Overexpression of Synaptophysin Enhances Neurotransmitter Secretion at *Xenopus* Neuromuscular Synapses

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Previous studies have suggested the importance of synaptophysin (p38), a major integral membrane protein of the synaptic vesicle, in transmitter secretion, but few have directly addressed its functional role at intact synapses. In the present study, injection of synthetic mRNA for synaptophysin into one of the early blastomeres of a *Xenopus* embryo resulted in elevated synaptophysin expression in 1 and 2 d embryos and in cultured spinal neurons derived from the injected blastomere, as shown by immunocytochemistry. At neuromuscular synapses made by neurons overexpressing synaptophysin [p38(+)] in 1 d cell cultures, the spontaneous synaptic currents (SSCs) showed a markedly higher frequency, as compared to control synapses. This increase in frequency was not accompanied by a change in the mean amplitude or the amplitude distribution of the SSCs, suggesting that synaptophysin is not involved in determining the size of transmitter quanta. The impulse-evoked synaptic currents (ESCs) of synapses made by p38(+) neurons showed increased amplitude as well as reduced fluctuation and delay of onset of ESCs. Under high-frequency tetanic stimulation at 5 Hz, the rate of tetanus-induced depression was faster for p38(+) neurons. Taken together, these results suggest a role for synaptophysin in the late steps of transmitter secretion, affecting the probability of vesicular exocytosis and/or the number of synaptic vesicles initially docked at the active zone.

[Key words: synaptophysin, overexpression, synaptic vesicle, neuromuscular synapse, embryonic neurons, transmitter secretion]

Recent progress in molecular cloning has led to the identification of a number of proteins associated with the synaptic vesicle (Bennett and Scheller, 1993; Kelly, 1993). The role of these proteins in neurotransmitter packaging and in vesicle mobilization and exocytosis remains largely unknown. One approach to address their functional roles is to manipulate the level of expression of a protein at presynaptic nerve terminals and examine the consequences of the manipulation on various properties of synaptic transmission. The present study is the first demonstration of overexpression of a synaptic vesicle protein,

synaptophysin, in developing *Xenopus* neurons. The effect of overexpression on synaptic function was examined in nerve-muscle cultures prepared from the injected embryos.

The method of overexpression or ectopic expression of messages by blastomere injection of *Xenopus* embryos has been used extensively to study the role of a number of proteins in embryo development and morphogenesis, including basic fibroblast growth factor (Amaya et al., 1991; Kimelman and Maas, 1992), homeobox proteins (Ruiz i Altaba and Melton, 1989; Niehrs et al., 1993), N-cadherin (Detrick et al., 1990; Fujimori et al., 1990), G proteins (Otte et al., 1992), and myogenic factors (Rashbass et al., 1992). Recently, the blastomere injection method was also used to manipulate proteins involved in synaptogenesis and synaptic function (Lu et al., 1991; Alder et al., 1992b). Injection of macromolecules into one of the early blastomeres of a *Xenopus* embryo results in an effective loading of the injected molecules into a subpopulation of the embryo's spinal neurons and myotomal myocytes, which can later be dissociated and cultured (Jacobson, 1982; Sanes and Poo, 1989). The synaptic functions at neuromuscular synapses formed by these nerve and muscle cells can then be assayed and compared to those of control synapses derived from the uninjected blastomere.

Synaptophysin is an integral membrane protein of synaptic vesicles found in both neurons and endocrine cells (Jahn et al., 1985; Wiedenmann and Franke, 1985). Although it has been used extensively as a marker for presynaptic sites, its function remains unknown. It contains four transmembrane regions and a cytoplasmic C-terminal domain with 10 copies of a tyrosine- and proline-rich pentapeptide repeat (Südhof et al., 1987; Johnston et al., 1989a). Several synaptophysin subunits form a homooligomer in the vesicle membrane (Rehm et al., 1986; Thomas et al., 1988; Johnston and Südhof, 1990), and synaptophysin has been shown to bind to an oligomeric presynaptic plasma membrane protein (Thomas and Betz, 1990). Purified synaptophysin reconstituted in lipid bilayers forms voltage-dependent channel structures whose properties can be altered by an antibody to synaptophysin (Thomas et al., 1988). Similar channels have been observed in synaptic vesicles and neurosecretory granules (Rahamimoff et al., 1988; Lee et al., 1992), which can also be blocked by a synaptophysin antibody (Lemos et al., 1992). Based on these *in vitro* properties of the isolated protein, it has been suggested that synaptophysin may function in formation of the fusion pore during exocytosis and/or vesicle docking (Monck and Fernandez, 1992).

