3 METHODS

3.1 PREPARATION OF SYNAPTOSOMES AND SYNAPTIC VESICLES FROM RAT BRAIN

3.1.3 PREPARATION OF SYNAPTOSOMES

Isolated nerve terminals (synaptosomes) were prepared from adult and embryonic Wistar rat whole brains or isolated brain areas following the procedure described by Edelmann et al. (1995). Mostly, pregnant female rats and their embryos were used, although synaptosomes were also occasionally prepared from male rats. Usually, one rat was sufficient for a synaptosomal preparation. The rat was anaesthetised with ether and immediately sacrificed by decapitation. The adult brain was subsequently removed as quickly as possible and instantly placed in ice-cold sucrose solution (see Section 2.3). Pregnant females generally carried between 15 and 18 embryoes, which were removed and placed on foil-covered ice. Embryonic brains were isolated as fast as possible and pooled in ice-cold sucrose solution. All subsequent steps were then performed on ice or at 4 °C.

Adult or embryonic rat brains were homogenised in 15 ml sucrose solution using a Dounce homogeniser (900 rpm, 9 strokes), and subsequently centrifuged at 1,200 g for 2 minutes in a Beckman 70 Ti rotor. The resultant pellet containing nuclei and unlysed cells was usually discarded, and the supernatant (postnuclear supernatant, or PNS) centrifuged for 12 minutes at 13,000 g to obtain a crude synaptosomal pellet (pellet 2, or P2).

To prepare purified synaptosomes, the crude synaptosomal pellet was reconstituted in sucrose solution and loaded onto a Ficoll gradient made up of three layers containing 6% (4 ml), 9 % (1 ml) and 13 % (4 ml) Ficoll® 400, respectively, in sucrose solution buffered with 5 mM Hepes, pH 7.4. The gradient was then centrifuged in a Beckman SW 40 Ti rotor at 90.000 g for 35 minutes, causing the synaptosomal fraction to collect at the interface between the 9 % and the 13 % Ficoll layer. This fraction was collected, diluted using sodium buffer (see Section 2.3), and pelleted at 700 g for 3 minutes to obtain a purified synaptosomal pellet (from supernatant 3, or S3).

Experiments using crude adult synaptosomes and using purified adult synaptosomes yielded comparable results. However, gradient centrifugation of embryonic samples proved to

be difficult (refer to Section 4.1 for detailed discussion). Therefore, most experiments were performed on adult and embryonic crude synaptosomal fractions rather than their purified equivalent. Crude synaptosomal fractions were also prepared from adult and embryonic mouse brain. For these experiments, NMRI mice were sacrificed by stretching. Mouse whole brains or isolated brain areas were then treated as described for the rat brains.

3.1.4 PREPARATION OF CRUDE SYNAPTIC VESICLES

Crude synaptic vesicles were prepared from adult and embryonic rat brain crude synaptosomes following the procedure described by Huttner et al. (1983). All steps were performed on ice or at 4 °C.

Adult or embryonic rat brains were isolated as described above (Section 3.1.1) and homogenised in 15 ml sucrose solution (900 rpm, 9 strokes) in the presence of the protease inhibitors PMSF (0.2 mM), pepstatin (2 µg / ml), leupeptin (2 µg / ml) and aprotinin (1 µg / ml). The homogenate was subsequently centrifuged at 800 g for 10 minutes, and the resultant supernatant centrifuged for 15 minutes at 9,200 g. The pellet was then washed by resuspension in 15 ml sucrose solution and centrifugation at 9,200 g for 15 minutes. The washed synaptosomal pellet was resuspended in a minimum amount of sucrose solution (usually 1.0 – 1.5 ml), and lysed by the addition of 9 volumes of ice-cold distilled H_2O followed by homogenisation in a Dounce homogeniser (2,000 rpm, 3 strokes). The lysate was buffered with 10 mM HEPES, pH 7.4. A subsequent centrifugation step at 25,000 g for 20 minutes resulted in the first lysate pellet (LP1, containing mainly plasma membranes) and the corresponding first lysate supernatant (LS1). Depending on sample size, the supernatant was then either centrifuged at 350,000 g for 30 minutes in a Beckman TLA 100.4 rotor (tube volume approximately 3 ml), or at 250,000 g for 2 hours in the Beckman 70 Ti rotor (tube volume approximately 30 ml). This last centrifugation step resulted in a crude synaptosomal pellet (LP2) and its corresponding dilute synaptosomal supernatant (LS2). For resuspension, the synaptic vesicle fraction was drawn through a 27 gauge needle 5 times.

