5 DISCUSSION

5.1 THE SNARE PROTEINS AND SYNAPTOPHYSIN

Synapses are specialised points of contact between neurons where information is transferred from one cell to the next by the regulated release of neurotransmitters in response to calcium ion signals. Neurotransmitters are stored in small synaptic vesicles. When an action potential arrives at the nerve terminal, voltage-gated calcium ion channels open and cause an influx of calcium ions. This influx triggers the fusion of synaptic vesicles with the plasma membrane. A fusion pore is formed, and neurotransmitter molecules are released into the synaptic cleft where they diffuse and subsequently bind to and activate specific receptors on the postsynaptic cell membrane. The synaptic vesicle is then endocytosed and reloaded with neurotransmitter, ready for another round of exocytosis. These exocytosis-endocytosis cycles can be repeated many times and are independent of the nerve cell body. Thus, the synapse contains all elements necessary for vesicle exocytosis and recycling.

For exocytosis to occur, the synaptic vesicle membrane has to fuse with the plasma membrane, thereby forming an aqueous fusion pore through which neurotransmitter is released. The fusion of the vesicle with the plasma membrane does not occur spontaneously because the repulsive energy between the two phospholipid bilayers is too high at close distances. Fusion proteins that bind to each other and overcome this repulsive energy are therefore essential prerequisites of exocytosis. At the synapse these fusion proteins are synaptobrevin on the synaptic vesicle, and syntaxin 1a and SNAP 25 at the plasma membrane. These three proteins are extremely reactive and form a highly stable ternary complex. Formation of this complex occurs spontaneously *in vitro* and is resistant to heat or detergent denaturation. In fact, binding of the SNARE proteins is virtually irreversible in the absence of the cellular chaperone NSF. Even when NSF is present, separated SNARE complexes will immediately reform *in vitro*.

The release of neurotransmitters at the synapse has to be a highly regulated event as faulty signal transduction could be detrimental to the organism. The extreme reactivity of the SNARE proteins thus probably necessitates the presence of regulatory proteins that control and fine-tune the interactions of synaptobrevin, syntaxin and SNAP 25 with each other and with other binding partners. While recent data has greatly increased our understanding of the molecular mechanisms underlying SNARE complex formation, little is known concerning the

regulation of this complex, both in the adult brain and during development. A few proteins that interact with the SNAREs have been identified. However, in most cases, not much is known about their function or regulatory significance.

One of the most intriguing candidate regulatory proteins is the synaptic vesicle protein synaptophysin which interacts with synaptobrevin. Synaptophysin was one of the first synaptic proteins to be identified (Bock and Helle, 1977; Jahn et al., 1985; Wiedenmann and Franke, 1985), but up until now its exact function has remained controversial. Its interaction with synaptobrevin is specific and exclusive, meaning that synaptobrevin that is bound to synaptophysin cannot at the same time enter the SNARE complex, and vice versa (Calakos and Scheller, 1994; Edelmann et al., 1995). The presence of two non-overlapping pools of synaptobrevin suggests a mechanism whereby synaptophysin regulates the availability of synaptobrevin to the SNARE complex. It was the aim of the present study to increase our knowledge concerning the function of synaptophysin and of the synaptophysin-synaptobrevin complex. Specifically, a possible regulation of the synaptophysin-synaptobrevin complex during development was to be examined.

5.2 DEVELOPMENTAL UPREGULATION OF THE SYNAPTOPHYSIN-SYNAPTOBREVIN COMPLEX

In this current study, immunoprecipitation procedures revealed that the synaptophysin-synaptobrevin complex was present in adult rat brain but was absent in embryonic brain. Indeed, whereas the SNARE complex could be detected as early on as embryonic day 14, the synaptophysin-synaptobrevin complex was absent in embryonic brain and only started to appear postnatally. This very interesting result was verified using a range of control experiments.

