedenmann and Franke 1985; Kivelä et al. 1989).

Next, detailed electron microscopy was performed on the synaptic-vesicle-rich glomeruli of the granular cerebellar cell layer, a region known to contain little or no synaptoporin (Marquèze-Pouey et al. 1991; Fykse et al. 1993). Such regions were easily identified in synaptophysin-deficient mice (Fig. 4g; for a comparison, see Rose et al. 1995) and shown to be packed with synaptic-like vesicles at a density of 166 vesicles/μm^2 comparable to that found in wild-type mice (187 vesicles μm^2). These vesicles were homogeneous in size, with a mean diameter of 39.9 nm and an electron-translucent interior, and were very similar to those in normal mice (mean diameter of 42.5 nm; compare Fig. 4h and i). Some vesicles were docked at active zones that were

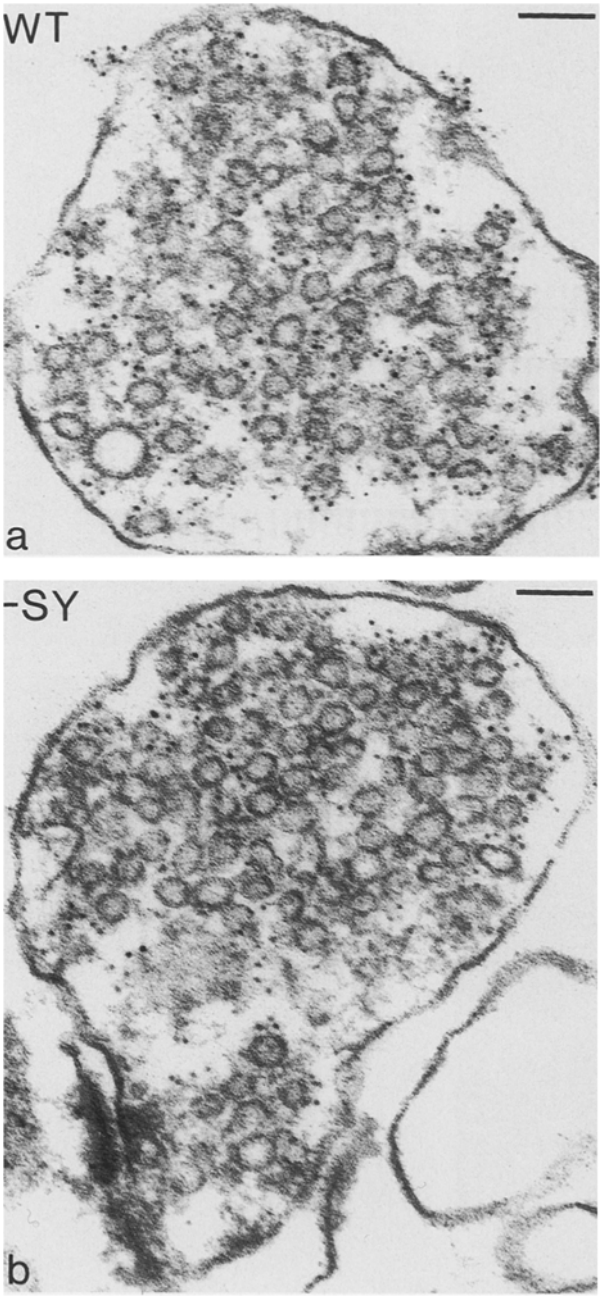

Fig. 6a, b. Immuno-electron microscopy of synaptosomes showing the presence of synaptobrevin 2 in synaptic vesicles of either a normal mouse (*WT*, **a**) or a mouse lacking synaptophysin (*-SY*; **b**). Note the similar vesicle size in both preparations and the specific labeling of some vesicles with immunogold particles. *Bars:* 100 nm

next to synaptic clefts with typical postsynaptic densities (compare regions demarcated by arrows in Fig. 4g, h and Fig. 4i).

To determine whether the abundant vesicles seen in synaptic regions of synaptophysin-deficient mice were also compositionally related to synaptic vesicles in normal mice, immuno-electron microscopy was performed using antibodies against different vesicle proteins. Synaptosomes containing vesicles that were specifically labeled by antibodies directed against synaptobrevin 2 were compared. No differences could be seen (Fig. 6a, b).