While the above work provides circumstantial evidence that synaptophysin participates in vesicular exocytosis, a direct dem-

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Figure 1. Plasmid used for *in vitro* transcription of synaptophysin mRNA. Rat synaptophysin cDNA (Buckley et al., 1987) was subcloned into the HindIII site of pSP64(polyA). The plasmid was then linearized with FspI and *in vitro* transcribed using SP6 RNA polymerase.

onstration of a role for synaptophysin in transmitter secretion at a functional synapse is lacking. In the present study we found that overexpression of synaptophysin markedly increases both spontaneous and evoked transmitter secretion at neuromuscular synapses in cell cultures, and the characteristics of the synaptic changes support the notion that synaptophysin is involved in determining the probability of vesicular exocytosis and/or the immediate availability of synaptic vesicles for exocytosis.

Materials and Methods

In vitro transcription and embryo injection. Capped mRNA was prepared from a pBluescript (SK)+/synaptophysin plasmid (provided by R. Kelly, UCSF), linearized with HincII (New England Biolabs, Beverly, MA), and transcribed *in vitro* using T3 RNA polymerase (Ambion, Austin, TX). Alternatively, the synaptophysin cDNA was subcloned into the HindIII site of a pSP64(polyA) vector (Promega, Madison, WI) and transcribed *in vitro*, after linearization with FspI (New England Biolabs, Beverly, MA), with SP6 RNA polymerase (Ambion, Austin, TX). β -Globin mRNA was generated from the Triplescript globin control template (Ambion, Austin, TX). Capped mRNAs were extracted twice with phenol/chloroform and chloroform, precipitated with ethanol, washed with 70% ethanol, and dried. *In vitro* translation was performed (Promega, Madison, WI) and the products were analyzed by 12% SDS-PAGE (Laemmli, 1970) followed by autoradiography. Female *Xenopus laevis* (*Xenopus* I, Ann Arbor, MI) were induced to lay eggs using human chorionic gonadotropin (Sigma, St. Louis, MO) and the eggs were fertilized artificially with testis homogenates. The mRNA was resuspended in RNAase-free H₂O, mixed with rhodamine-conjugated dextran (Sigma, St. Louis, MO) to a final mRNA concentration of 1 μ g/ μ l, and ~8 ng was injected into two-cell stage embryos as described previously using a Picospritzer (General Valve Co., Fairfield, NJ) with a pressure of 400 kPa (Lu et al., 1991).

Culture preparation. *Xenopus* nerve-muscle cultures were prepared as previously described (Tabti and Poo, 1991). Briefly, neural tubes and the associated myotomal tissue of 1-d-old *Xenopus* embryos (stage 20–22 according to Nieuwkoop and Faber, 1967) were dissociated in Ca²⁺/Mg²⁺-free saline supplemented with EDTA [115 mM NaCl, 2.6 mM KCl, 0.5 mM EDTA, 10 mM HEPES (pH 7.6)] for 15–20 min. The cells were plated on glass coverslips and were used for experiments after a 24 hr incubation at room temperature. The culture medium consisted (v/v) of 50% Leibovitz L-15 medium (Sigma, St. Louis, MO), 1% fetal calf serum (GIBCO, Gaithersburg, MD), and 49% Ringer's solution [115 mM NaCl, 2 mM CaCl₂, 2.5 mM KCl, 10 mM HEPES (pH 7.6)].