5.2.1 Control experiments verify the developmental upregulation of the synaptophysin-synaptobrevin complex

As a first control experiment, immunoprecipitates from adult and embryonic brain extracts which had been analysed using monoclonal antibodies against synaptobrevin and synaptophysin, were analysed using polyclonal antibodies. The idea was that polyclonal antibodies, which usually recognise more antigen epitopes than their monoclonal equivalents, might detect a form of synaptophysin or synaptobrevin that is not recognised by the

monoclonal antibodies. However, the polyclonal antibodies yielded similar results to their monoclonal equivalents, namely the absence of the synaptophysin-synaptobrevin complex in embryonic brain. In a second control experiment approximately ten times more of the embryonic than the adult immunoprecipitates with respect to total protein content was analysed. This might have revealed the presence of synaptophysin in the synaptobrevin immunoprecipitates that was previously below the detection limit. However, no synaptophysin-synaptobrevin complex was detected even in this case in embryonic brain. Thus, the absence of the synaptophysin-synaptobrevin complex in embryonic brain was also substantiated by this control experiment.

Immunoprecipitation of protein complexes was always performed in the presence of detergent. Detergents solubilise membrane proteins, and thereby release them from conformational restraints that may have been imposed by their membrane location. Detergent extraction procedures might therefore led to the formation of protein complexes that did not exist prior to solubilisation of membrane proteins, or to the loss of interactions that were present before solubilisation. In order to verify that the lack of synaptophysin-synaptobrevin interaction in embryonic detergent extracts was not due to a detergent artefact, extractions were performed at three different protein to detergent ratios ranging from 0.5 mg/ml to 5 mg/ml. The synaptophysin-synaptobrevin complex was absent at all three protein to detergent ratios tested, again substantiating the initial results obtained.

5.2.2 Cross-linking of the synaptophysin-synaptobrevin complex in adult but not in embryonic brain

For the final control experiment an approach was chosen to analyse protein interactions without having to use detergent solubilisation at all. Proteins that are in very close proximity to each other can be cross-linked chemically, and the complex can then be immunodetected on Western Blots. Employing this approach, synaptic proteins from adult and embryonic brain were cross-linked in their natural membrane environments using the membrane-permeable cross-linker disuccinimidyl suberate. Whole membranes were subsequently collected by centrifugation and analysed. A 56 kDa band corresponding to the synaptophysin-synaptobrevin complex was detectable only in synaptosomal samples from adult brain but not from embryonic brain. Similar results were obtained when cross-linking proteins in synaptic vesicle fractions (not shown). Thus, the absence of the synaptophysin-synaptobrevin complex

in embryonic brain had now been substantiated using a range of experiments, both in the presence and in the absence of detergent.

Interestingly, only traces of the synaptobrevin homodimer were detected in cross-linked embryonic brain samples. The function of the synaptobrevin homodimer has not yet been elucidated. However, Laage and Langosch (1997) were able to show that synaptobrevin homodimerises via its transmembrane domains and that appearance of the dimer is concentration dependent. Thus, an increase in the concentration of synaptobrevin on the vesicle probably leads to increased dimer formation. Less synaptobrevin dimer formation in the embryonic brain could therefore indicate that fewer synaptobrevin molecules are present on embryonic vesicles than on adult vesicles. Whether synaptophysin binds to the dimerised form of synaptobrevin is not known. In the present study, no such heterotrimers were detected on Western Blots after cross-linking. Perhaps synaptobrevin homodimerisation serves as a mechanism that links individual SNARE complexes into a larger ring-like structure that lines the fusion pore during regulated exocytosis.

5.2.3 Regional differences in synaptophysin-synaptobrevin complex formation with respect to development?

Experiments so far were performed on whole brains and therefore yielded whole brain averages as results. To check for regional differences, proteins were immunoprecipitated from different brain areas of adult and embryonic rat including bulbus olfactorius, cortex and cerebellum. The results mirrored those obtained for whole brain, namely the presence of the synaptophysin-synaptobrevin complex in all adult brain areas tested and its absence in all embryonic brain areas tested. Thus, the developmental upregulation of the synaptophysin-synaptobrevin complex seems to apply to most if not all areas of the brain. This upregulation was also detected in mouse brain. As was the case in rat brain, the synaptophysin-synaptobrevin complex was absent in the embryonic mouse brain even though it was clearly present in the adult mouse brain. Furthermore, the developmental upregulation could also be seen in mouse hippocampal primary tissue culture, where the synaptophysin-synaptobrevin complex was not detected at day six *in vitro* but was clearly present at day thirteen *in vitro*. This observation is interesting because it means that the developmental cue(s) that induce synaptophysin-synaptobrevin complex formation in the postnatal brain also occur in tissue culture, at least by day thirteen.