Neuroendocrine tissues in synaptophysin-deficient mice

Synaptophysin is abundantly expressed in the adrenal medulla where it occurs not only in SET vesicles, but also in certain catecholamine-containing, small dense-core vesicles (see Bauerfeind et al. 1995). Immunoreactivity to synaptobrevin 2 is also restricted to the adrenal medulla in mutant mice (Fig. 7e). Finally, pancreatic islet cells as a typical neuroepithelial cell type have also been examined by double immunofluorescence microscopy using different antibodies. Again, a restrictive multipunctate distribution is seen in islet cells and in neurons with synaptobrevin 2 antibodies in normal and in synaptophysin-deficient mice (Fig. 8).

Discussion

We have shown that the abundant vesicle protein synaptophysin is not essential; neuronal and neuroendocrine tissues develop normally and contain apparently normal synaptic vesicles in mice that exhibit no synaptophysin. This is an unexpected finding given the abundant and ubiquitous expression of synaptophysin in synaptic vesicles and certain secretory vesicles of neuroendocrine tissues, although a similar lack of dramatic phenotypic alterations has been observed also for other synaptic vesicle proteins that are, however, only reversibly attached to synaptic vesicles (Rosahl et al. 1993, 1995; Geppert et al. 1994). It is also not clear why antibodies against synaptophysin or synaptophysin antisense oligonucleotides reduce transmitter secretion significantly (Alder et al. 1992a, b), whereas the complete absence or synaptophysin in our experiments does not result in overt phenotypic alterations.

Since synaptophysin is a member of a polypeptide family (cf. Leube 1994), it is possible that related polypeptides substitute for the deleted functions in our mutant mice. Although we have not detected dramatic changes in the mRNA levels of synaptoporin and pantophysin, it cannot be excluded that differences in polypeptide levels may compensate for the gene loss. Nevertheless, expression of the structurally related SCAMPs (Brand and Castle 1993) is also not affected (Fig. 2). It is possible, however, that other, as yet unidentified, members of the synaptophysin gene family (for synaptogyrins as potential candidates, see Südhof 1995) exist or that, alternatively, unrelated molecules may act in this phenotypic rescue.

Another outcome of the knockout experiment is that synaptic vesicles of characteristic dimension, morphology, and polypeptide composition are formed even in the absence of synaptophysin. Although synaptophysin had been shown to induce the formation of certain vesicles in non-neuroendocrine cells (Leube et al. 1989, Leube et