3.2 Determination of protein concentrations

3.2.1 BICINCHONINIC ACID METHOD TO DETERMINE PROTEIN CONCENTRATION

Protein concentrations were usually determined according to the bicinchoninic acid (BCA) method of Smith et al. (1985). A standard curve was prepared using seven duplicate serial dilutions of bovine serum albumin (BSA) ranging from 20 µg / ml to 300 µg / ml. BSA was usually dissolved in sucrose solution or PBS in correspondence with the sample buffer.

Standards and appropriately diluted samples were pipetted in duplicate into the wells of a 96-well microtiter plate (20 µl / well). 200 µl of reaction solution (solution A and solution B, 50:1 v/v; see Section 2.3) was added to each well, and the plate incubated at 60 °C for 30 minutes. The plate was then allowed to cool for 10 minutes before the absorbance of the samples was measured in an Elisa-reader at 550 nm. Sample protein concentrations were estimated from the standard curve.

3.2.2 BRADFORD METHOD TO DETERMINE PROTEIN CONCENTRATION

Alternatively, protein concentrations were determined by the method of Bradford (Bradford, 1976) using the Roti-quant kit (see Section 2.2). A standard curve was prepared in sample buffer using serial dilutions of BSA ranging from 0.2-1.0 mg / ml. The Roti-quant Coomassie Brilliant Blue-G250 solution was prepared and diluted as recommended by the manufacturer, and 5 ml of the 1-times solution added to 100 µl of sample or standard. The sample and standards were then mixed and their optical densities (ODs) measured after 5-30 minutes at 595 nm. Sample protein concentrations were estimated from the standard curve.

3.3 Immunoprecipitation of protein complexes

To analyse the interactions between the SNARE proteins and synaptophysin, proteins were first extracted from their lipid membranes. If membranes were present as pellets, these were suspended in extraction buffer containing Triton X-100 (1 % v/v) (usually at a concentration of 1-2 mg / ml) (see Section 2.3), while, if membranes were present in suspension, Triton X-100 (20 % stock solution in PBS) was added directly to the samples to a final concentration of 1% (v/v). The binding studies with recombinant synaptobrevin were performed using the detergent CHAPS (2 % v/v) for protein solubilisation. All membrane

samples were incubated with detergent for one hour on ice while shaking. Insoluble particles were subsequently removed by centrifugation at 700 g for 5 minutes.

2.5 µl of ascites fluid (corresponding to about 7-10 µg of IgG) of the monoclonal antibodies against synaptobrevin, synaptophysin, or syntaxin were added to 200 µl of extraction supernatant. The incubations were performed for 16-18 hours at 4°C under rotation. 25 µl of Protein G Sepharose suspension (diluted 3:1 in extraction buffer) was subsequently added to bind the immunoprecipitates, and the samples incubated for a further hour at 4 °C while shaking. The beads were then collected by centrifuging at 200 g for one minute. Beads were subsequently washed three times using extraction buffer and analysed together with their corresponding supernatants via SDS polyacrylamide gel electrophoresis and immunoblotting (see Sections 3.4 and 3.5 below).

3.4 Protein gel electrophoresis

Protein samples were electrophoresed on a denaturing SDS-polyacrylamide gel system under discontinuous conditions according to the method of Laemmli (1970). Proteins were separated mostly on 12 % gels using 0.75 mm spacers, unless the weight of the proteins to be analysed required lower or higher percentage gels. Samples were dissolved in sample buffer (see Section 2.3) and heated at 95°C for 5 minutes prior to electrophoresis. Low molecular weight marker standards (LMW, see Section 2.2) ranging from 14.4 kDa to 94 kDa were added to the gel runs.