Surprisingly, when culturing neurons from mouse hypothalamus, the synaptophysin-synaptobrevin complex was still absent at thirteen days *in vitro*. In one out of three preparations, however, some complex formation could be detected by thirteen days *in vitro*, perhaps due to "contamination" by presumably GABAergic neurons that were not removed during dissection of the very small hypothalamic area in the mouse embryonic brain. Mouse hypothalamic neurons are peptidergic, and it was therefore very interesting to note that, when immunoprecipitating proteins from various peptide-secreting neuroendocrine cell-lines, the synaptophysin-synaptobrevin complex was also absent. Furthermore, studies performed by Anne Drenckhahn in our laboratory have shown that the synaptophysin-synaptobrevin complex is likewise absent in neuroendocrine tissues including the neurohypophysis, adrenal gland, and adenohypophysis (Becher et al., 1999b). These results show that neuroendocrine cells, and possibly also peptide-secreting neurons, are comparable to embryonic neurons with respect to the synaptophysin-synaptobrevin interaction.

5.3 POSSIBLE FACTORS REGULATING THE DEVELOPMENTAL UPREGULATION OF THE SYNAPTOPHYSIN-SYNAPTOBREVIN COMPLEX

Once the lack of synaptophysin-synaptobrevin interaction in embryonic brain had been established, possible reasons for the absence of this complex were examined. At least two potential explanations existed. Either synaptophysin and synaptobrevin were functionally quite able to interact but did not do so because of different localisations. This would be the case if synaptophysin and synaptobrevin occurred in different neurons or on different vesicle populations during development. Alternatively, synaptophysin and synaptobrevin could occur on the same vesicle population in the embryonic brain but be functionally unable to interact, perhaps because of a posttranslational modification.

5.3.1 Synaptophysin and synaptobrevin occur on the same vesicle population during development

Immunofluorescence analyses of embryonic cerebellum (kindly performed by Dr Ingrid Pahner) showed a perfect co-localisation of synaptophysin and synaptobrevin. This result indicated that synaptophysin and synaptobrevin were at least present in the same neurons in the embryo. Immunoisolation techniques were then used to analyse whether synaptophysin and synaptobrevin localised to the same vesicle population in embryonic brain. Using beads

coated with antibody against synaptophysin, only those vesicles that contained synaptophysin were isolated. Analysis of the embryonic vesicle fractions recovered by this method revealed the presence of synaptobrevin, indicating that synaptophysin and synaptobrevin occurred on the same vesicle population in the embryonic brain. A difference in localisation could therefore not explain the absence of the synaptophysin-synaptobrevin complex in embryonic brain.

5.3.2 Observed differences in lipid modification of synaptobrevin cannot explain the developmental upregulation of the synaptophysin-synaptobrevin complex

If synaptophysin and synaptobrevin occur on the same vesicle population in embryonic brain, then their lack of interaction is probably due to a functional constraint. A possible explanation for the absence of the synaptophysin-synaptobrevin complex in embryonic brain could be that synaptophysin and / or synaptobrevin need to be modified posttranslationally before binding can occur.

Posttranslational fatty acid modifications have been shown to mediate protein-membrane and protein-protein interactions (Dunphy and Linder, 1998). Yeast synaptobrevin homologues are palmitoylated posttranslationally (Couve et al., 1995), therefore the palmitoylation state of synaptobrevin from adult and embryonic rat brain was analysed. Surprisingly, while synaptobrevin from adult brain was palmitoylated, synaptobrevin from embryonic brain seemed not to be. Although this was initially considered as a possible control mechanism for the synaptophysin-synaptobrevin interaction, later studies using recombinant and therefore unpalmitoylated full-length synaptobrevin showed that synaptophysin from adult brain was perfectly capable of binding to this unpalmitoylated form of synaptobrevin whereas synaptophysin from embryonic brain was not. In addition, unpalmitoylated recombinant synaptobrevin was also able to interact with its SNARE binding partners syntaxin and SNAP 25 both from adult and from embryonic brain. These results suggest that the palmitoylation state of synaptobrevin does not interfere with its binding to synaptophysin. Neither is palmitoylation of synaptobrevin a prerequisite for its binding to syntaxin and SNAP 25. Thus, the posttranslational fatty acid modification probably has other functions, perhaps anchoring synaptobrevin to very specific membrane sites on adult but not embryonic synaptic vesicles.