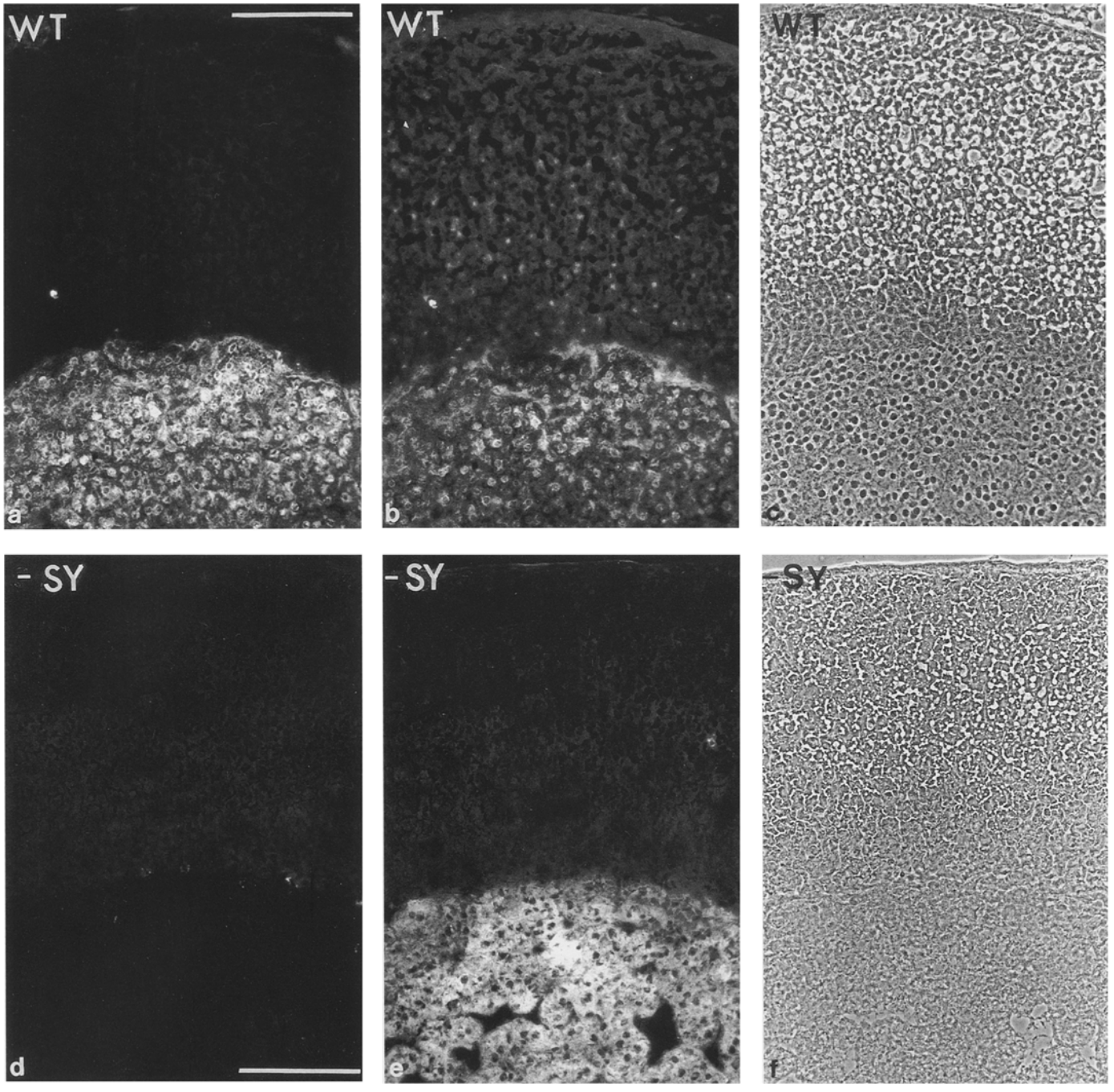

Fig. 7a–f. Double-label immunofluorescence microscopy of adrenal glands (formaldehyde fixation) depicting the co-localization of synaptophysin (**a**, **d**) and synaptobrevin 2 (**b**, **e**) in the adrenal medulla in a normal mouse (*WT*) compared with a mouse lacking synaptophysin (*-SY*). Note the strong reactivity of the medulla with synaptobrevin 2 antibodies (**e**) in contrast to the absence of synaptophysin immunoreactivity (**d** rabbit antibodies against the cytoplasmic carboxyterminus). Phase-contrast images are shown in **c** and **f**. *Bars:* 50 μm

al. 1994), it is clearly not needed for the formation of synaptic vesicles in neurons. These seemingly contradictory results may be attributable to the structural and functional redundancy in neurons that guarantees the maintenance of vital body functions, such as synaptic transmission, which relies on the presence of synaptic vesicles.

Our mutant mice should help to clarify the significance of the recently reported strong association of synaptobrevins, especially of synaptobrevin 2, with synaptophysin (Calakos and Scheller 1994; Edelmann et al. 1995; Washbourne et al. 1995). Remarkably, the distribution and localization of synaptobrevin 2 is not altered in mutant mice. Only an increased intensity of synapto-

