4 RESULTS

4.1 ESTABLISHMENT OF EXPERIMENTAL PROCEDURES

Techniques used for this study included immunoprecipitation and immunoisolation, chemical cross-linking, and binding analyses. Most experiments were analysed using SDS-PAGE and immunoblotting procedures. It was therefore essential to confirm the specificity of the antibodies prior to use.

Postnuclear supernatants were prepared from adult and embryonic rat whole brains. The protein concentration was determined using the BCA assay, and equal amounts of total protein were subsequently electrophoresed on SDS-PAGE, and immunoblotted. As can be seen from Figure 1, all antibodies tested were specific, producing only one band of immunoreactivity, which was at the correct position. Similar results were obtained when proteins were immunoprecipitated prior to immunoblotting analysis (not shown). The published molecular weights of the respective antigens are 38 kDa for synaptophysin (Jahn et al., 1985; Wiedenmann and Franke, 1985), 18 kDa for synaptobrevin (Südhof et al., 1989), 25kDa for SNAP 25 (Mollinedo and Lazo, 1997), 35 kDa for syntaxin (Bennett et al., 1992), 60 kDa for src-kinase (Pang et al., 1988), and 65 kDa for Munc-18 (Okamoto et al., 1997). These correspond with the estimated molecular weights obtained here when comparing the antigen's position on the immunoblot to that of the molecular weight marker bands (indicated on the left in Figure 1). Note that the results obtained in neurons when using monoclonal antibodies against synaptophysin and synaptobrevin (Cl 7.2 and Cl 69.1, respectively) are comparable to those from their polyclonal equivalents (G 96 and 106.5, respectively). In contrast, for reasons not yet fully understood, synaptophysin from certain neuroendocrine tissues and cell lines (for example, some batches of GT 1.7 cells, see Figure 14) is occasionally only recognised by the polyclonal G 96 antibody. This may perhaps be due to a difference in posttranslational modification in synaptophysin from neurons and neuroendocrine cells.

All synaptic proteins tested were present at higher levels in the adult compared to the embryonic day 20 (E 20) samples. It is important to note that the increase in synaptic proteins shown in Figure 1 is relative to total protein content in the postnuclear supernatant. Thus, although adult brain contains more total protein than E 20 brain, the levels of synaptic proteins are selectively increased relative to this general increase. The increase in synaptic

protein levels during development was to be expected, since a large number of synapses is formed only postnatally. Interestingly, the enzyme src-kinase, which is located on synaptic vesicles and phosphorylates synaptophysin, seemed to be present at higher levels in the embryonic sample, an observation that was confirmed in further experiments (not shown). Whether this implies that synaptophysin is more highly phosphorylated in embryonic than in adult brains remains to be established. In the present study, anti-phosphotyrosine antibodies were used to try to evaluate differences in synaptophysin phosphorylation during development, but no conclusive results were obtained.

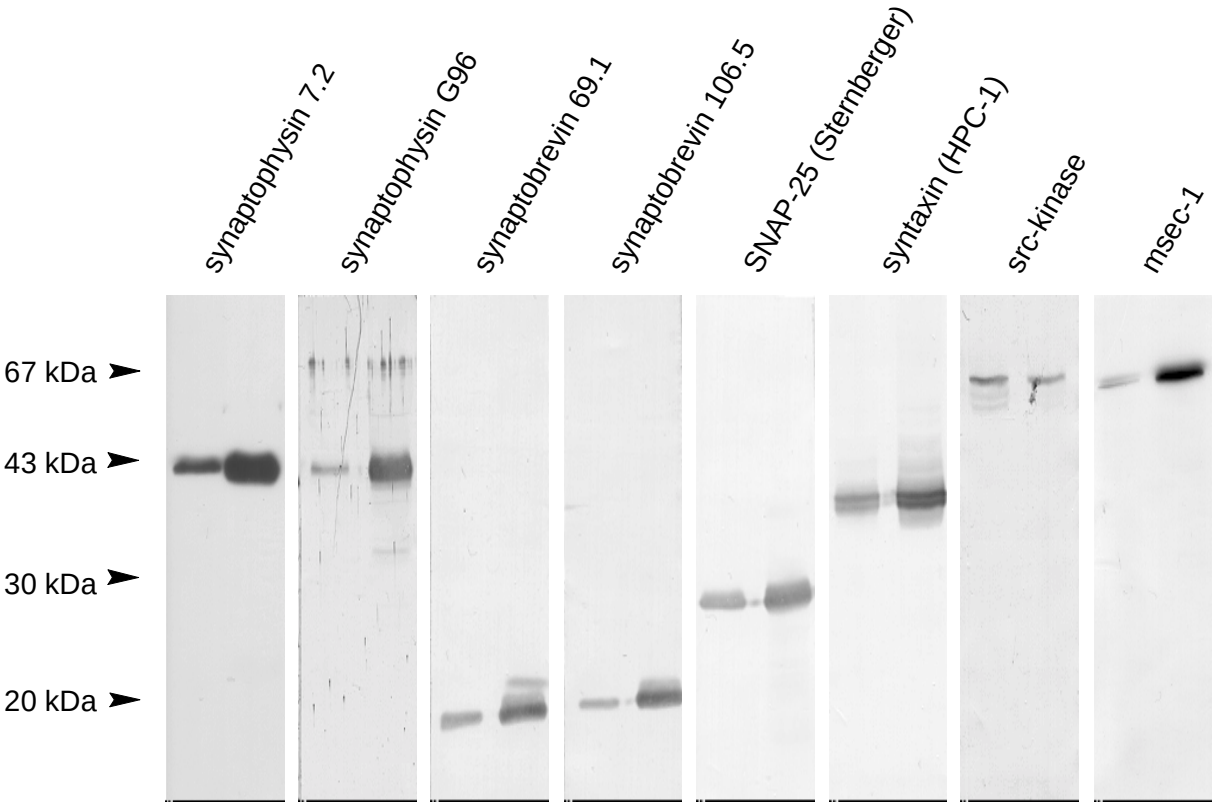

Figure 1
Antibody specificity

Equal amounts of total protein of adult (right lanes) and embryonic day 20 (E 20) (left lanes) rat brain postnuclear supernatants were analysed via SDS-PAGE and immunoblotting. Note that all proteins tested were present in higher amounts in the adult compared to the E 20 brains. One exception was the synaptophysin-phosphorylating enzyme src-kinase which was present in higher amounts in the embryonic brain.

Once the specificity of the antibodies had been confirmed, subsequent Western blots could be divided into strips and exposed to more than one antibody if needed. This procedure saved antibody and allowed for more flexibility when wanting to vary incubation times and procedures.

Crude synaptosomes or crude synaptic vesicle fractions purified from rat whole brain homogenates were often used as starting material in this study (see Section 3.1). For preparation of these fractions, a postnuclear supernatant (PNS) was obtained from the brain homogenate by a low-speed centrifugation step. The corresponding pellet contained mainly unlysed cells and nuclei, and was usually discarded. Centrifugation of the PNS yielded a crude synaptosomal pellet (pellet two, or P2).

Synaptic vesicles were isolated via hypo-osmotic lysis of the synaptosomal pellet. The plasma membranes were then pelleted (lysis pellet one, or LP1) and the resulting supernatant centrifuged at high speed to obtain the crude synaptic vesicle fraction (lysis pellet two, or LP2). As can be seen in Figure 2, when equal amounts of total protein were analysed, synaptosomal proteins increased in relation to total protein amounts during these purification steps. Also, although the method described was developed for the isolation of adult synaptosomes and synaptic vesicles (Huttner et al., 1983; Edelmann et al., 1995) it could be applied equally well to rat embryonic brains (Figure 2, left panel). Note that the plasma membrane SNARE protein SNAP 25 was present in high amounts in the crude synaptic vesicle fraction. Although this may be due partially to plasma membrane contamination in this crude fraction, the main reason is that both SNAP 25 and syntaxin really are present in substantial amounts on synaptic vesicles (Walch-Solimena et al., 1995; Otto et al. 1997), where they are thought to accumulate after vesicular exocytosis-endocytosis cycles.

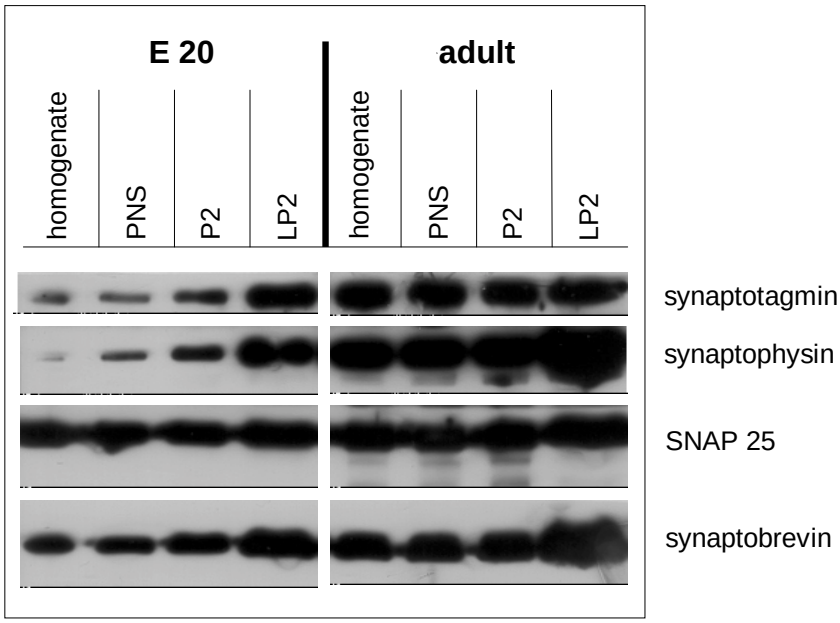

Figure 2
Relative increase in synaptic protein amounts during purification
Crude synaptic vesicle fractions (lysis pellet two, or LP2) were prepared from embryonic day 20 (E 20) and adult rat whole brain homogenates as described in Section 3.1. The intermediate fractions (postnuclear supernatant, or PNS, and crude synaptosomal pellet, or pellet two, or P2) were analysed for the indicated synaptic proteins via SDS-PAGE and immunoblotting. Equal amounts of total protein were loaded. Note that the indicated proteins increased in relation to total protein amounts during the purification procedures. Note also that the SNARE protein SNAP 25 was present in high amounts in the synaptic vesicle fraction, indicating its presence on synaptic vesicles.

To analyse the interactions between the SNAREs and synaptophysin, proteins were extracted from synaptosomal or synaptic vesicle fractions using the detergent Triton X-100, and were subsequently immunoprecipitated. This method has been described by Edelmann et al. (1995) for immunoprecipitation of both the SNARE complex and the synaptophysin-synaptobrevin complex from purified synaptosomal fractions derived from adult rat brain. Purified synaptosomal fractions (supernatant three, or S3) are obtained when crude synaptosomal fractions are centrifuged on a Ficoll gradient. In the present study, immunoprecipitation from crude and from purified adult synaptosomes (Figure 3) yielded comparable results. In either case, when immunoprecipitating with the antibody against synaptobrevin, both the SNARE complex binding partners SNAP 25 and syntaxin, and the vesicular binding partner synaptophysin, were precipitated together with synaptobrevin.

Immunoprecipitating with the antibody against syntaxin yielded only the SNARE complex proteins, while precipitating with the antibody against synaptophysin yielded only the synaptophysin-synaptobrevin complex, thus showing that the two complexes were mutually exclusive. The SDS control experiment, whereby the SNARE complex is shown to be SDS resistant whereas the synaptophysin-synaptobrevin complex is not (Edelmann et al., 1995), could also be reproduced without difficulty in adult crude synaptosomal extracts (Figure 3). An overview of the various interactions of the SNARE proteins and of synaptophysin that have been described in the literature is given in Table 1.