Immunocytochemistry. Embryos were prepared for whole-mount staining by a modification of a procedure of Dent et al. (1989). Briefly, embryos were fixed for 2 hr in 20% dimethyl sulfoxide (Sigma, St. Louis, MO) and 80% methanol, after manual removal of the vitelline membrane. They were then bleached in 10% H₂O₂ (Fisher Scientific, Fair Lawn, NJ) in fixative for 2 d and stored in 100% methanol at –20°C. Nerve-muscle cultures were fixed 24 hr after plating in 4% formaldehyde (Ladd Research Industries, Inc., Burlington, VT) in 0.1 M phosphate buffer (pH 7.4) for 30 min. The procedure for staining both embryos and cultures was as follows. Embryos or cultures were washed in a solution containing 10 mM Tris (pH 7.0), 150 mM NaCl, and 0.05% Tween-20 (TBST) and blocked with 20% calf serum (GIBCO, Gaithersburg, MD) in TBST for 2 hr on a Nutator. They were then incubated in polyclonal anti-frog synaptophysin antibodies (Valtorta et al., 1988) (1:100) in blocking solution overnight at 4°C. Embryos and cultures were washed five times for 1 hr each with TBST and then incubated in donkey anti-rabbit antibody conjugated to alkaline phosphatase (Jackson ImmunoResearch Labs, Inc., West Grove, PA) (1:5000) in blocking solution overnight at 4°C. Following five washes in TBST for 1 hr each, the embryos and cultures were washed in 100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 0.2% Tween-20, and 1% levamisole (Vector Laboratories, Inc., Burlingame, CA) (AP solution) three times for 5 min each. They were then stained with 4.55 μ l/ml nitroblue tetrazolium chloride and 3.55 μ l/ml 5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt (Boehringer Mannheim Biochemica, Indianapolis, IN), in AP solution for 10 min and then refixed in 0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM MgSO₄, 3.7% formaldehyde (MEMFA) overnight at 4°C. Dark-field pictures of embryos were taken on a dissecting microscope and photographs of cultures were taken on a Zeiss microscope at 40 $\times$.

Electrophysiology. Whole-cell patch-clamp recordings (Hamill et al., 1981) were made in culture medium after 1 d of incubation at room temperature (20–22°C). The solution inside the whole-cell recording pipette contained 150 mM KCl, 1 mM NaCl, 1 mM MgCl₂, and 10 mM HEPES (pH 7.2). The membrane current in all recordings was monitored by a patch-clamp amplifier (EPC-7, List, Great Neck, NY). Data were stored on a videotape recorder for later playback onto a storage oscilloscope (5113, Tektronix, Beaverton, OR) and oscillographic recorder (RS 3200, Gould, East Rutherford, NJ), and for analysis by the scan program (kindly provided by Dr. J. Dempster, Strathclyde University, Glasgow, UK). Due to culture to culture variation, synaptophysin-overexpressing cells were compared to control cells in the same culture dish for all data collection.

Results

Overexpression of synaptophysin

To overexpress synaptophysin (p38) in presynaptic neurons, capped synthetic synaptophysin mRNA prepared from one of two plasmids [pBluescript (SK)+ or pSP64(polyA)] (Krieg and Melton, 1984) containing rat synaptophysin cDNA (Buckley et al., 1987; Leube et al., 1987; Fig. 1) was injected into one of the blastomeres of two-cell stage *Xenopus* embryos, using a blastomere injection method (Jacobson, 1982; Sanes and Poo, 1989; Lu et al., 1991; Alder et al., 1992b). *In vitro* translation using a rabbit reticulocyte lysate system confirmed that a protein of correct size (MW 38 kDa) was synthesized from the mRNA. To demonstrate that exogenous synaptophysin was expressed, embryos that had been injected in one blastomere with synaptophysin mRNA at the two-cell stage were fixed 1 or 2 d after injection. Immunocytochemical staining of the whole-mounted embryos with anti-synaptophysin antibody showed that each embryo stained positively for synaptophysin exclusively on one side of the body (Fig. 2A–F), indicating that progeny cells of the injected blastomere overexpressed synaptophysin. The two types of synthetic synaptophysin mRNAs gave similar differential staining of the embryo. These results are consistent with previous studies in which cell lines transfected with synaptophysin cDNA or microinjected with its mRNA expressed the exogenous protein for up to several days (Johnston et al., 1989b;