Gels were made up as follows:
(refer to Section 2.2 for recipes of buffer and stock solutions)

separating gel

	7.5 %	**10 %**	**12 %**	**15 %**
separating gel buffer	1.5 ml	1.5 ml	2.00 ml	1.5 ml
acrylamide stock solution	1.5 ml	2.0 ml	3.20 ml	3.0 ml
bisacrylamide stock solution	0.6 ml	0.8 ml	1.28 ml	1.3 ml
H_2O	2.4 ml	1.7 ml	1.52 ml	0.2 ml
TEMED	8 µl	8 µl	8 µl	8 µl
10 % ammonium persulfate	75 µl	75 µl	75 µl	75 µl

stacking gel

	3.75 %
separating gel buffer	500.0 µl
acrylamide stock solution	250.0 µl
bisacrylamide stock solution	100.0 µl
H2O	1150.0 µl
TEMED	2.6 µl
10 % ammonium persulfate	20.0 µl

3.5 Western blotting and immunodetection

Proteins were transblotted from SDS gels onto Hybond C nitrocellulose membranes (see Section 2.2) at 0.3 A / gel for 20 minutes in a semi-dry transfer chamber using Western blotting buffer (see Section 2.3) for semidry transfer.

Following Western blotting, the membranes were immersed in Ponceau-S solution (see Section 2.3) to visualise the molecular weight marker and to check for protein integrity, and were then destained using dH_2O. Using the molecular weight marker as a guide, blots were divided horizontally into wide strips carrying the proteins of interest. These strips were then carefully labelled with a pencil, rinsed in TS buffer, and incubated in blocking solution for 1 hour at room temperature or overnight at 4 °C. Incubation with the primary antibody was performed at 4 °C overnight or for 1-2 hours at room temperature in antibody solution (see Section 2.3). Incubation with the secondary antibody was performed for 1 hour at room temperature in antibody solution. After each incubation blots were washed 4 times for 15 minutes in blocking solution. All incubations and washes were performed on a shaker.

The method of immunodetection depended on the type of secondary antibody used. To stain for alkaline phosphatase activity, blot strips were pre-incubated for 5-10 minutes in AP-buffer and then developed in AP-developing solution. The reaction was stopped with two H_2O rinses.

To detect horseradish peroxidase activity, blot strips were bathed in ECL solution (see Section 2.2) for 1 minute and then exposed to film. Following ECL development, the nitrocellulose blots could be stained in colour development solution (see Section 2.3).

3.6 CHEMICAL CROSS-LINKING USING DSS

Synaptic proteins were cross-linked using the chemical cross-linker DSS. For this procedure, synaptosomes were resuspended in Krebs-Ringer buffer (see Section 2.3) at a concentration of 1 mg / ml and prewarmed at room temperature for 10 minutes. DSS in DMSO (see Section 2.2) was added to yield a final concentration of 0.5 M cross-linker and 5 % (v/v) solvent. After a 45 minute incubation at room temperature while shaking, the reaction was quenched by the addition of Tris-HCl pH 7.4 (final concentration 100 mM) and incubated for a further 30 minutes. The membranes were then pelletted at 350,000 g for 30 minutes, resuspended in non-denaturing sample buffer, and analysed by SDS-PAGE and immunoblotting

3.7 PREPARATION OF CELL LINE AND PRIMARY TISSUE CULTURE SAMPLES

Membrane fractions were prepared from the rat pheochromocytoma cell line PC 12 (Ahnert-Hilger et al., 1998), the rat hypothalamic neuronal cell line GT1.7 (Martinéz de la Escalera et al., 1992), and the mouse cholecystokinin-secreting cell line STC-1 (Glassmeier et al., 1998). Additionally, mouse hippocampus and hypothalamus primary tissue cultures were used. For sample preparation, the culture medium was decanted from the petri dishes, and cells were rinsed with cold PBS, and then gently removed from the base of the petri dishes and resuspended in PBS using a disposable cell scraper or a blue-tip (approximately 1 ml PBS per dish with an 8 centimeter diameter). Unless otherwise stated, cells were centrifuged for 10 minutes at 1,200 g, and the resultant pellets pooled, resuspended in PBS, and homogenised in a 2 ml Dounce homogeniser (900 rpm, 9 strokes). The homogenate was then centrifuged at 350,000 g for 30 minutes in a Beckman TLA 100.4 rotor to obtain a postnuclear membrane fraction.