Table 1
Complexes formed between synaptophysin, synaptobrevin, SNAP 25 and syntaxin

	synaptophysin	**synaptobrevin**	**SNAP 25**	**syntaxin**
synaptophysin	yes[1]	yes[2] (sensitive to SDS)	no[2]	no[2]
synaptobrevin	yes[2] (sensitive to SDS)	yes[3]	only as part of fusion complex[4]	only as part of fusion complex[4]
SNAP 25	no[2]	only as part of fusion complex[4]	-	yes[5]
syntaxin	no[2]	only as part of fusion complex[4]	yes[5]	-

[1] Rehm et al., 1986; Thomas et al., 1988; Johnston and Südhof, 1990
[2] Edelmann et al., 1995
[3] Laage and Langosch, 1997
[4] Jahn and Südhof, 1994
[5] Fasshauer et al., 1997 (syntaxin-SNAP 25 complex stoichiometry of 2:1)

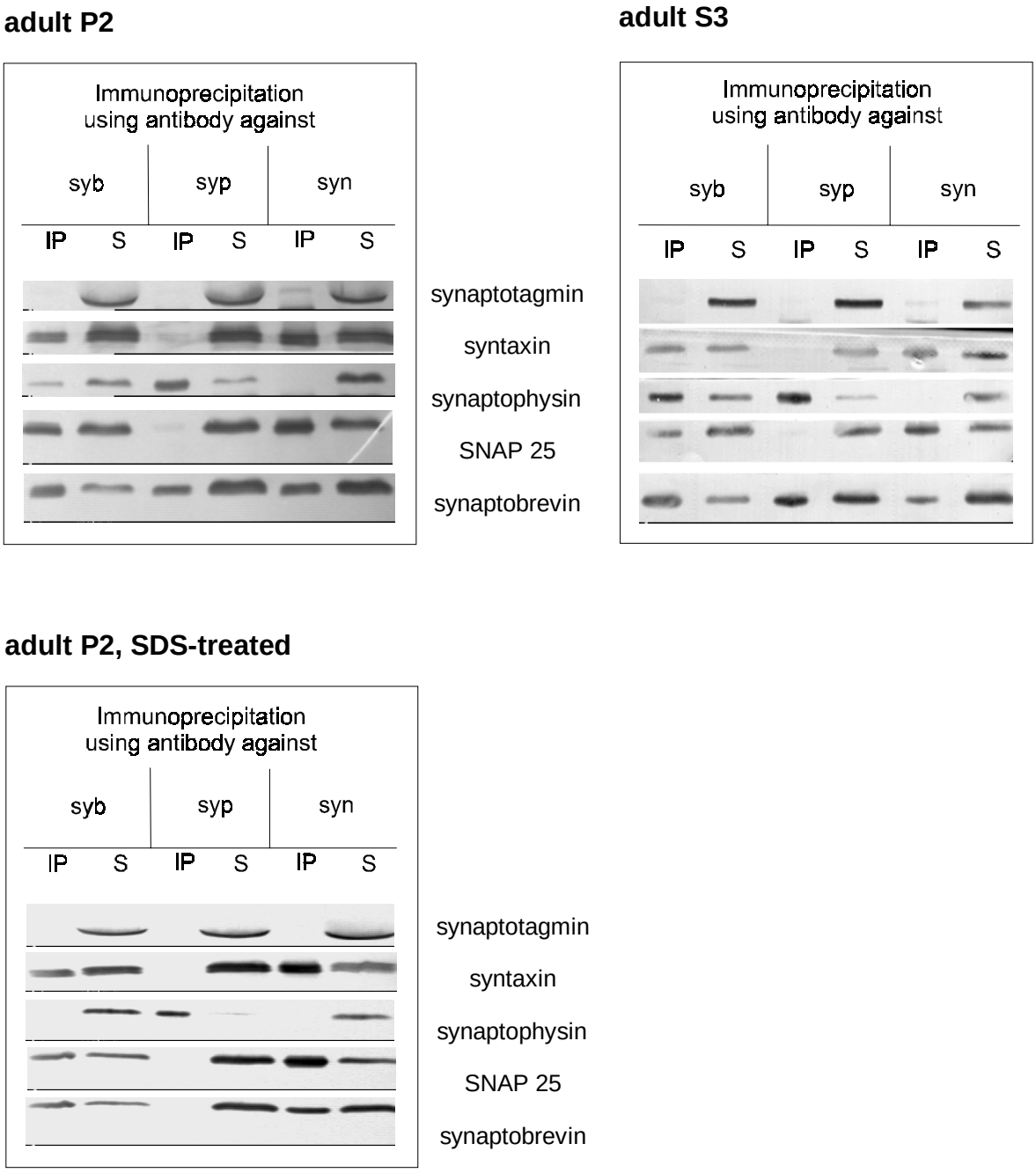

Figure 3
Immunoprecipitation of synaptosomal protein complexes from adult brain
Proteins were immunoprecipitated from adult rat brain crude synaptosomal extracts (pellet two, or P2) (in the absence and presence of 10 % w/v SDS) and purified synaptosomal extracts (supernatant three, or S3) using antibodies against synaptobrevin (syb) (Cl 69.1), synaptophysin (syp) (Cl 7.2), and syntaxin (syn) (HPC-1). Immunoprecipitates (IP) and supernatants (S) were analysed for the indicated synaptic proteins via SDS-PAGE and immunoblotting. Note that the SNARE complex could be detected in P2 and S3 samples and was SDS resistant. The synaptophysin-synaptobrevin complex could also be detected both in P2 and in S3, but was SDS-sensitive.

Whereas in the adult, purified synaptosomal fractions could be prepared easily, gradient centrifugation of embryonic samples proved to be more difficult. This was probably due to a different myelination state of embryonic neurons leading to a different buoyant density of synaptosomes and therefore to a change in behaviour on the Ficoll gradient. Additionally, the lower yield of crude synaptosomes from embryonic brain made sample recovery after gradient centrifugation even more difficult. Unfortunately, experimenting with different Ficoll concentrations did not yield any better results. Therefore, seeing that both crude and purified vesicles could be used for immunoprecipitation studies in the adult but only crude synaptosomes could be prepared in sufficient quantities from embryonic brains, subsequent studies were performed on adult and embryonic crude synaptosomal fractions rather than their purified equivalent. Immunoprecipitation from crude synaptic vesicles, where gradient centrifugation is not necessary for purification procedures, could be performed equally well from embryonic and from adult samples.

4.2 THE SYNAPTOPHYSIN-SYNAPTOBREVIN COMPLEX AND THE SNARE COMPLEX IN EMBRYONIC brain and neuroendocrine cells

4.2.1 The synaptophysin-synaptobrevin complex and the SNARE complex in embryonic rat brain

Are the exocytotic SNARE complex and the synaptophysin-synaptobrevin complex already present in embryonic brains? To answer this question, protein complexes were immunoprecipitated from embryonic crude synaptosomal extracts using an adaptation of the methods described by Edelmann et al. (1995). Interestingly, although the SNARE complex could be detected as early on as embryonic day 14, the synaptophysin-synaptobrevin complex was absent in both embryonic day 14 and embryonic day 20 rat brains (Figure 4). When immunoprecipitating with the antibody against synaptobrevin, the SNARE complex binding-partners SNAP 25 and syntaxin were present in the immunoprecipitate together with synaptobrevin, but synaptophysin was detected only in the supernatant. Similarly, when immunoprecipitating with the antibody against synaptophysin, synaptobrevin was present in the supernatant only. As in the adult, immunoprecipitating with the antibody against syntaxin yielded the SNARE complex but no synaptophysin. Thus, while the SNARE complex could already be detected at early stages of development, the vesicular synaptophysin-synaptobrevin complex was absent in embryonic brain.

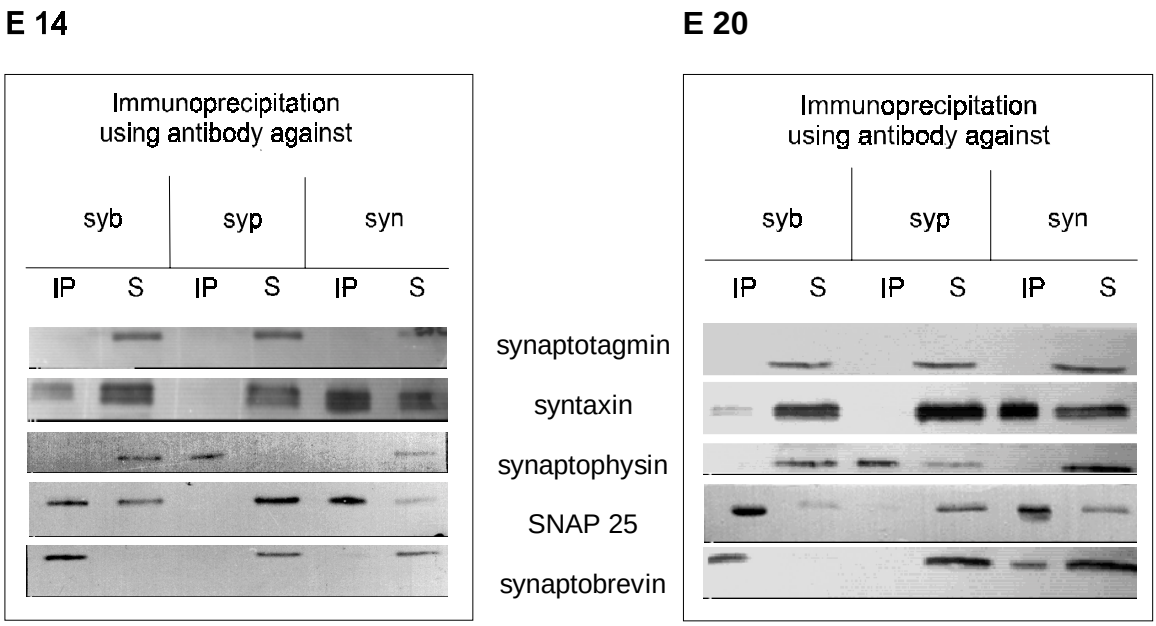

Figure 4
Immunoprecipitation of synaptosomal protein complexes from embryonic brain
Proteins were immunoprecipitated from embryonic day (E) 14 and E 20 rat brain crude synaptosomal extracts using antibodies against synaptobrevin (syb) (Cl 69.1), synaptophysin (syp) (Cl 7.2), and syntaxin (syn) (HPC-1). Immunoprecipitates (IP) and supernatants (S) were analysed for the indicated synaptic proteins via SDS-PAGE and immunoblotting. Note that while the SNARE complex was present already at E 14, the synaptophysin-synaptobrevin complex could not be detected in embryonic brain.

In order to confirm the absence of the synaptophysin-synaptobrevin complex in embryonic brain, control experiments were performed. First, the detergent to protein ratio used for extraction and immunoprecipitation in embryonic samples was varied in order to test for detergent-induced artefacts. As can be seen in Figure 5, the synaptophysin-synaptobrevin complex was absent at all three detergent to protein ratios tested. When immunoprecipitating with the antibody against synaptobrevin, only synaptobrevin but no synaptophysin was found in the precipitates. Similarly, when immunoprecipitating with the antibody against synaptophysin, only synaptophysin but no synaptobrevin was found in the immunoprecipitates.

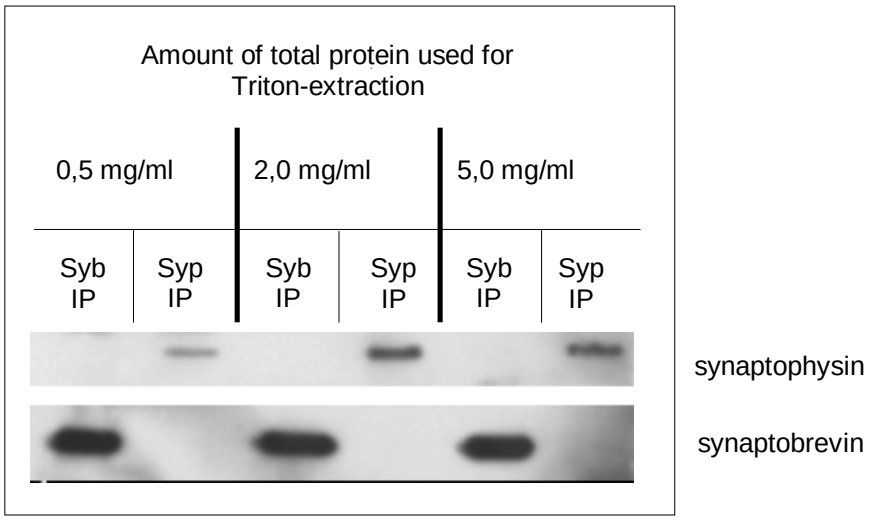

Figure 5
Varying the detergent / protein ratio for immunoprecipitation
Crude synaptosomes were prepared from E 20 rat brain, and proteins were extracted at different detergent / protein ratios prior to immunoprecipitation with antibodies against synaptobrevin (syb) (Cl 69.1) and synaptophysin (syp) (Cl 7.2). Immunoprecipitates (IP) were analysed for the indicated synaptic proteins via SDS-PAGE and immunoblotting. Note that the synaptophysin-synaptobrevin complex was absent at all three detergent to protein ratios tested.

A second approach to confirming the absence of the synaptophysin-synaptobrevin complex in embryonic brain was to use polyclonal as well as monoclonal antibodies against synaptophysin and synaptobrevin to analyse the immunoprecipitates on Western blots (Figure 6). Seeing that polyclonal antibodies usually recognise more antigen epitopes than their monoclonal equivalents, the idea was that they might detect a form of synaptophysin or synaptobrevin that is not recognised by the monoclonal antibodies. However, both polyclonal antibodies produced the same results as those obtained when using monoclonal antibodies, namely the absence of synaptophysin in synaptobrevin precipitates and, similarly, the absence of synaptobrevin in synaptophysin precipitates.