Figure 2. Overexpression of synaptophysin in *Xenopus* embryos injected with synaptophysin mRNA. Embryos were injected at the two-cell stage, and synaptophysin expression was examined by immunocytochemical staining using alkaline phosphatase-conjugated secondary antibodies. *A* and *D*, Dorsal view of 1-d-old embryos showing positive staining for synaptophysin (purple) on one half only. *B* and *C*, *E* and *F*, Lateral views of 2-d-old embryos, expressing exogenous synaptophysin only on one side. *Left and right photographs* are opposite lateral views of the same embryo. *G–I*, Same as *A–C*, except that the embryos were not injected with synaptophysin mRNA. In the 2-d-old embryo (*H* and *I*), weak staining was visible in the spinal cord and other neuronal structures, but no difference in staining intensity was found between the two sides of the embryo. *J* and *K*, Nerve-muscle cultures prepared from 1-d-old embryos injected with synaptophysin mRNA. Some neurons and their processes (marked by solid arrows) are more darkly stained than others (open arrows). Staining is also visible in some myocytes. *L*, Culture prepared from a control, uninjected embryo. All neurons (open arrow) stained with the same low intensity, and no myocytes showed significant staining. *M* and *N*, Myocytes in nerve-muscle cultures prepared from embryos injected with synaptophysin mRNA. Some myocytes (marked by arrowheads) expressed synaptophysin while others (unmarked) are unstained. *O*, Nerve-muscle culture from an mRNA-injected embryo stained with preimmune serum instead of synaptophysin antibodies. No background staining is visible in either the neurons (open arrow) or myocytes. Scale bars: 0.5 mm for 1 d embryos, 0.7 mm for 2 d embryos, and 30 μ m for cultures.

Figure 3. Overexpression of synaptophysin enhances spontaneous synaptic current (SSC) frequency. *A*, Continuous trace depicts membrane current recorded from whole-cell voltage-clamped ($V_h = -70$ mV) myocytes that were innervated either by a control neuron (*top*) or by a neuron overexpressing synaptophysin [p38 (+)] (*bottom*). Inward currents are shown as downward deflections at a slow (*left*) and a fast (*right*) time scale. The *right* panels show superimposed traces of the events recorded during a 3 min period. Calibration: 0.25 nA, 40 sec, and 0.25 nA, 10 msec for the *slow* and *fast* traces, respectively. *B*, SSC frequency for p38(+) synapses and control synapses (*left*) and β -globin(+) and control synapses (*right*). Each data point (*top*) represents the SSC frequency at a single synapse during a 10 min recording period, and the scattering of the data along the abscissa is for display only. Mean SSC frequency of all synapses + SEM is shown in the bar graphs below. The mean SSC frequency of p38(+) synapses (*open bars*) is higher than that of control synapses (*solid bars*) ($p < 0.05$, t test), but that of β -globin(+) synapses is not significantly different from the controls in the same culture ($p > 0.05$, t test).

Leube et al., 1989; Linstedt and Kelly, 1991). Control, uninjected embryos showed very little endogenous synaptophysin staining at 1 d (Fig. 2*G*); however, by 2 d, the spinal cord and other neuronal structures showed positive staining (Fig. 2*H,I*).

To examine synaptophysin overexpression in spinal neurons, immunocytochemistry was performed on nerve-muscle cultures prepared from 1-d-old mRNA-injected embryos. One day after plating the cells, at the time physiological studies of acetylcho-

line (ACh) secretion were performed (see below), many neurons in the culture were found to stain more darkly than others, indicating overexpression of synaptophysin (Fig. 2*J,K*). By contrast, in cultures prepared from noninjected embryos all neurons stained with the same low-level intensity, reflecting the low level of endogenous synaptophysin expression (Fig. 2*L*). Since the progeny cells derived from the injected blastomere consist of both spinal neurons and myotomal myocytes, some of the my-

ocytes in the culture would be expected to express synaptophysin as well. Indeed, some myocytes stained positively while others did not (Fig. 2*M,N*) and none of the myocytes in cultures prepared from uninjected embryos showed any significant staining (Fig. 2*L*). No staining was observed in embryos or cultures that were incubated with preimmune serum instead of the synaptophysin antibody (Fig. 2*O*). Thus, the exogenous synaptophysin was present in cultured neurons 2 d after mRNA injection, at the time of physiological studies of synaptic function.