5.3.3 Synaptophysin from embryonic brain, from PC12 cells, and from transfected CHO cells does not bind to recombinant synaptobrevin

As mentioned above, synaptophysin from adult brain extracts was able to bind to full-length recombinant synaptobrevin whereas synaptophysin from embryonic brain was not. Synaptophysin from the pheochromocytoma cell line PC 12, and from transfected Chinese hamster ovary (CHO) cells behaved similarly to synaptophysin from embryonic brain in that it also did not bind to recombinant synaptobrevin. From these results it is deduced that a posttranslational modification of synaptophysin regulates its binding to synaptobrevin: Seeing that both synaptobrevin from adult brain and unmodified recombinant synaptobrevin were able to bind to synaptophysin from adult brain, it seems probable that no posttranslational modification of synaptobrevin is necessary to bring about binding in the adult brain. Furthermore, it seems unlikely that embryonic synaptobrevin is posttranslationally modified to inhibit binding, since then synaptophysin from embryonic brain should be able to bind to the unmodified recombinant synaptobrevin. These results suggest that synaptobrevin does not need to be posttranslationally modified in order to participate in the synaptophysin-synaptobrevin complex, and that possibly a posttranslational modification of synaptophysin controls its interaction with synaptobrevin.

5.3.4 Disulphide bridges in synaptophysin from adult and embryonic brain

Synaptophysin transverses the synaptic vesicle membrane four times and has both its amino-terminus and its carboxy-terminus in the cytoplasm. In this way, two intravesicular loops are formed which are stabilised by disulphide bridges, one within each loop. These disulphide bridges have been shown previously to be important for synaptophysin homodimerisation (Johnston and Südhof, 1990). In the present study it was shown that these disulphide bridges were also important for the synaptophysin-synaptobrevin interaction. Probably they confer a conformational structure upon synaptophysin that is necessary to allow binding to synaptobrevin. Incubation of synaptic vesicles from adult brain with the reducing agent β-mercaptoethanol abolished the interaction of synaptophysin with synaptobrevin. This observation led to the idea that a possible difference in disulphide bridge-formation in the embryonic brain might perhaps account for the lack of synaptophysin-synaptobrevin interaction. However, when non-reduced synaptic vesicle samples from adult and embryonic brain were analysed via SDS-polyacrylamide gel electrophoresis and immunoblotting procedures, synaptophysin from adult and from embryonic brain showed identical running

patterns (not shown). Furthermore, exposing synaptic vesicles from embryonic brain to a reduction-oxidation cycle did not induce synaptophysin-synaptobrevin binding. These results indicate that both synaptophysin from adult brain and from embryonic brain contain disulphide bridges and that disulphide bridge-formation is therefore not a probable mechanism controlling the synaptophysin-synaptobrevin interaction.

5.3.5 Possible binding partners for the synaptophysin-synaptobrevin complex

In summary, the synaptophysin-synaptobrevin complex does not occur in the embryonic brain, even though synaptophysin and synaptobrevin localise to the same vesicle population. Therefore, their lack of interaction is probably due to a functional constraint. Could it be that an additional protein is needed to mediate binding, and that synaptophysin and synaptobrevin only bind in a ternary complex with this third protein? The synaptophysin-synaptobrevin complex has been described to interact with the c subunit of the vacuolar proton pump (Galli et al., 1996), and with the organelle-associated motor protein brain myosin V (Prekeris and Terrian, 1997). In experiments performed for the current study the c subunit of the vacuolar proton pump was often absent under conditions where the synaptophysin-synaptobrevin complex could be immunoprecipitated (not shown). These results suggest that the interaction of synaptophysin with synaptobrevin does not depend on the presence of the vacuolar proton pump. The interaction of the synaptophysin-synaptobrevin complex with brain myosin V was not analysed in the present study. However, only a minority of vesicles carrying the synaptophysin-synaptobrevin complex are complexed with brain myosin V (Prekeris and Terrian, 1997). Furthermore, brain myosin V is an important component of the neuronal growth cone motility system (Wang et al., 1996), where the synaptophysin-synaptobrevin complex does not occur (present study). Therefore, myosin V is highly unlikely to play a role in regulating the synaptophysin-synaptobrevin interaction. In the search for further proteins that might interact with the synaptophysin-synaptobrevin complex, immunoprecipitates from adult and embryonic brain were compared on silver-stained SDS polyacrylamide gels (not shown). However, no conspicuous differences in protein band patterns were noted between adult and embryonic samples, at least not in the molecular weight range tested.