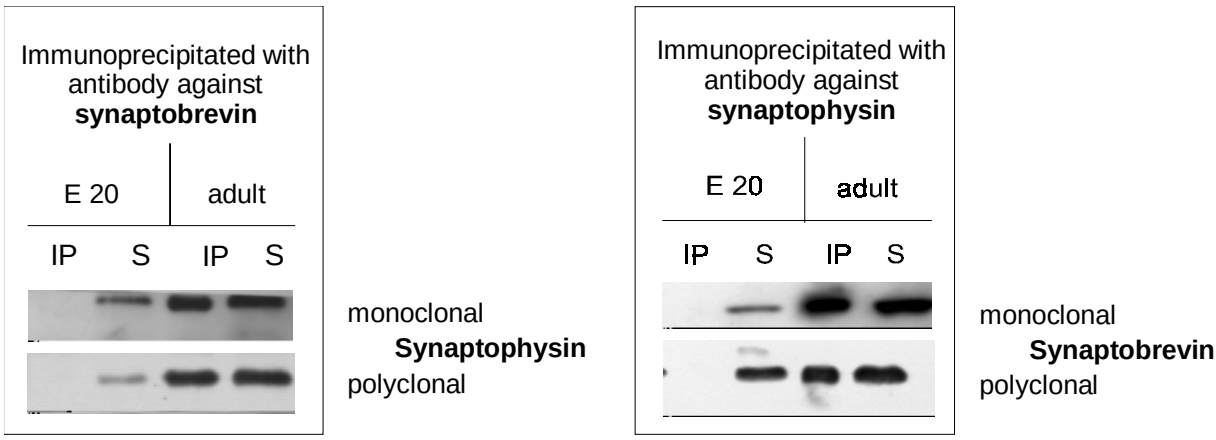

Figure 6
Using both monoclonal and polyclonal antibodies for immunodetection
Proteins were immunoprecipitated from adult and embryonic day (E) 20 rat brain crude synaptosomal extracts using antibodies against synaptobrevin (syb) (Cl 69.1) and synaptophysin (syp) (Cl 7.2). Immunoprecipitates (IP) and supernatants (S) were analysed via SDS-PAGE and immunoblotting using both monoclonal and polyclonal antibodies against synaptobrevin and synaptophysin. Both polyclonal antibodies produced the same results as those obtained when using monoclonal antibodies.

In a third control experiment, the amount of embryonic protein used for immunoblotting analysis was approximately ten times greater than that used for adult. Because the protein amounts were determined prior to extraction and immunoprecipitation procedures, the amount of protein present in the immunoprecipitates and thus the ten times greater amount of embryonic sample loaded compared to adult could only be an estimate. Again, no synaptophysin-synaptobrevin complex was detected (Figure 7).

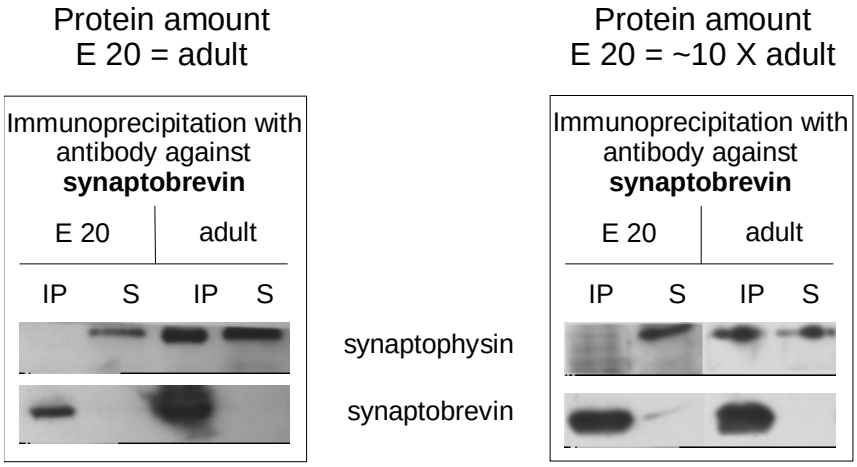

Figure 7
Varying the amount of sample analysed by SDS-PAGE and immunoblotting
Proteins were immunoprecipitated from adult and embryonic day (E) 20 rat brain crude synaptosomal extracts using antibodies against synaptobrevin (syb) (Cl 69.1) and synaptophysin (syp) (Cl 7.2). Immunoprecipitates (IP) and supernatants (S) were analysed via SDS-PAGE and immunoblotting. In the right panel, the amount of total protein loaded was about 10 times greater for the E 20 sample than for the adult sample. Because the protein amounts were determined prior to extraction and immunoprecipitation procedures, the amount of protein present in the immunoprecipitates and thus the ten times greater amount of embryonic sample loaded compared to adult can only be an estimate. No synaptophysin-synaptobrevin complex was detected.

A different approach to studying protein interactions is their chemical cross-linking using the membrane-permeable cross-linker disuccinimidyl suberate (DSS). No detergent extraction is necessary when using this method. After DSS treatment, the membranes containing the cross-linked proteins are simply pelleted and subsequently analysed by immunoblotting, thus allowing for protein interactions to be studied in their natural membrane environment.

Western Blots of adult cross-linked samples and non-cross-linked controls were probed for synaptobrevin immunoreactivity (Figure 8A). In the control lanes only one band representing the synaptobrevin monomer was present (left lanes of E 20 and adult in Figure 8A). In the cross-linked samples, two additional bands were noticeable besides that for the synaptobrevin monomer (Figure 8A, right lanes). One band was located at between 30 kDa and 40 kDa and represents the synaptobrevin dimer (which has a calculated molecular weight of 36 kDa). The other band was located at between 40 kDa and 60 kDa and represents the synaptophysin-synaptobrevin complex (which has a calculated molecular weight of 56 kDa). No synaptophysin-synaptobrevin complex could be detected in the cross-linked embryonic samples. Surprisingly, the synaptobrevin dimer formation also seems to increase during neuronal development with only trace amounts being present at E 20 compared to adult (Figure 8A, left lanes). In order to confirm the identity of the upper cross-linked band in adult samples, the blots were probed for synaptophysin. Unfortunately, the synaptophysin-synaptobrevin complex seemed to be more difficult to detect with the synaptophysin antibody than with the synaptobrevin antibody, probably because the antibody binding site becomes less accessible on immunoblots after cross-linking. Immunoprecipition of cross-linked samples was therefore performed using antibodies against synaptobrevin and synaptophysin in order to increase the amount of complex present and thus making it easier to detect. As shown in Figure 8B, the synaptophysin-synaptobrevin complex was present in both adult cross-linked immunoprecipitates (right lanes) but in neither of the embryonic immunoprecipitates (left lanes). Thus, the results obtained through cross-linking studies resembled those obtained by immunoprecipitation alone, namely that the synaptophysin-synaptobrevin complex is present in adult brain but is absent in embryonic brain.

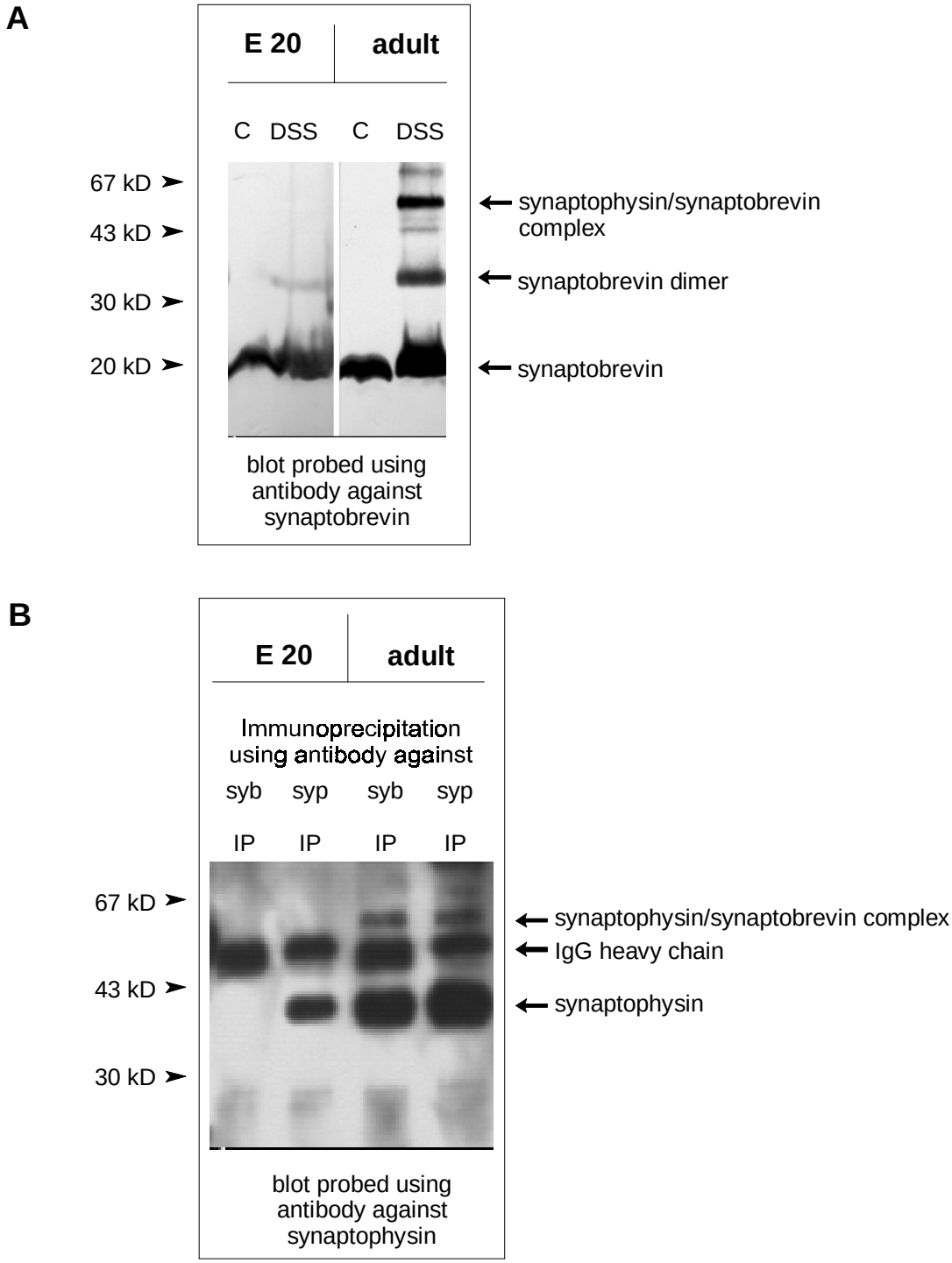

Figure 8
Chemical cross-linking of synaptosomal proteins

Proteins from embryonic day (E) 20 and adult rat whole brain crude synaptosomal fractions were cross-linked using the chemical cross-linker DSS (as described in Section 3.7). Membranes were then either pelleted and analysed via SDS-PAGE and immunoblotting (**A**), or proteins were extracted and immunoprecipitated (IP) using antibodies against synaptobrevin (syb) (Cl 69.1) and synaptophysin (syp) (Cl 7.2) (**B**). Note that the synaptophysin-synaptobrevin complex was present in adult cross-linked samples but was absent in embryonic cross-linked samples.

Synaptic vesicles contain an isoform of synaptophysin called synaptoporin (Fykse et al., 1993), or synaptophysin II. Synaptoporin has been shown previously to bind to synaptobrevin in adult synaptosomes (Edelmann et al., 1995). Could it be that synaptoporin substitutes for synaptophysin in embryonic brains? To test for this possibility, synaptobrevin immunoprecipitates from embryonic day 14 and 20 brains, and from adult brain, were tested for the presence of synaptoporin. As can be seen in Figure 9, the synaptoporin-synaptobrevin complex was present in adult but not in embryonic brain extracts. Thus, synaptoporin did not substitute for synaptophysin in embryonic brains. Rather, the synaptoporin-synaptobrevin complex followed the same developmental pattern observed for the synaptophysin-synaptobrevin complex.

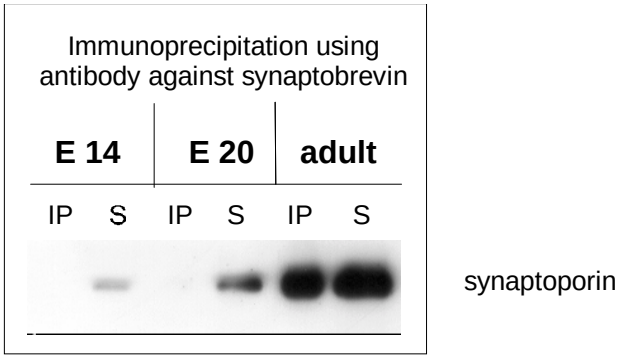

Figure 9
Synaptoporin and the synaptoporin-synaptobrevin complex in embryonic and adult synaptosomes
Proteins were immunoprecipitated from embryonic day (E) 14, E 20 and adult rat brain crude synaptosomal extracts using the anti-synaptobrevin antibody (Cl 69.1). Immunoprecipitates (IP) and supernatants (S) were analysed for synaptoporin via SDS-PAGE and immunoblotting. Note that the synaptoporin-synaptobrevin complex was present in adult but not in embryonic brain extracts.

Synaptic vesicles additionally contain an isoform of synaptobrevin II called synaptobrevin I. Synaptobrevin II is the major synaptobrevin isoform occurring in brain, and synaptobrevin I is less ubiquitously expressed (Archer et al., 1990). Immunoprecipitates from embryonic brain were analysed to check whether embryonic synaptophysin might bind to synaptobrevin I and thereby be unavailable for binding to synaptobrevin II. However, no such binding was observed (not shown).