Effects on spontaneous synaptic currents

The functional consequences of synaptophysin overexpression were examined by recording synaptic currents in 1 d nerve-muscle cultures prepared from embryos injected with synaptophysin mRNA. Rhodamine-conjugated dextran was co-injected with the mRNA and used as a convenient vital marker for cells that were overexpressing synaptophysin [p38(+)] (Sanes and Poo, 1989; Lu et al., 1991; Alder et al., 1992b). Synaptic currents in isolated myocytes innervated by either p38(+) or control neurons from the same culture were measured using the whole-cell recording method (Hamill et al., 1981). The frequency of spontaneous synaptic currents (SSCs) at synapses made by p38(+) neurons was markedly higher than at those made by control neurons (Fig. 3*A*). In the case of mRNA transcribed from the pBluescript (SK)+ plasmid, the mean SSC frequency was $33.9 \pm 13.7/\text{min}$ ($\pm\text{SEM}$; $n = 18$) and $5.4 \pm 2.1/\text{min}$ ($\pm\text{SEM}$; $n = 19$), respectively, for p38(+) and control neurons. The difference was significant at $p < 0.05$ (t test; Fig. 3*B*). Neurons loaded with synaptophysin mRNA transcribed from the pSP64(polyA) plasmid also yielded a higher SSC frequency ($17.0 \pm 3.0/\text{min}$, $\pm\text{SEM}$; $n = 12$) than controls ($3.6 \pm 1.1/\text{min}$, $\pm\text{SEM}$; $n = 13$; $p < 0.01$, t test). As a control for the blastomere injection method, β -globin mRNA was loaded into *Xenopus* embryos instead of synaptophysin mRNA. We found no significant difference ($p > 0.05$, t test) between the mean SSC frequency of β -globin(+) neurons ($7.0 \pm 2.6/\text{min}$, $\pm\text{SEM}$; $n = 14$) and that of control neurons ($6.1 \pm 2.2/\text{min}$, $\pm\text{SEM}$; $n = 14$; Fig. 3*B*). The presence of synaptophysin in the postsynaptic myocyte had no influence on SSC frequency, since when the data were grouped according to the presence or absence of exogenous synaptophysin in the postsynaptic myocyte, no significant difference in SSC frequency was found (data not shown).

Despite the marked increase in frequency, the distribution of the SSC amplitudes at synapses made by p38(+) neurons remained the same as that of control synapses. In both cases, we observed a monotonic, skewed amplitude distribution typical of developing synapses in these cultures (Evers et al., 1989) (Fig. 4*A*). The mean SSC amplitude was 0.34 ± 0.07 nA ($\pm\text{SEM}$; $n = 15$) and 0.34 ± 0.08 nA ($\pm\text{SEM}$; $n = 11$) for p38(+) and control synapses, respectively. No significant difference ($p > 0.05$, t test) was found between the two groups either for the mean SSC amplitude or for any particular amplitude bin. Similarly, no significant difference was found in the rise times of SSCs (Fig. 4*B*). These results strongly suggest that overexpressing synaptophysin in these neurons affects presynaptic ACh release by increasing the availability and/or the probability of exocytosis of synaptic vesicles without changing the quantal size.

Effects on evoked synaptic currents

To determine whether exogenous synaptophysin also affects impulse-evoked synaptic transmission, we measured the evoked synaptic currents (ESCs) elicited in the postsynaptic myocyte

Figure 4. Overexpression of synaptophysin has no effect on SSC amplitudes or rise times: SSC amplitude (*A*) and rise time (*B*) distributions for p38(+) (solid line) and control (dashed line) synapses. Curves represent averaged amplitude or rise time distributions from 15 p38(+) and 11 control synapses. The cumulative frequency refers to the proportion of total events with amplitudes or rise times smaller than a given value. For clarity, error bars ($\pm\text{SEM}$) are shown only for some points along the solid lines. No significant difference was found between the p38(+) and control synapses ($p > 0.05$, t test) at any point along the amplitude or rise time distributions.