5.3.6 A small peptide from adult cytosol induces formation of the synaptophysin-synaptobrevin complex

If a posttranslational modification of synaptophysin controls its interaction with synaptobrevin, this mechanism could operate in one of two ways: either synaptophysin is modified in the adult brain to induce synaptobrevin-binding, or it is modified in the embryo to inhibit binding. Such a modification could be caused by a factor residing on the vesicle or in the cytosol. If the factor should reside in the cytosol, then incubation of synaptophysin and synaptobrevin with this cytosol might change their binding behaviour. A set of experiments that was designed to try and test these possibilities yielded exciting results, namely that a factor was present in adult cytosol that caused binding of synaptophysin to synaptobrevin. When embryonic synaptic vesicles were incubated with adult synaptic cytosol, synaptophysin-synaptobrevin complex formation was induced. These results indicate that the synaptophysin-synaptobrevin interaction is modulated by a posttranslational modification of synaptophysin in the adult brain.

When performing the cytosolic incubation experiments the other way around, namely incubating adult synaptic vesicles with embryonic synaptic cytosol, this did not disrupt the synaptophysin-synaptobrevin complex on adult vesicles. Thus, the embryonic cytosol did not contain a factor that inhibited formation of the synaptophysin-synaptobrevin complex. Therefore, synaptophysin seems to be modified posttranslationally in mature neurons, and this modification facilitates its binding to synaptobrevin. Similar results were obtained when incubating PC 12 cells with adult rat synaptic cytosol. Here, again, binding of synaptophysin to synaptobrevin was induced upon cytosol incubation. Also, when incubating synaptophysin-transfected CHO cells with adult synaptic cytosol and then with recombinant synaptobrevin, binding of synaptophysin to the recombinant synaptobrevin was induced. These results suggest that synaptophysin from neuroendocrine cells and transfected CHO cells, and that from embryonic brain are comparable with respect to their synaptobrevin-binding behaviour.

The cytosolic factor that induces synaptobrevin-synaptophysin binding still needs to be characterised. Preliminary results showed that the activity was trypsin-sensitive and could be dialysed through a 3 kDa membrane, indicating that it was a short peptide of probably less than 20 amino acids in length. In addition, adult cytosol could be boiled for up to fifteen minutes without reduction of activity, suggesting either that the peptide is unfolded in its native condition, or that it easily refolds subsequent to heat denaturation. High performance liquid chromatography (HPLC) of the less than 3 kDa cytosolic fractions from embryonic and

adult brain (kindly performed by Dr Thomas Jöns and Dr Klaus Buchner) revealed a double-peak at 280 nm that was present only in adult cytosol (not shown). Whether this peak represents the cytosolic activity that induces synaptophysin-synaptobrevin binding remains to be established. Because of its small size, the cytosolic factor is probably not an enzyme but perhaps rather a short linker peptide that binds to synaptophysin and thereby changes its interaction domain in such a way that binding to synaptobrevin can occur, possibly by changing the charge or neutralising a steric hindrance.

5.4 POSSIBLE FUNCTIONS OF SYNAPTOPHYSIN AND THE SYNAPTOPHYSIN-SYNAPTOBREVIN INTERACTION

Before synaptogenesis, synaptophysin and synaptobrevin do not interact although they reside on the same vesicle population. In neuroendocrine cells, synaptophysin and synaptobrevin do not interact at all. It is therefore probable that synaptophysin has more than one function, one involving its interaction with synaptobrevin, and the other being independent of synaptobrevin. A range of possible functions for synaptophysin has been suggested in the literature. The initially proposed roles in binding calcium (Rehm et al., 1986), and in forming an exocytotic fusion pore (Thomas et al., 1988) could not be substantiated in later studies (see Südhof and Jahn, 1991; Brose et al., 1992). Unfortunately, synaptophysin knock-out mice have not been able to shed additional light on the role that synaptophysin plays during exocytosis (Eshkind and Leube, 1995; McMahon et al., 1996). These mice seem very similar to their normal litter mates in general behaviour and appearance. Possibly they have certain defects in vesicle recycling and synaptic transmission that are not crucial for vital functions but may still perform important specific tasks not detected with the methods used in these studies. Additionally, the synaptophysin isoform synaptoporin and the distantly related synaptogyrin (a synaptic vesicle protein with a structure similar to synaptophysin) (Stenius et al., 1995) may compensate for the absence of synaptophysin. Therefore double and triple knock-outs of synaptophysin with synaptoporin and / or synaptogyrin would probably yield more conclusive results.