In order to identify the time-point of developmental upregulation of the synaptophysin-synaptobrevin complex, synaptosomal extracts were prepared from rat brains at various postnatal stages starting at birth. As shown in Figure 10, the synaptophysin-synaptobrevin complex started to appear only after birth and reached adult levels around postnatal day 15.

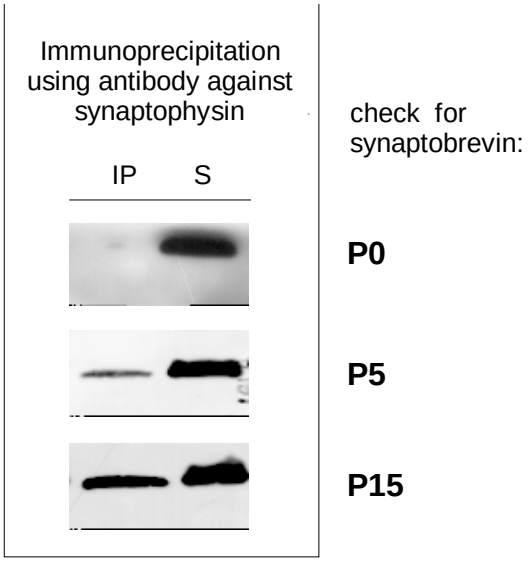

Figure 10
Synaptophysin and the synaptophysin-synaptobrevin complex in postnatal synaptosomes
Proteins were immunoprecipitated from P 0, P 5 and P 15 rat brain crude synaptosomal extracts using the anti-synaptophysin antibody (Cl 7.2). Immunoprecipitates (IP) and supernatants (S) were analysed for synaptobrevin via SDS-PAGE and immunoblotting. Note that the synaptophysin-synaptobrevin complex started to appear only after birth and reached adult levels around postnatal day 15.

4.2.2 The synaptophysin-synaptobrevin complex and the SNARE complex in embryonic mouse brain

The developmental upregulation of the synaptophysin-synaptobrevin complex as well as its SDS-susceptibility in the adult rat brain were also observed in the mouse (Figure 11).

When immunoprecipitating with an antibody against synaptobrevin, both synaptobrevin and synaptophysin could be detected in precipitates from adult mouse brain, but only synaptobrevin could be detected in precipitates from embryonic brain. Here, synaptophysin was present in the supernatant only. Similarly, when immunoprecipitating with an antibody against synaptophysin, both synaptophysin and synaptobrevin could be detected in adult brain precipitates, but only synaptophysin could be detected in embryonic brain precipitates, with synaptobrevin being present in the supernatant only. Precipitating in the presence of 10 % SDS abolished the synaptophysin-synaptobrevin interaction in adult brain extracts (Figure 11A).

These results were confirmed by cross-linking and immunoprecipitation procedures (Figure 11B). As was the case with rat brain, the synaptophysin-synaptobrevin dimer was only present in cross-linked samples from adult mouse brain and not in embryonic mouse brain. Also, as with rat brain, the synaptobrevin dimer was clearly present in adult mouse samples but only faintly visible in embryonic mouse samples.

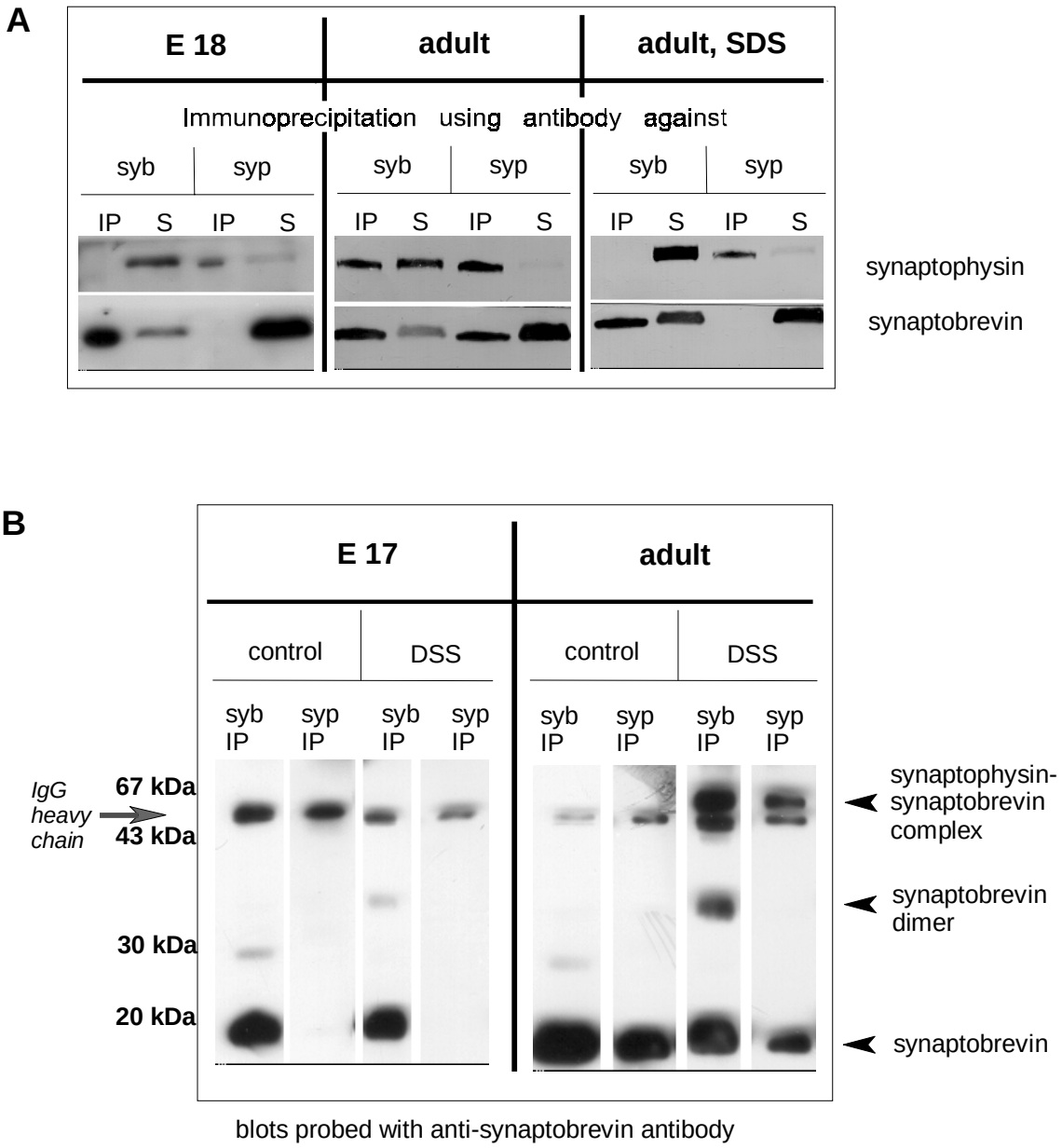

Figure 11
Immunoprecipitation of synaptosomal protein complexes from mouse brain

A. Proteins were immunoprecipitated from adult (in the absence and presence of 10 % w/v SDS) and embryonic day (E) 18 mouse brain crude synaptosomal extracts using antibodies against synaptobrevin (syb) (Cl 69.1) and synaptophysin (syp) (Cl 7.2). Immunoprecipitates (IP) and supernatants (S) were analysed for synaptophysin and synaptobrevin via SDS-PAGE and immunoblotting.

B. Proteins from E 17 and adult mouse whole brain crude synaptosomal fractions were cross-linked using the chemical cross-linker DSS (as described in Section 3.7). Proteins were extracted and immunoprecipitated (IP) using antibodies against synaptobrevin (syb) (Cl 69.1) and synaptophysin (syp) (Cl 7.2). Samples were analysed for synaptobrevin via SDS-PAGE and immunoblotting.

The experiments so far had been performed on samples obtained from whole brain only. To test for regional differences, various brain areas were prepared and subjected to extraction and immunoprecipitation procedures. In the rat, the developmental upregulation of the synaptophysin-synaptobrevin complex could be followed in bulbus olfactorius, cortex and cerebellum (results not shown). In the mouse, the complex was present in adult hippocampus and hypothalamus, but was absent in these regions in tissue culture when tested after six days in vitro and only became detectable after 13 days in vitro in hippocampus. In the hypothalamus, the complex was absent also after 13 days in vitro (Figure 12). However, in one out of three precipitations the complex could be detected after 13 days in vitro. This discrepancy may be due to differences in the dissection of the very small hypothalamic area in the mouse embryonic brain, where variable amounts of presumably GABAergic neurons may "contaminate" the peptidergic neurons and generate synapses where the synaptophysin-synaptobrevin complex occurs.

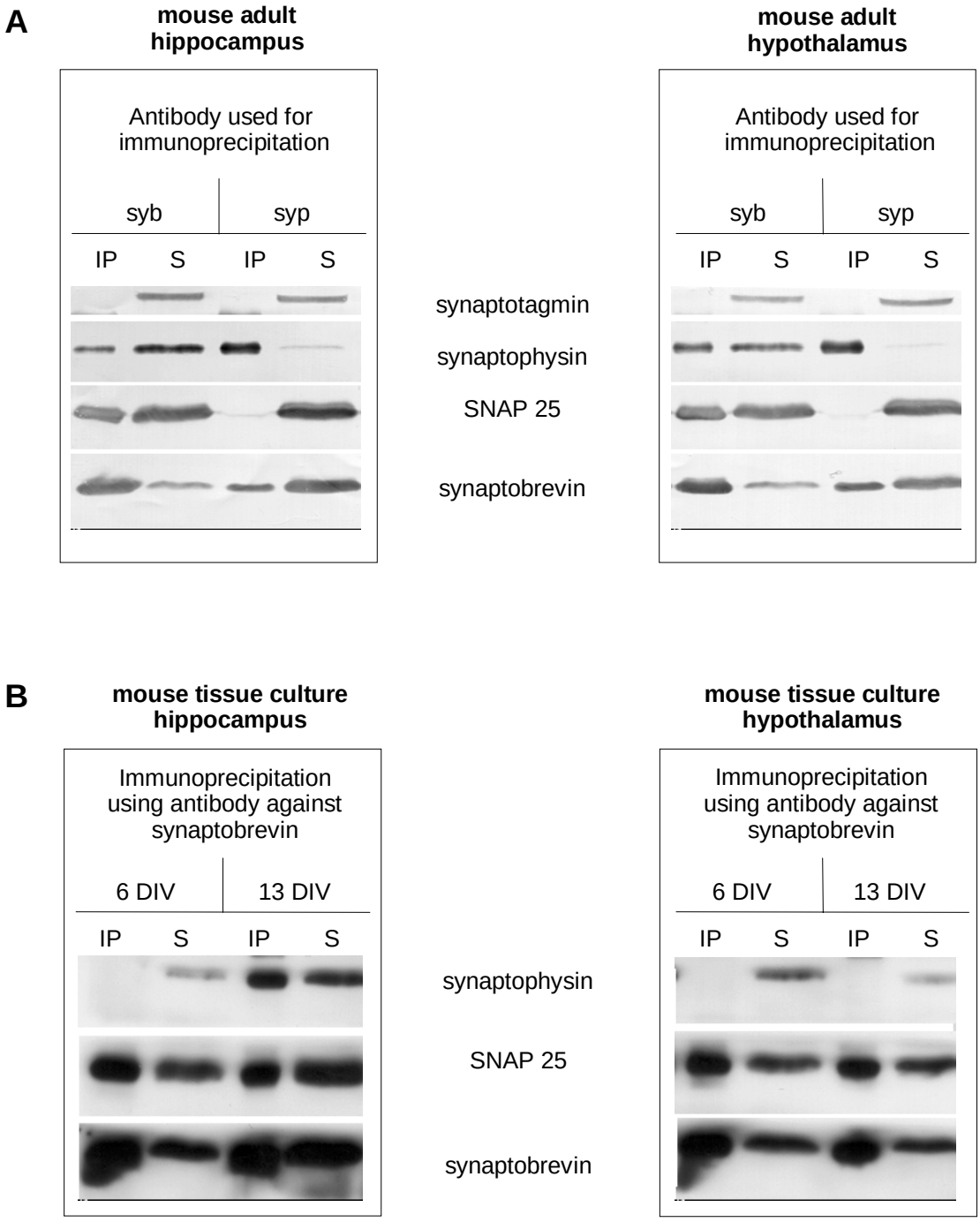

Figure 12
Immunoprecipitation of synaptosomal protein complexes from specific areas of mouse brain and from primary tissue culture

A. Proteins were immunoprecipitated from adult mouse hippocampus or hypothalamus crude synaptosomal extracts using antibodies against synaptobrevin (syb) (Cl 69.1) and synaptophysin (syp) (Cl 7.2). Immunoprecipitates (IP) and supernatants (S) were analysed for the indicated proteins via SDS-PAGE and immunoblotting.