3.8 IMMUNOISOLATION OF SYNAPTIC VESICLES

Whole brain homogenates were prepared in 0.32 M sucrose solution using a Dounce homogenizer (2000 rpm, 9 strokes) and subsequently centrifuged at 35,000 g for 25 minutes. A suspension of Eupergit C1Z beads coupled to monoclonal antibodies against synaptobrevin or synaptophysin, or to glycine (as a control for non-specific binding) was added to the resultant supernatant at a concentration of 1-2 µl beads / 300 µl supernatant. The incubation

was carried out at 4 °C for 35 minutes on a shaker. The beads were subsequently collected by centrifuging at 7,500 g for 2 minutes, and washed 3 times in sucrose solution. Proteins in the bead pellet and their corresponding supernatants were analysed by SDS-PAGE and immunoblotting (Sections 3.4 and 3.5).

3.9 RECOMBINANT SYNAPTOBREVINS

Full-length rat synaptobrevin II (residues 1-116) was subcloned into the Nde 1 and EcoR1 sites of the vector pHO2c (Fasshauer et al., 1997). The cytoplasmic domain of synaptobrevin II (residues 1-96) was subcloned into TrcHisA (Invitrogen) (Chapman et al., 1994). Both clones were kind gifts of Prof. Dr. R. Jahn (Göttingen, Germany).

Bacterial colonies were grown on LB agar, and then in LB medium, both containing 150 µg ampicillin / ml. The bacterial suspension was subsequently diluted 1:10 in TB medium and grown for a further 4 hours, and then induced for 2 - 2.5 hours using iPTG (0.8 mM) (see Sections 2.2 and 2.3).

The bacteria were then pelleted at 3,500 g for 40 minutes and subsequently resuspended in a small volume of bacterial extraction buffer (Section 2.3) and frozen at -80 °C. After thawing, small amounts of DNAse I, lysozyme and PMSF were added, and the suspension incubated for 20 minutes at room temperature. The suspension was then sonicated on ice, incubated for 20 minutes on ice in the presence of cholic acid (1.5 %), and subsequently centrifuged at 23,000 g (20 minutes). The supernatant was recentrifuged and the second supernatant exposed to Ni^{2+}-Sepharose beads under rotation for one hour at 4 °C to bind the His6-tagged fusion proteins. The beads were then centrifuged at 1,100 g for 2 minutes and washed three times in ½-times bacterial extraction buffer containing 1.5 % cholate. Beads were subsequently loaded onto a Polyprep chromatography column and fractions eluted using increasing amounts of imidazole (up to 400 mM) in 1.5 % cholate and ½-times bacterial extraction buffer. Fractions were collected and analysed by SDS-PAGE and Coomassie staining. The fractions containing high amounts of purified His6-tagged fusion protein were then pooled and dialysed overnight at 4 °C against dialysing buffer (see Section 2.3).

To check for the presence of recombinant protein, the dialysed fractions were separated via SDS-PAGE (Section 3.4) and stained using Coomassie blue solution (see

Section 2.3). Gels were heated in the microwave for 3 minutes in the presence of the dye and subsequently washed and destained by heating several times in H_2O.

For synaptophysin-binding studies, the recombinant synaptobrevins were immobilised on Ni-beads via their His-tag. Incubations were performed in PBS containing CHAPS (1,5 % w/v) or Triton X-100 (1 % v/v) for 16-18 hours at 4 °C, after which the beads were pelletted and washed three times. The proteins in the bead pellets and their corresponding supernatants were analysed by SDS-PAGE and immunoblotting (Sections 3.4 and 3.5).