5.4.1 Synaptophysin probably plays a role in the genesis and stabilisation of synaptic vesicles

A range of studies has shown that synaptophysin plays a role in the formation and structural maintenance of synaptic vesicles (Jahn and Südhof, 1994). When nonneuroendocrine cells are forced to express synaptophysin, the protein is integrated into a novel type of vesicle (Leube et al., 1989), suggesting that synaptophysin is involved in vesicle genesis. In addition, Daly and Ziff (1997) have shown that the rate of synaptophysin synthesis is proportional to the rate of synaptic vesicle formation. Evidence that synaptophysin functions in maintaining the high curvature of synaptic vesicles comes from the observation that synaptophysin cannot be reconstituted in liposomes in an inside-out orientation (Jahn and Südhof, 1994). Furthermore, peripherin, which has an overall structure very much like that of synaptophysin, similarly functions in maintaining the high curvature of the rim region of photoreceptor discs (Arikawa et al., 1992).

5.4.2 Synaptophysin may provide a readily available pool of synaptobrevin for exocytosis

What is the function of the synaptophysin-synaptobrevin complex? The synaptophysin-synaptobrevin interaction is absent in embryonic brain and in neuroendocrine cell lines and tissues. Complex formation is therefore not a prerequisite for exocytosis. During neuronal development, the synaptophysin-synaptobrevin complex is upregulated, starting to appear postnatally. Considering its time of appearance, the complex most likely plays a role only during or after synapse formation. As has already been suggested by Edelmann et al. (1995), synaptophysin probably regulates the availability of synaptobrevin to the SNARE complex. Such candidate regulatory proteins have also been described for syntaxin and SNAP 25. For example, the hydrophilic Munc-18 binds to syntaxin when it is not part of the SNARE complex (Hata et al., 1993), while the proteins Munc-13 (Betz et al., 1997) and tomosyn (Fujita et al., 1998) bind syntaxin when it is part of the SNARE complex. The ATPase Hrs-2 binds to SNAP 25 in a calcium-dependent manner (Bean et al., 1997)

Synaptophysin probably regulates the availability of synaptobrevin to the fusion reaction by keeping synaptobrevin from entering inactive "cis" SNARE complexes on the synaptic vesicle. Previous studies have shown that the SNARE complex does not exist only in a “trans” orientation with synaptobrevin on the vesicle and syntaxin and SNAP 25 on the

plasma membrane, but also in an inactive “cis” orientation where all three proteins reside on the vesicle membrane (Walch-Solimena et al., 1995; Kretschmar et al., 1996; Otto et al., 1997). The functional significance of the vesicular syntaxin and SNAP 25 is not clear. Most likely, they accumulate on the vesicle after being internalised subsequent to exocytosis. NSF, which has been shown to act prior to membrane fusion (Bittner and Holz, 1992; Banerjee at el., 1996; Robinson and Martin, 1998), dissociates the "cis" SNARE complex on the vesicle. Its action, however, is short-lived and dissociated complexes readily reassemble at least *in vitro* (Otto et al., 1997). What keeps the "cis" SNAREs from reassembling *in vivo*? It can be envisioned that synaptophysin scavenges synaptobrevin that is released from the "cis" SNARE complex subsequent to NSF hydrolysis, thereby preventing it from re-entering this inactive, "dead-end" complex and making it available for binding to plasma membrane SNAREs. Perhaps formation of the "cis" SNARE complex is energetically less favourable than formation of its "trans" equivalent owing to the different positioning of the proteins. Under these conditions, synaptophysin might keep synaptobrevin from re-entering the "cis" SNARE complex but allow it to enter its "trans" equivalent. Thus, the synaptophysin-synaptobrevin interaction would speed up the process of synaptic vesicle fusion and thereby enhance neurotransmitter secretion. It is not known what happens to syntaxin and SNAP 25 once they are on the synaptic vesicles. Perhaps they are pulled back to the plasma membrane by interacting with their non-SNARE binding partners during subsequent rounds of exocytosis