B. Proteins were extracted from E 15 primary tissue culture after 6 days in vitro (DIV) and 13 DIV, and immunoprecipitated using an antibody against synaptobrevin (Cl 69.1). Immunoprecipitates (IP) and supernatants (S) were analysed for the indicated proteins via SDS-PAGE and immunoblotting.

4.2.3 The synaptophysin-synaptobrevin complex and the SNARE complex in neuroendocrine cells

The synaptophysin-synaptobrevin complex could not be detected in the rat pheochromocytoma cell line PC 12 (Ahnert-Hilger et al., 1998), the rat hypothalamic gonadotropin-releasing hormone neuronal cell line GT 1.7 (Martinéz et al., 1992), or the murine cholecystokinin-secreting gut cell line STC-1 (Glassmeier et al., 1998) (Figure 13). When immunoprecipitating in these neuroendocrine cell lines with an antibody against synaptobrevin, only the SNARE complex but no synaptophysin was precipitated, although synaptophysin was clearly visible in the supernatants.

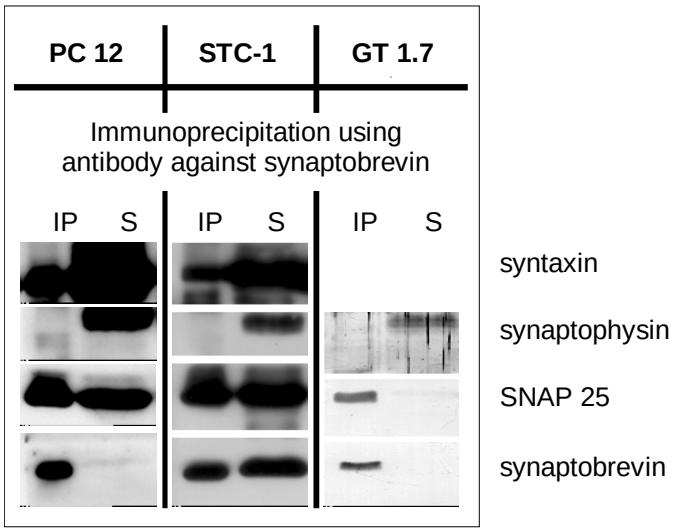

Figure 13
Analysis of the synaptophysin-synaptobrevin interaction in PC 12 cells and other neuroendocrine cell lines
Proteins were extracted and immunoprecipitated from PC 12, GT 1.7 and STC-1 cells using an antibody against synaptobrevin (syb) (Cl 69.1). Immunoprecipitates (IP) and supernatants (S) were analysed for synaptophysin and synaptobrevin via SDS-PAGE and immunoblotting.

4.3 LOCALISATION OF SYNATOPHYSIN AND SYNAPTOBREVIN IN EMBRYONIC AND ADULT BRAIN

Why do synaptophysin and synaptobrevin not interact in the embryonic brain? Are they perhaps not in close enough proximity of one another? This would be the case if, during development, synaptophysin and synaptobrevin were expressed in different subsets of neurons, or on different populations of synaptic vesicles. To test this possibility, dual immunofluorescence experiments were performed on rat cerebellar slices. Immunoprecipitation data of adult and embryonic cerebellum had revealed that the synaptophysin-synaptrobrevin complex was present in adult cerebellum but absent in embryonic cerebellum (results not shown, see Becher et al., 1999a). This correlated with the immunoprecipitation result obtained for whole brains. Immunofluorescence data, however, showed a perfect co-localisation of the two proteins in the embryonic cerebellum (Figure 14). These results indicate that synaptophysin and synaptobrevin are present in the same nerves in embryonic brain and that their lack of interaction is not due to a difference in location patterns in the brain.

No difference in location of synaptophysin and synaptobrevin was observed at the light microscopic level. Thus, the two proteins are definitely expressed within the same neurons. However, perhaps developing neurons contain immature subtypes of synaptic vesicles that do not carry both synaptobrevin and synaptophysin? Then the two proteins would be unable to interact even though they were expressed within the same cell. To test this possibility, a different, biochemical approach was used. Native vesicles were immunoisolated using Eupergit beads coated with an antibody against synaptophysin. Note that no detergent was used during this procedure and that, therefore, whole vesicles rather than solitary proteins and protein complexes were immunoisolated.

Using this approach, one can distinguish whether synaptophysin and synaptobrevin are present on the same vesicle or on different vesicle types. Imagine that the two proteins are present on different vesicle types (Figure 15, bottom). Then, when immunoisolating vesicles with an antibody against synaptophysin, only synaptophysin but no synaptobrevin would be present in the bead pellet. If, however, both proteins were present on the same vesicle (Figure 15, top) then immunoisolating with an antibody against synaptophysin would yield both proteins.

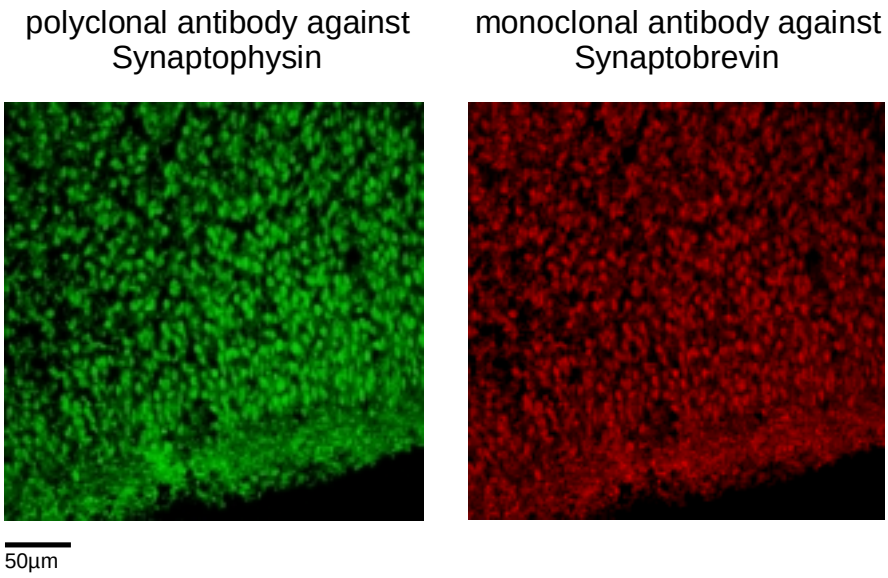

Figure 14
Synaptophysin, synaptobrevin, and the synaptophysin-synaptobrevin complex in embryonic cerebellum
Confocal laser microscopic analysis of synaptophysin and synaptobrevin in rat E 18 cerebellar cortex (courtesy of Dr Ingrid Pahner).

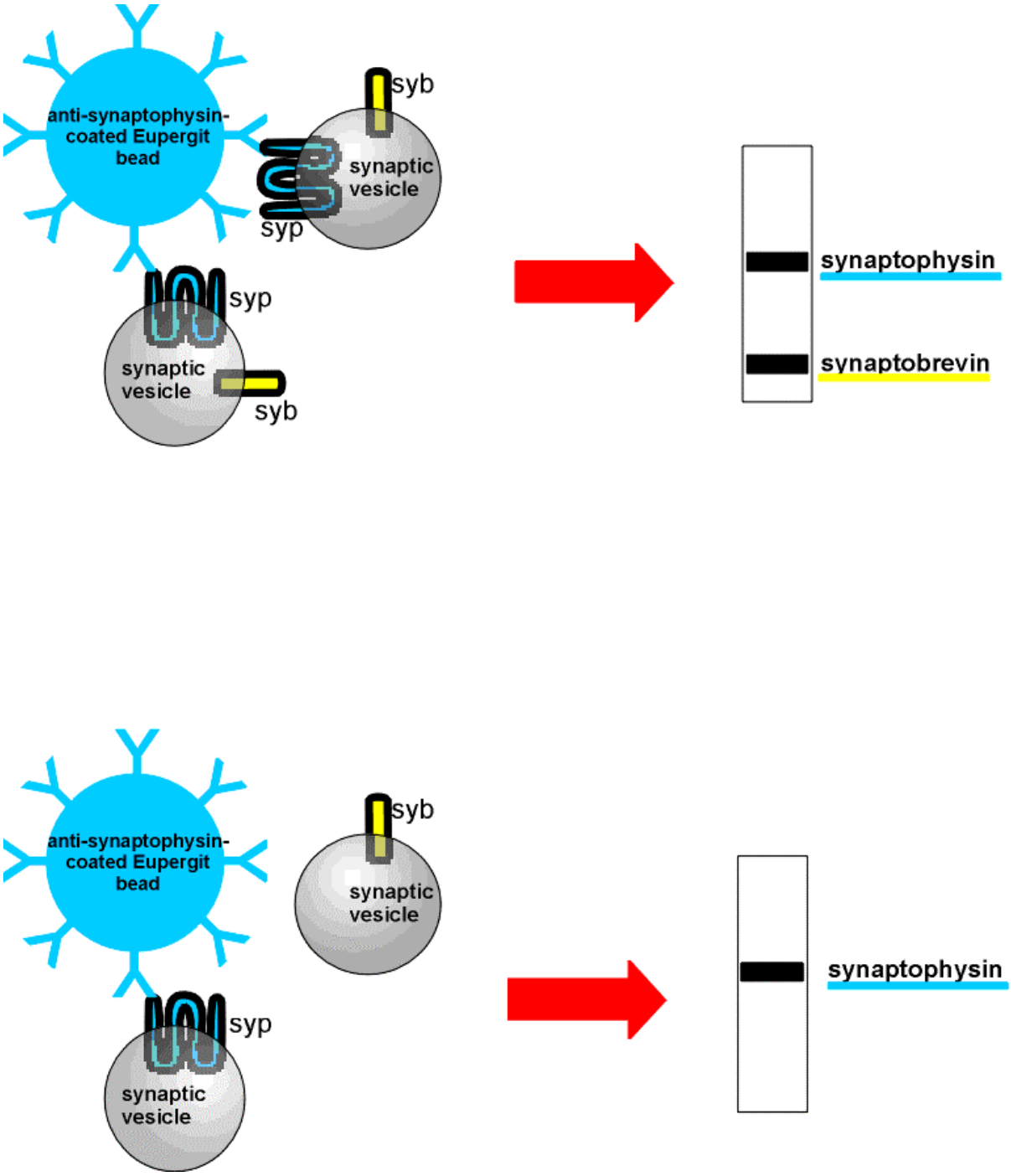

Figure 15
Schematic diagram of immunoprecipitation technique
Eupergit beads coated with an antibody against synaptophysin are used to distinguish whether synaptophysin and synaptobrevin are present on the same vesicle or on different vesicle types (see text for details).

Synaptic vesicles were isolated from adult and embryonic day 20 rat brains using Eupergit beads coated with an antibody against synaptophysin. As was to be expected, both synaptophysin and synaptobrevin could be detected in the bead pellets from adult brain (left lane of "adult" in Figure 16). Interestingly, the same results were obtained when immunoisolating synaptic vesicles from embryonic brain (left lane of "E 20" in Figure 16). Thus, synaptophysin and synaptobrevin are present on the same vesicles in embryonic brain. A difference in location can therefore not explain their lack of interaction.

To confirm the specificity of the immunoisolation experiment, two types of controls were performed. First, Eupergit beads coated with glycine were used to test for non-specific binding. No binding was observed (second lanes in "E 20" and "adult"), although synaptophysin and synaptobrevin were clearly detected in the supernatants. As a second control, binding of the α-subunit of the plasma membrane channel protein Kv 1.6 to the Eupergit beads was tested. No binding was observed (top lanes) although the protein was clearly detected in the supernatants, indicating that the immunoisolation procedure was specific for synaptic vesicles.

These results show that synaptophysin and synaptobrevin occur on the same vesicle population both in adult and in embryonic brain and that lack of binding is therefore not due to a differential sorting process.

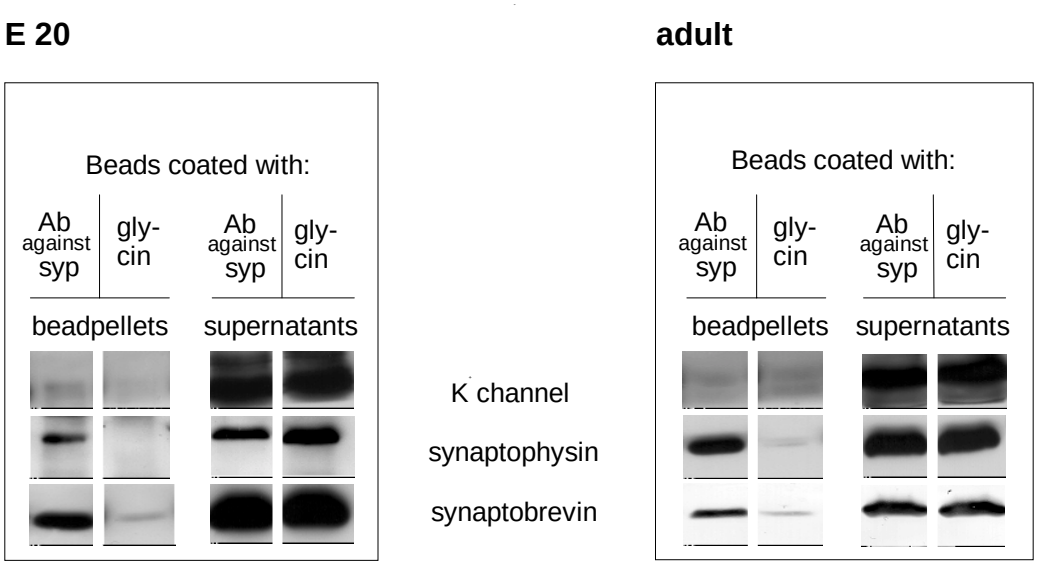

Figure 16
Immunoisolation of synaptic vesicles from E 20 and adult rat brain using antibody-coated Eupergit beads

Eupergit beads coated either with an antibody against synaptophysin, or with glycine as negative control, were incubated with E 20 and adult rat whole brain crude synaptic vesicle fractions (described in Section 3.8). Beadpellets and their corresponding supernatants were analysed for synaptophysin and synaptobrevin, and for the α-subunit of the Kv 1.6 channel as control, via SDS-PAGE and immunoblotting.