by low-frequency (0.05 Hz), suprathreshold stimulation of the presynaptic neuron at the soma with an extracellular patch electrode. Synapses made by neurons overexpressing synaptophysin exhibited a higher mean ESC amplitude than those made by control neurons in the same culture prepared from the same embryo (Fig. 5*A,B*; Table 1). Injection of β -globin mRNA instead of synaptophysin mRNA in the early embryo, on the other hand, did not result in any significant difference between the β -globin(+) and control synapses (Fig. 5*B*, Table 1). In addition, the delay of onset, as defined by the time between the end of 0.5 msec stimulus at the soma and the onset of ESCs, also showed a significant reduction at p38(+) synapses (Table 1). The fluctuation of the ESC amplitude was assessed by calculating the coefficient of variation (CV), or SD/mean , of the ESC

Figure 5. Enhanced evoked synaptic currents (ESCs) resulting from overexpression of synaptophysin in presynaptic neurons. **A**, Continuous trace depicts the membrane current recorded from myocytes that were innervated either by a control neuron (*top*) or by a neuron overexpressing synaptophysin [*p38 (+)*] (*bottom*). The neurons were extracellularly stimulated (0.5 msec duration, 0.05 Hz) to generate action potentials. Recorded ESCs are shown as downward deflections spaced at regular intervals among randomly occurring SSCs, at a slow (*left*) and a fast (*right*) time scale. Samples of oscilloscope traces of three ESCs are shown on the *right*. Calibration: 0.5 nA, 40 sec, and 1 nA, 10 msec for the *slow* and *fast* traces, respectively. **B**, Evoked responses for *p38(+)* synapses and control synapses (*left*) and β -globin(+) and control synapses (*right*). Each data point (*top*) represents the average ESC amplitude at a single synapse and the scattering of the data along the abscissa is for display only. Mean ESC amplitude of all synapses (+SEM) is shown in the bar graphs *below*. The mean ESC amplitude of *p38(+)* synapses (*open bars*) is higher than that of control synapses (*solid bars*) ($p < 0.01$, *t* test) but the mean ESC amplitude of β -globin(+) synapses is not significantly different from that of controls in the same culture ($p > 0.05$, *t* test).

amplitude observed at each synapse. The CV values were found to be significantly lower in *p38(+)* synapses than in control synapses (Table 1). In all parameters examined, β -globin(+) synapses showed no difference compared to their controls. Again, all these effects were found to be due to presynaptic overexpres-

sion of synaptophysin, since analyzing the data according to the presence or absence of exogenous synaptophysin in the postsynaptic myocyte yielded no significant difference (data not shown). These effects on evoked synaptic transmission may reflect an increase in the probability of exocytotic fusion and/or

increased vesicle availability at the active zone. The reduced delay of onset of evoked response specifically suggests increased efficiency of excitation–secretion coupling in p38(+) synapses at the active zone, perhaps by increasing the probability of vesicle fusion.

Effects on synaptic depression during tetanus

Depression of evoked responses following repetitive synaptic activation at high frequency reflects depletion of available synaptic vesicles and is characteristic of many synapses (Zucker, 1989). We have compared the rate of synaptic depression during high-frequency tetanic stimulation at synapses made by p38(+) neurons with that at control synapses. Suprathreshold stimulation applied to the presynaptic neuron at 5 Hz for 1 min led to an average of $38 \pm 21\%$ ($\pm$ SEM; $n = 8$) and $42 \pm 14\%$ ($\pm$ SEM; $n = 8$) reduction of the ESC amplitude in control and p38(+) synapses, respectively. As shown in Figure 6, p38(+) neurons had a larger mean ESC amplitude at the onset of the tetanus and during the first 48 sec of tetanus ($p < 0.05$, t test), but the amplitudes of the control and p38(+) responses were not significantly different at the end of the 1 min tetanus. The best-fitted linear slope of the data points for p38(+) synapses was found to be 2.5-fold of that of control synapses. Thus, it appears that the rate of depression was faster in the p38(+) neurons. Overall, our results suggest an increased probability of vesicle fusion in p38(+) cells without a change in the rate of vesicle replenishment. This increased probability of fusion could also be accompanied by a higher number of vesicles already docked at the active zone.

Discussion

There are two main findings of the present study. First, this is the first demonstration that the level of a synaptic vesicle protein in the developing embryo can be manipulated by injecting its mRNA into early blastomeres. The exogenous protein can be detected during the first 2 d after fertilization and physiological characteristics of the synapses can be assayed. Second, we have shown that increased levels of synaptophysin significantly elevate the efficacy of both spontaneous and evoked transmitter secretion, in support of a direct involvement of synaptophysin in transmitter secretion.