The relevance of synaptophysin for an enhanced neurotransmitter secretion has been shown in an elegant set of experiments performed by J. Alder and his co-workers. For their study, Alder et al. (1992a) used *Xenopus* oocytes injected with rat cerebellar mRNA. These oocytes were capable of secreting glutamate in a calcium ion-dependent manner, a property which was shown to depend on the expression of synaptophysin. When antisense oligonucleotides to synaptophysin were coinjected together with the cerebellar mRNA, the synaptophysin expression was decreased, which in turn caused a reduction in calcium-dependent secretion. In a different approach neuromuscular synapses were loaded with antibodies to synaptophysin, leading to a reduction in the frequency of spontaneous synaptic currents and an inhibition of transmitter secretion (Alder et al., 1992b.). Overexpression of synaptophysin, on the other hand, led to enhanced neurotransmitter secretion (Alder et al., 1995).

The results obtained by Alder et al. correlate very well with the proposed role of synaptophysin in assuring the availability of synaptobrevin to the fusion reaction. Antisense oligonucleotides and antibodies against synaptophysin mRNA and protein, respectively, probably decreased the amount of synaptophysin that was available for interacting with synaptobrevin. This led to an increased entrapment of synaptobrevin in inactive "cis" SNARE complexes and thus to a decrease of synaptobrevin available for the fusion complex. Therefore, vesicle fusion and neurotransmitter release was decreased when less synaptophysin was available for interacting with synaptobrevin. Similarly, when synaptophysin was overexpressed, more synaptobrevin was scavenged from the "cis" SNARE complex and became available for the fusion reaction, thus enhancing the efficiency of neurotransmitter release.

If synaptophysin ensures the availability of synaptobrevin to the fusion complex, then the presence of the synaptophysin-synaptobrevin interaction should increase the efficiency of synaptic transmission. Thus, an increase in the synaptophysin-synaptobrevin complex might be observed during periods of high synaptic activity, such as under repeated stimulation. Indeed, preliminary experiments performed for the present study yielded exciting results. Kindled rats which display a stimulus-produced seizure activity and serve as a model for epilepsy, were analysed with respect to the synaptophysin-synaptobrevin complex. When comparing immunoprecipitates in the kindled rat with those in an unstimulated control, the synaptophysin-synaptobrevin complex was shown to be upregulated in the cortex and hippocampus of the kindled rat. Although further experiments are needed to verify these results, it seems that an increase in synaptic activity is paralleled by an increase in synaptophysin-synaptobrevin complex formation. The mechanism of kindling may be similar to that of long-term potentiation (Martin, 1991), leading to the tentative suggestion that the synaptophysin-synaptobrevin complex might perhaps play a role in learning and memory formation.

In conclusion, the synaptophysin-synaptobrevin complex is absent in the embryonic brain and is upregulated during neuronal development. Before synaptogenesis, the two proteins do not interact even though they reside on the same synaptic vesicle population. A small cytosolic peptide that is expessed or active only in adult brain can induce the interaction of synaptophysin and synaptobrevin. The synaptophysin-synaptobrevin complex is not an essential prerequisite for regulated exocytosis. Hypothalamic neurons at day three in culture undergo regulated neurosecretion (Ahnert-Hilger et al., 1996; Kraszewiski et al., 1996) as do

neuroendocrine PC 12 cells (Ahnert-Higer and Weller, 1993), but neither contain the synaptophysin-synaptobrevin complex (this study). It is probable that the synaptophysin-synaptobrevin interaction fine-tunes synaptic responses by regulating the availability of synaptobrevin to the fusion complex in a positive manner. According to this view, the complex provides a readily available pool of synaptobrevin for exocytotic membrane fusion. Synaptophysin would recruit synaptobrevin after disassembly of the "cis" SNARE complex subsequent to vesicle endocytosis and release it when it is required for another round of rapid exocytosis. Support for such a role comes from experiments performed by Alder et al. (1992a, 1992b, 1995) where increased availability of synaptophysin correlated with increased efficiency of neurotransmitter secretion. Furthermore, the synaptophysin-synaptobrevin complex was upregulated in kindled rat brain (preliminary result of this study) indicating that increased synaptic activity is associated with increased complex formation. Thus, the synaptophysin-synaptobrevin interaction adds a vesicular component to synaptic plasticity both during development and in the adult brain.