4.4 POSTTRANSLATIONAL MODIFICATIONS

Why do synaptophysin and synaptobrevin not interact in embryonic brain even though they are present on the same vesicle type? A possible explanation could be that either synaptophysin or synaptobrevin need to be modified posttranslationally before binding can occur. In this case, a specific enzyme or factor should be present in the adult brain to induce this posttranslational modification. Alternatively, a posttranslational modification of either synaptophysin or synaptobrevin could inhibit binding. In this case, the enzyme or factor would probably be present in the embryonic brain.

4.4.1 Palmitoylation

The failure of synaptophysin and synaptobrevin to interact with each other in embryonic brain might be due to differences in fatty acid modification. Covalent lipid modifications anchor proteins to specific membrane sites. These modifications mediate protein-membrane and protein-protein interactions (for review, see Dunphy and Linder, 1998). SNAP 25, for example, is anchored to the plasma membrane by palmitoylation of cysteine residues (Hess et al., 1992). A negative regulatory role for palmitoylation has been shown for the interaction of the G-protein subunit $G_{z\alpha}$ with G_zGAP, a regulator of G-protein signalling, whereby palmitoylation of $G_{z\alpha}$ inhibited G_zGAP activity (Tu et al., 1997).

The yeast synaptobrevin homologues Snc1 and Snc2 have been shown to be palmitoylated posttranslationally (Couve et al., 1995). To test whether rat synaptobrevin 2 is also palmitoylated, synaptic vesicle fractions from adult and E 20 brain were incubated with radioactive palmitate. Membranes were then either pelleted and stored, or proteins were extracted and immunoprecipitated with antibodies against synaptobrevin or synaptotagmin. The immunoprecipitates and stored membrane fractions were subsequently separated by gel electrophoresis. Two equivalent gels were run, and one was Western-Blotted and incubated with antibodies against synaptobrevin and synaptotagmin, while the other was exposed to film (Figure 17, left and right panels, respectively). Note that these are two different approaches whereby both labelled and unlabelled synaptobrevin and synaptotagmin are visible on the Western Blot, but only radioactively labelled proteins are detectable on the Fluorogram. When precipitating with an antibody against synaptobrevin in adult samples, a band was visible both on the Western Blot and on the Fluorogram. This indicates that radioactive palmitate was incorporated into synaptobrevin from adult brain. Similar results were obtained for synaptotagmin (a finding that has already been published by Chapman et al., 1996, and by Veit et al., 1996). When precipitating with an antibody against synaptobrevin in embryonic samples, a band was visible on the Western Blot but not on the Fluorogram, indicating that synaptobrevin from embryonic brain did not incorporate radioactive palmitate. Similarly, synaptotagmin from embryonic brain was also not palmitoylated. Comparable results were obtained when loading whole membranes without immunoprecipitating (Western blot and Fluorogram, left lanes). Note that the band intensities on the Western Blot indicate that similar amounts of synaptobrevin and synaptotagmin were present in the adult and embryonic samples.

Synaptobrevin 2 and synaptotagmin were palmitoylated in adult but not embryonic rat brain. Indeed, the level of palmitoylation was altogether much lower in embryonic compared to adult rat brain vesicle proteins (Fluorogram, left lanes). However, this does not seem to interfere with SNARE complex formation, which has been shown to occur as early as embryonic day 14 (Figure 4). Could it be that the lack of synaptobrevin palmitoylation inhibits its interaction with synaptophysin? As will be shown below (Section 4.4.3), synaptophysin from adult rat brain is able to bind to recombinant, unpalmitoylated synaptobrevin. It is therefore highly unlikely that the lack of palmitoylation of synaptobrevin in embryonic brain hinders its interaction with synaptophysin in vivo.

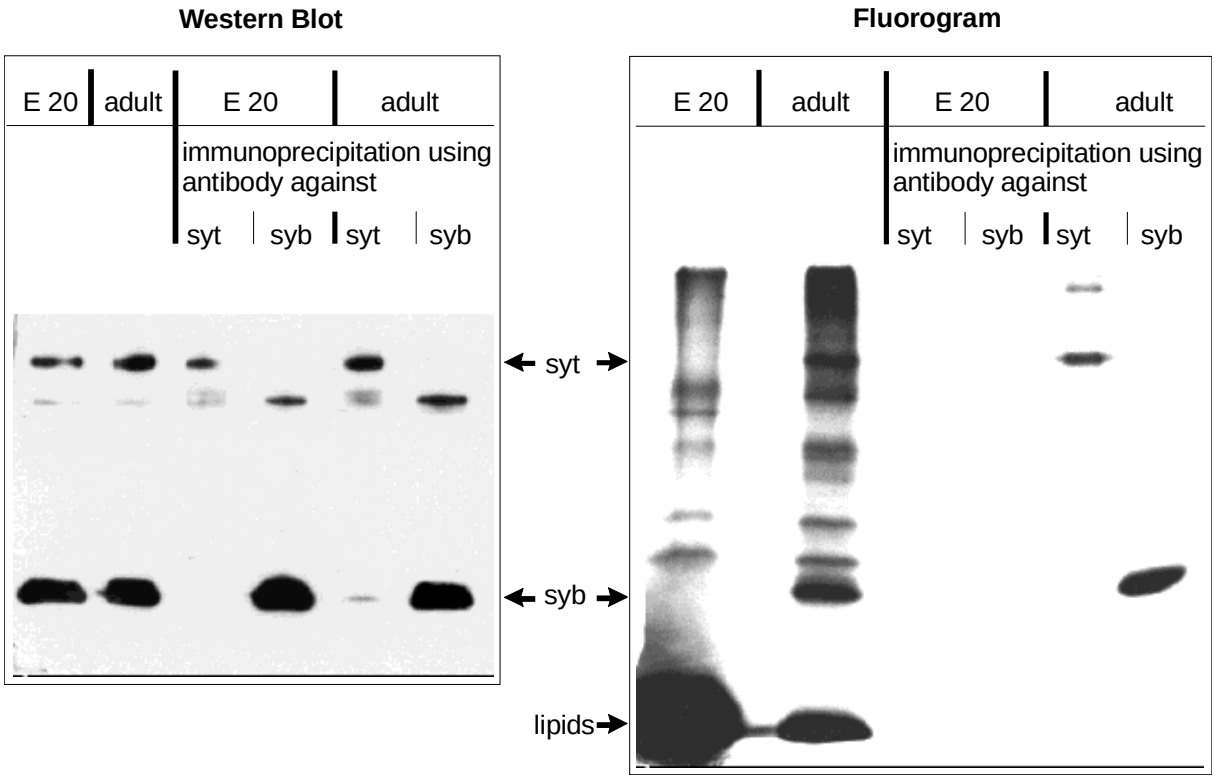

Figure 17
Palmitoylation of synaptobrevin in adult and embryonic synaptic vesicles
E 20 and adult rat whole brain crude synaptic vesicle fractions were incubated with radioactive palmitate, and subsequently pelleted and immediately analysed (two left lanes), or extracted and immunoprecipitated using antibodies against synaptobrevin (syb) and synaptotagmin (syt) and then analysed (four right lanes). Analysis was performed via SDS-PAGE and immunoblotting (whole, uncut Western Blot exposed to antibodies against synaptobrevin (syb) and synaptotagmin (syt), left), or via SDS-PAGE and exposure to film (Fluorogram, right, courtesy of Dr Michael Veit). Note that comparable amounts of synaptobrevin were used for the embryonic and adult experiments.

4.4.2 Reduced and oxidised forms of synaptophysin

The synaptophysin molecule transverses the vesicle membrane four times and has its amino- and carboxy-terminals in the cytoplasm. In this way, two intravesicular loops are formed, which are stabilised by two disulphide bridges, one within each loop. These disulphide bridges have been shown to be essential for synaptophysin homodimerisation, probably because they shape the conformation of synaptophysin (Johnston and Südhof, 1990). The disulphide bridges can be dispersed and reformed via reduction and oxidation procedures in synaptic vesicles isolated from rat adult whole brain homogenates (Johnston and Südhof, 1990). Figure 18 shows that the synaptophysin-synaptobrevin interaction was abolished in the presence of the reducing agent β-mercaptoethanol, indicating that the disulphide bonds of synaptophysin are important for its interaction with synaptobrevin. The interaction of synaptophysin with synaptobrevin could also be inhibited using high salt conditions (Figure 18A, and previously shown by Edelmann et al., 1995).

When exposing synaptic vesicles from embryonic rat brain to reduction and oxidation procedures, no induction of synatophysin-synatobrevin complex formation occurred (Figure 18B). Thus, the lack of synaptophysin-synaptobrevin interaction in embryonic brain does not seem to be due simply to a possible difference in the redox environment of synaptophysin.

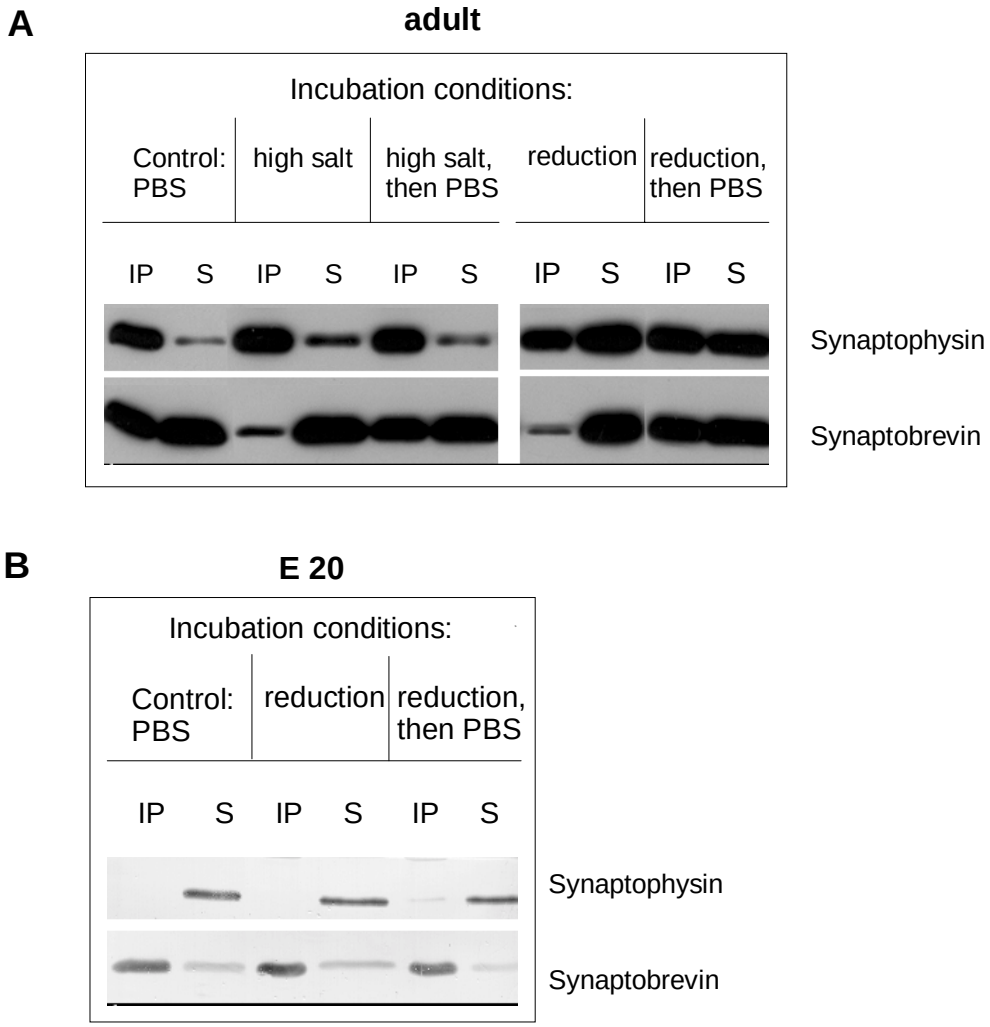

Figure 18
Reversibility of the synaptophysin-synaptobrevin interaction using reduction / oxidation or high salt conditions
Crude synaptic vesicles were isolated from E20 (**B**) or adult (**A**) rat brain and were incubated for 30 minutes at room temperature with PBS, β-mercaptoethanol (10 % v/v in PBS), or 450 nM NaCl (adult samples only). Proteins were then either immediately extracted and immunoprecipitated, or membranes were first washed, resuspended and incubated with PBS prior to protein extraction and immunoprecipitation.