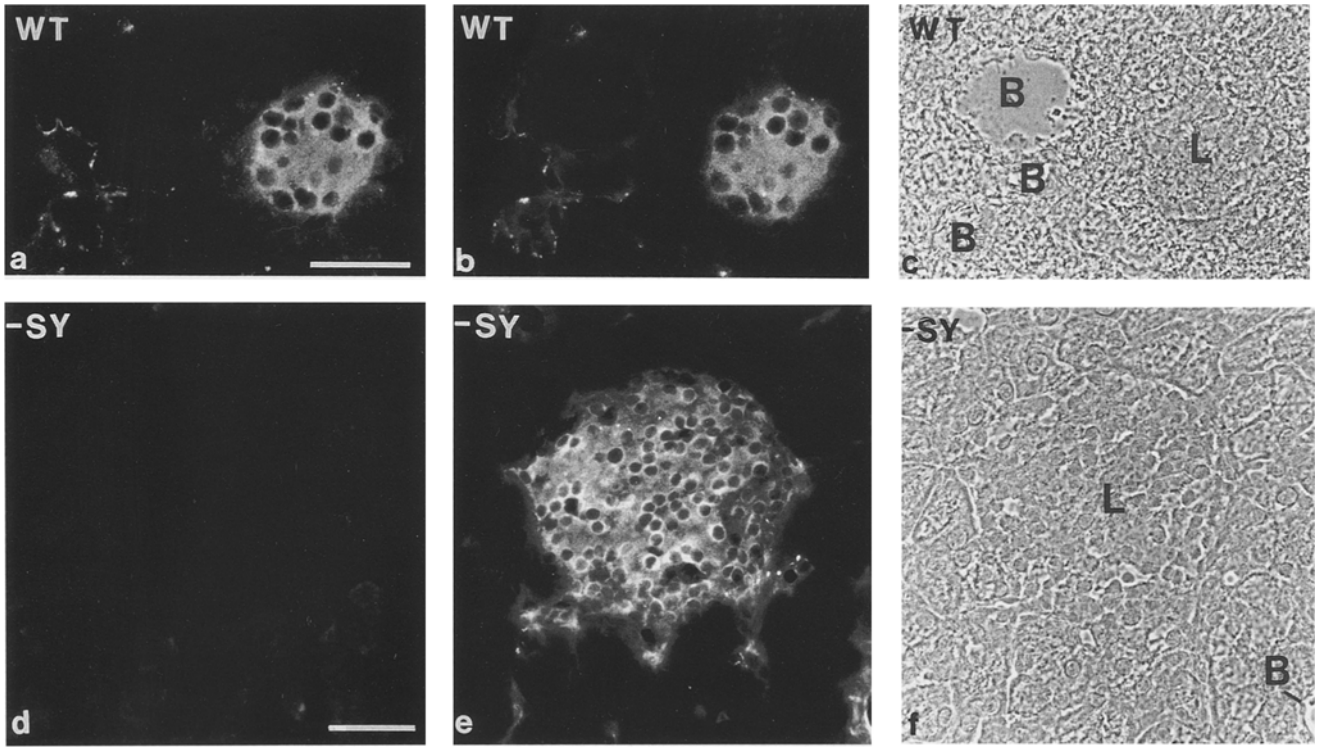

Fig. 8a–f. Localization of synaptophysin (**a**, **d**) and synaptobrevin 2 (**b**, **e**) by double-label immunofluorescence microscopy in formaldehyde-fixed sections of pancreas taken either from a normal (*WT*) or a mutant (*-SY*) mouse. Blood vessels (*B*) containing nerve fibers and the islets of Langerhans (*L*) are labeled; cf. the phase-contrast images (**c**, **f**). Note that, whereas synaptophysin cannot be detected in the mutant mouse with specific rabbit antibodies against the carboxyterminus (**d**), the large islet and the nerve fiber (*lower right corner*) are still strongly labeled by synaptobrevin 2 antibodies in **e**. *Bars:* 50 μm

brevin staining has been noted in synaptophysin-deficient mice, most markedly in the adrenal medulla (compare Fig. 7b and e). It will therefore be of interest to compare the quantity of synaptobrevin 2 molecules in different tissues between normal and synaptophysin-deficient mice. Furthermore, is synaptobrevin 2 bound to an alternative partner, such as synaptoporin, pantophysin, a SCAMP, or another polypeptide in synaptophysin-deficient mice?

Nevertheless, in spite of their normal appearance, synaptophysin-deficient mice may have certain subtle defects in vesicle recycling and synaptic transmission that are not crucial for vital functions but that may still be important for specific tasks, such as certain forms of regulated secretion, neuroendocrine differentiation, or higher brain functions, and were not detectable by the methods used in this study.

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Note added in proof. After acceptance of the manuscript we obtained from Dr. Heinrich Betz (Max Planck Institute, Frankfurt, Germany) an aliquot of rabbit antiserum Cy[4] that was raised against a recombinant fusion protein containing the cytoplasmic carboxyterminus of rat synaptoporin (Knaus et al. 1990). This serum recognizes a polypeptide of M_r ~35,000 by immunoblotting at similar levels in total brain extracts from normal and synaptophysin-deficient mice.