4.4.3 Recombinant synaptobrevin

In order to test whether synaptophysin or synaptobrevin need to undergo posttranslational modifications before they can bind to each other, synaptophysin from embryonic and adult rat brain was exposed to recombinant synaptobrevin. If a posttranslational modification of synaptobrevin is necessary for binding, then recombinant (and therefore unmodified) synaptobrevin should not be able to bind to synaptophysin from adult or embryonic brain. If,

on the other hand, a posttranslational modification of synaptophysin is necessary for binding, then synaptophysin from adult brain (which can bind synaptobrevin from adult brain) should also be able to bind recombinant synaptobrevin.

As can be seen in Figure 19, synaptophysin from adult rat brain was able to bind recombinant synaptobrevin whereas synaptophysin from embryonic brain was not able to bind. Recombinant synaptobrevin was dotted onto nitrocellulose membranes, which were subsequently incubated with synaptosomal extracts from adult and embryonic brain. As is shown in Figure 19A, synaptophysin from adult brain but not from embryonic brain bound to the recombinant synaptobrevin dots. In a different experimental set-up, crude synaptic vesicle extracts from adult and embryonic brain were incubated with recombinant synaptobrevin bound to nickel beads. Again, only synaptophysin from adult brain bound (Figure 19B). These results show that recombinant, unmodified synaptobrevin is perfectly able to bind to synaptophysin from adult brain. Synaptophysin from embryonic brain, however, is unable to bind. This indicates that a posttranslational modification of synaptophysin controls its binding to synaptobrevin. As has been shown before (Edelmann et al, 1995), synaptophysin from adult brain was unable to bind to just the cytoplasmic domain of recombinant synaptobrevin.

In order to test whether neuroendocrine synaptophysin is comparable to that from embryonic brain, PC 12 cell synaptophysin was exposed to recombinant synaptobrevin (Figure 19C). As with embryonic brain synaptophysin, PC 12 synaptophysin did not bind to recombinant synaptobrevin. Synaptophysin from transfected CHO cells (CHOp38), which may lack a posttranslational modification, was also unable to bind to recombinant synaptobrevin (Figure 19C), again suggesting that a posttranslational modification of synaptophysin controls its binding behaviour.

Note that these experiments do not show whether it is embryonic or adult synaptophysin that is posttranslationally modified. Two possible scenarios exist: either synaptophysin from adult brain is modified to facilitate complex formation, or synaptophysin from embryonic brain is modified to inhibit it.

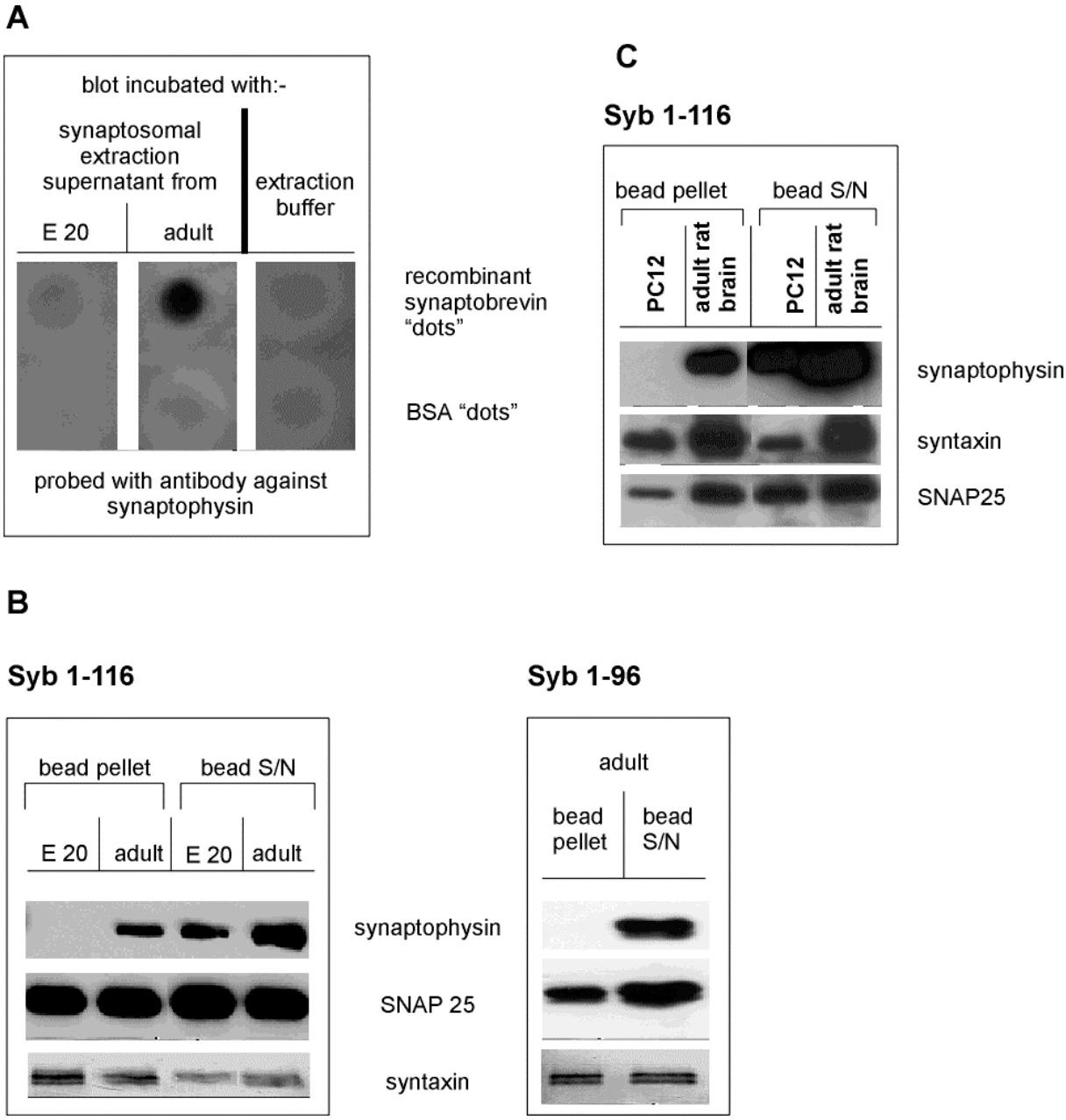

Figure 19
E 20 and adult rat synaptophysin binding to recombinant synaptobrevin

A. Full-length recombinant synaptobrevin was dot-blotted onto nitro-cellulose membranes and the non-specific binding sites were blocked. The membrane was then incubated with synaptosomal extraction supernatants from E 20 and adult rat whole brain crude synaptosomes, washed, and probed using an anti-synaptophysin antibody. Non-specific binding interactions were assessed using BSA dot-blots, and via incubation with extraction buffer alone.

B. Crude synaptic vesicle extracts from E 20 and adult rat brain were incubated with either full-length synaptobrevin (residues 1-116) or the N-terminal (cytoplasmic) part of synaptobrevin (residues 1-96) (see section 3.9). Beadpellets and their corresponding supernatants were analysed for the indicated proteins via SDS-PAGE and immunoblotting.

C. CHOp38 and PC 12 cell extracts were incubated with full-length recombinant synaptobrevin. Beadpellets and their corresponding supernatants were analysed for the indicated proteins via SDS-PAGE and immunoblotting.

4.5 A FACTOR THAT INDUCES BINDING

If synaptophysin is posttranslationally modified in the adult brain to facilitate synaptophysin-synaptobrevin binding, or in the embryonic brain to inhibit binding, then a factor that induces such a modification may be present in adult or embryonic synaptic cytosol. To test this hypothesis, adult or embryonic synaptic vesicle fractions were incubated with synaptic cytosol from adult or embryonic brain (Figure 20).

Interestingly, when incubating synaptic vesicles from embryonic brain with cytosol from adult brain, synaptophysin-synaptobrevin complex formation was induced (Figures 20A and 20B). This induction did not occur when synaptic vesicles from embryonic brain were incubated with cytosol from embryonic brain. Synaptic vesicles from adult brain contained the synaptophysin-synaptobrevin complex both after incubation with adult and with embryonic cytosol (Figure 20A). Additionally, a control incubation of synaptic vesicles from embryonic brain with PBS did not induce formation of the synaptophysin-synaptobrevin complex (Figure 20B). Thus, embryonic cytosol does not contain a factor that inhibits complex formation. Note that the adult synaptic cytosol was free of synaptophysin and synaptobrevin (Figure 20B).

In PC 12 cell extracts, the synaptophysin-synaptobrevin complex did not occur (Figure 13). Neither was it present in PC 12 cell vesicle fractions incubated with PBS (Figure 20C, left lanes). However, after incubation of PC 12 cell vesicle fractions with adult rat brain synaptosomal cytosol, complex formation was induced (Figure 20C, right lanes). Comparable results were also obtained for CHOp38 cells, where synaptophysin did not bind to recombinant synaptobrevin (Figure 19C). When CHOp38 cell membrane fractions were incubated with PBS prior to addition of recombinant synaptobrevin, no synaptophysin-synaptobrevin complex could be detected (Figure 19D). However, incubation of CHOp38 cell fractions with adult synaptosomal cytosol prior to addition of recombinant synaptobrevin induced complex formation. These results indicate that synaptophysin from neuroendocrine cells or from CHOp38 cells behaves similarly to, and therefore may be compared to, synaptophysin from embryonic brain.

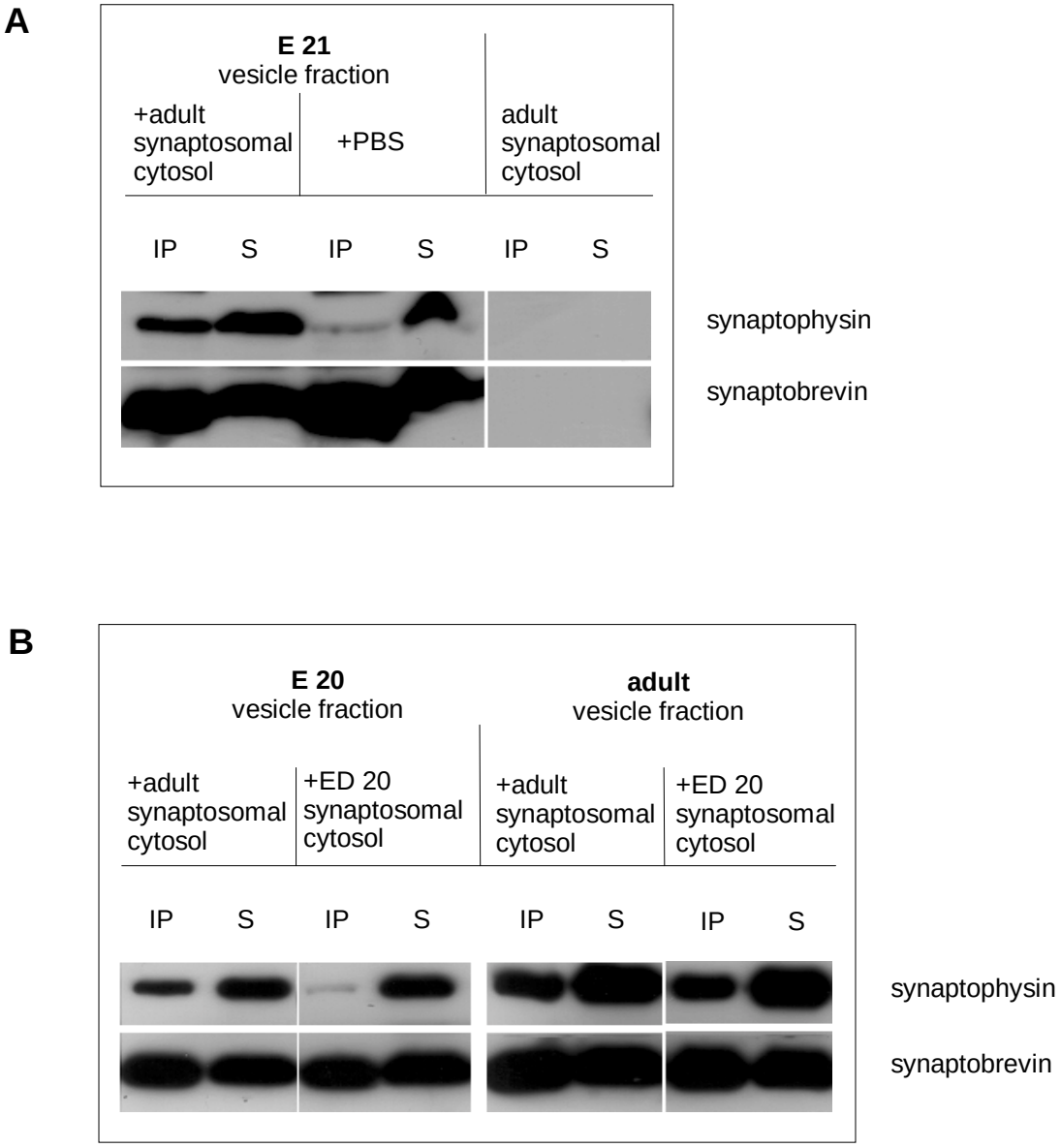

Figure 20
Incubation of adult and embryonic synaptic vesicles with synaptosomal cytosol

A. Crude synaptic vesicle fractions from E 20 or adult rat brain were incubated for 90 minutes at 37ºC with the synaptosomal cytosol fractions (LS2) obtained from either adult brain or from embryonic brain. Proteins were subsequently extracted and immunoprecipitated using an antibody against synaptobrevin (Cl 69.1). Immunoprecipitates (IP) and supernatants (S) were analysed for synaptobrevin and synaptophysin via SDS-PAGE and immunoblotting.

B. Crude synaptic vesicle fractions from E 20 rat brain were incubated for 90 minutes at 37ºC with a synaptosomal cytosol fraction (LS2) obtained from adult brain. Proteins were subsequently extracted and immunoprecipitated using an antibody against synaptobrevin (Cl 69.1). Immunoprecipitates (IP) and supernatants (S) were analysed for synaptobrevin and synaptophysin via SDS-PAGE and immunoblotting. Note that the adult synaptosomal cytosol alone did not contain synaptophysin or synaptobrevin (right panel).

C. and D. see following page

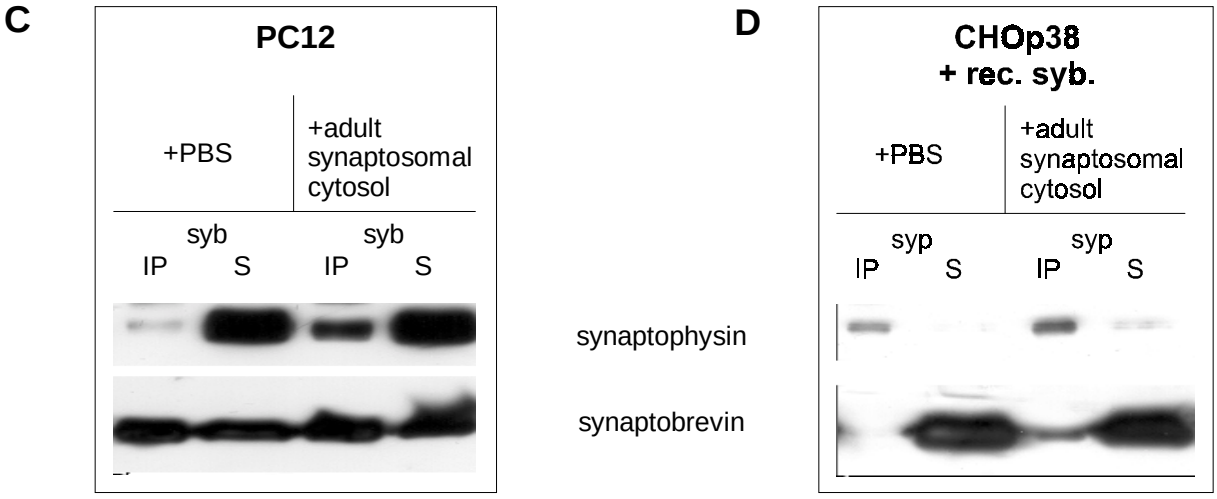

Figure 20 (continued from preceding page)

C. Crude vesicle fractions from PC 12 cells were incubated for 90 minutes at 37ºC with PBS or with a synaptosomal cytosol fraction (LS2) obtained from adult brain. Proteins were then extracted and immunoprecipitated using antibodies against synaptobrevin (Cl 69.1) and synaptophysin (Cl 7.2), and samples analysed as described above.

D. Membrane fractions from CHOp38 cells were incubated for 90 minutes at 37ºC with PBS or with a synaptosomal cytosol fraction (LS2) obtained from adult brain prior to extraction and addition of full-length recombinant synaptobrevin. Proteins were then immunoprecipitated using antibodies against synaptobrevin (Cl 69.1) and synaptophysin (Cl 7.2), and samples analysed as described above.

Adult brain synaptic cytosol seems to contain a factor that induces synaptophysin-synaptobrevin interaction, probably via a posttranslational modification of synaptophysin. In order to test whether this factor is a protein (or peptide), adult synaptic cytosol was trypsin-digested prior to incubation with embryonic synaptic vesicles (Figure 21). Whereas untreated adult synaptic cytosol induced synaptophysin-synaptobrevin complex formation both in embryonic synaptic vesicle fractions (Figure 21 A) and in permeabilised synaptosomal fractions (Figure 21 B), trypsin-digested cytosol failed to induce complex formation in either fraction. These results indicate that the complex-inducing factor present in adult brain synaptosomal cytosol is a protein or peptide.

The adult cytosol was subsequently fractionated according to molecular weight using Centricon Concentrators (see Section 2.4.2) with a molecular weight cut-off of 10 kDa and

3 kDa. The cytosolic activity that induced synaptophysin-synaptobrevin complex formation was still present in fractions containing only molecules smaller than 3 kDa (not shown), thus suggesting that the cytosolic factor is a peptide rather than a large protein. In addition, the activity was not affected by boiling of the sample (not shown), implicating that the active peptide is probably unfolded in its native condition. A few such natively unfolded peptides and proteins are known to exist at the synapse, for example synuclein, a protein implicated in Alzheimer's disease and learning (Weinreb et al., 1996). Further experiments including high performance liquid chromatography (HPLC) fractionation of the relevant cytosolic fractions are still required to identify and characterise the peptide responsible for the synaptophysin-synaptobrevin interaction.

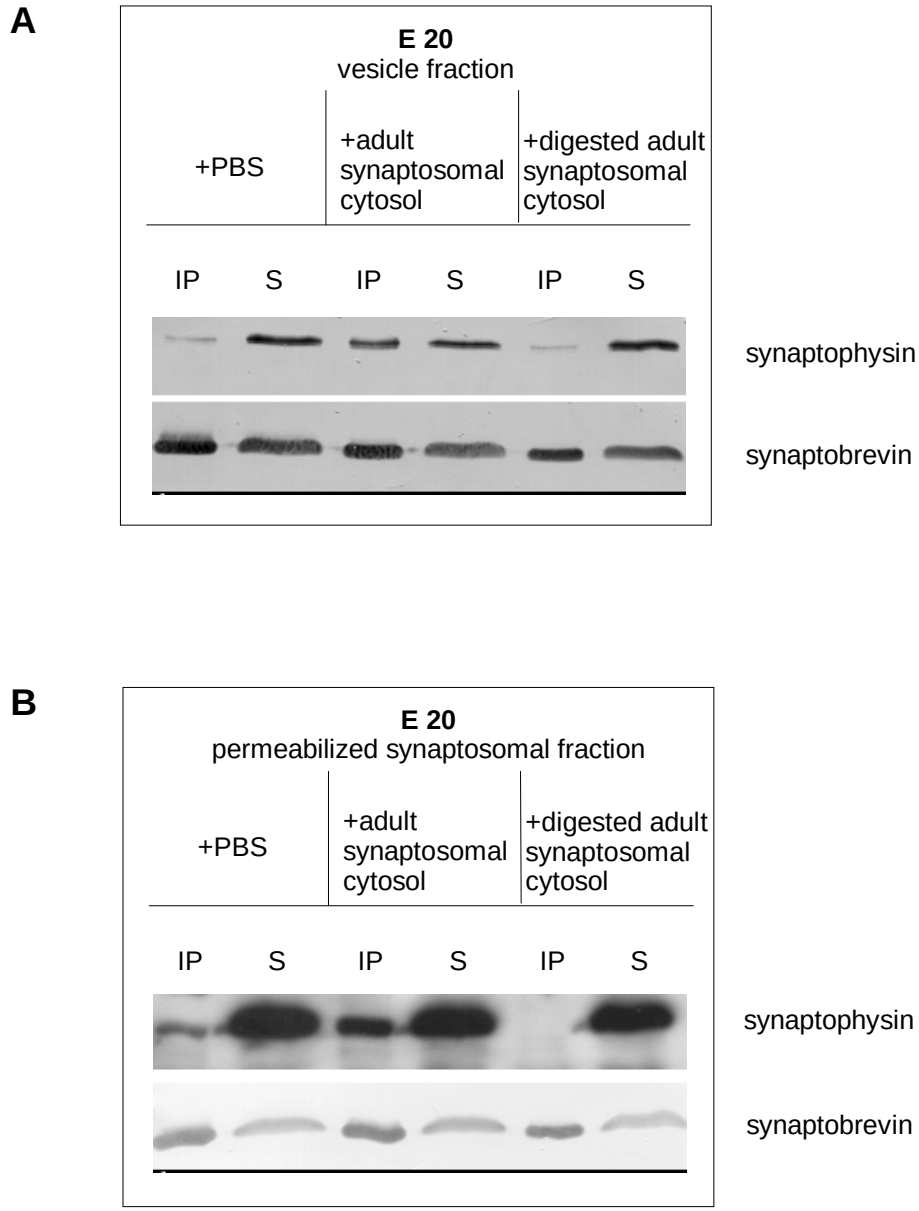

Figure 21
Trypsin-digestion of the adult LS2 fraction prior to incubation with E 20 synaptic vesicles or permeabilised synaptosomes

Crude synaptic vesicles (**A**) or SLO-permeabilised crude synaptosomes (**B**) from E 20 rat brain were incubated either with PBS, adult synaptosomal cytosol (LS2 fraction), or trypsin-digested adult synaptosomal cytosol for 90 minutes at 37ºC. Proteins were subsequently extracted and immunoprecipitated using an antibody against synaptobrevin (Cl 69.1). Immunoprecipitates (IP) and supernatants (S) were analysed for synaptobrevin and synaptophysin via SDS-PAGE and immunoblotting.

4.6 PHYSIOLOGICAL / PATHOPHYSIOLOGICAL RELEVANCE

Is the synaptophysin-synaptobrevin complex controlled only with respect to neuronal development, or are there additional situations in which the complex is regulated? Preliminary experiments with kindled rats, that serve as a model for epilepsy, showed an increase in synaptophysin-synaptobrevin complex formation in kindled rat brains compared to unstimulated controls (Figure 22). The term kindling refers to a stimulus-produced seizure activity which is progressively intensified by repeated high frequency stimulation. The mechanism may perhaps be similar to that of long-term potentiation, where a short period of intense activity gives rise to a persistent change in synaptic strength (Martin, 1991). If kindling should indeed produce an increase in synaptophysin-synaptobrevin complex formation, then this complex may play a role during periods of high synaptic activity. Further experiments are however necessary to verify the results obtained.

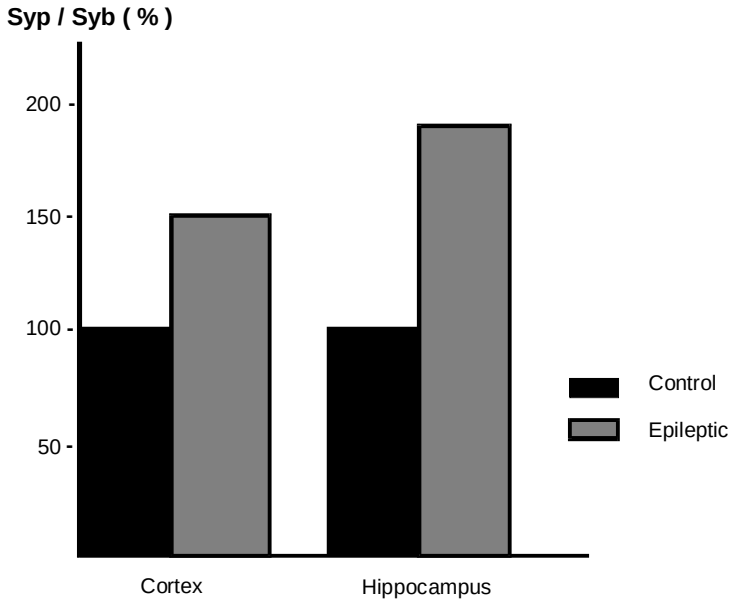

Figure 22
Immunoprecipitation of the synaptophysin-synaptobrevin complex from control and kindled rat cortex and hippocampus

Crude synaptosomal fractions were prepared from cortex and hippocampus of kindled and control rats (kindly supplied by Dr Andreas Draguhn). Proteins were extracted at a concentration of 1 mg / ml and immunoprecipitated using an antibody against synaptobrevin (Cl 69.1). Immunoprecipitates and supernatants were analysed for synaptobrevin and synaptophysin via SDS-PAGE and immunoblotting. Antibody bands were scanned and quantitated using ScanPacK 3.0. An increase in the synaptophysin / synaptobrevin ration (syp / syb) indicated an increase in synaptophysin-synaptobrevin complex formation.