The functional importance of synaptophysin in transmitter secretion was also shown previously in a reconstituted secretion

Figure 6. Effect of synaptophysin overexpression on tetanus-induced depression. Amplitudes of ESCs during a 1 min tetanus at 5 Hz were averaged in 6 sec bins. Each data point ($\pm$ SEM) represents the value averaged for eight p38(+) (solid line) and eight control (dashed line) synapses. A significant difference was found during the first 48 sec ($p < 0.05$, t test).

system in *Xenopus* oocytes (Alder et al., 1992a). In that study, injection of oocytes with either total cerebellar or *Torpedo* electric lobe mRNA resulted in Ca^{2+} -dependent secretion of glutamate or ACh, respectively. Inhibition of synaptophysin expression by antisense oligonucleotides or interference with synaptophysin function by antibody injection into the oocytes led to reduced glutamate and ACh release. However, exactly what step in transmitter secretion from the oocyte is affected by synaptophysin is not clear.

Using the same early blastomere injection method described here, Alder et al. (1992b) have shown that introduction of anti-synaptophysin antibodies into presynaptic spinal neurons reduced the frequency of SSCs and the amplitude of ESCs at these *Xenopus* neuromuscular junctions. Since the antibody effects could be due to steric hindrance of vesicular exocytosis by antibody binding rather than to a direct interference of synaptophysin's function in exocytosis, the present study of overexpressing synaptophysin represents an important step in elucidating the role of synaptophysin in intact neurons. Compared to the result of antibody treatment, overexpressing synaptophysin led to opposite effects on both SSC frequency and ESC amplitude. In

Table 1. The effects of synaptophysin overexpression on evoked synaptic currents

	Average ESC amplitude (nA)	Delay of onset ^a of ESC (msec)	Coefficient of variation for ESC	Number of cells examined
p38(+) ^b	$4.04 \pm 0.36^{**}$	$2.5 \pm 0.2^*$	$0.34 \pm 0.06^*$	20
Control	2.17 ± 0.32	3.1 ± 0.3	0.48 ± 0.05	22
p38(+) ^c	2.45 ± 0.41	$2.5 \pm 0.3^*$	$0.31 \pm 0.06^*$	13
Control	1.69 ± 0.41	3.2 ± 0.3	0.50 ± 0.07	14
β -globin(+) ^d	2.86 ± 0.69	2.5 ± 0.2	0.33 ± 0.07	9
Control	2.88 ± 0.69	2.2 ± 0.2	0.33 ± 0.07	9

Data are compared to their own control by Student's t test: *, $p < 0.05$; **, $p < 0.01$.

^a Delay of onset refers to the time between the end of the stimulation and the beginning of the ESC response.

^b Capped synaptophysin mRNA prepared from pBluescript (SK) + plasmid (Buckley et al., 1987).

^c Capped synaptophysin mRNA prepared from pSP64(polyA) plasmid.

^d Capped β -globin mRNA prepared from Triplascript plasmid (Ambion).

both cases, however, the SSC amplitude distribution remained unaffected, consistent with the idea that synaptophysin is not involved in determining the size of ACh quanta, but rather affects the probability of exocytosis and/or the number of vesicles available for exocytosis. In general, vesicle availability could be affected by a number of factors, including vesicle stability, rate of vesicle mobilization, docking of vesicles at the active zone, and rate of recycling. However, the reduction in the delay of onset of evoked responses in p38(+) cells suggests that synaptophysin enhances excitation–secretion coupling at the active zone and is likely to be involved in the final steps of secretion processes, for example, affecting the number of docked vesicles or the probability of vesicle fusion. This conclusion is further supported by the finding of a faster rate of depression during a high-frequency tetanus in p38(+) neurons, without an apparent increase in the rate of vesicle replenishment. The simplest explanation of this result is an increased probability of vesicle fusion, although this could also be accompanied by an increased number of vesicles initially docked at the active zone.

It is interesting to note that introduction of exogenous synapsin I or IIa, phosphoproteins associated with the synaptic vesicle membrane, into these embryonic presynaptic neurons also led to potentiation of spontaneous and evoked ACh secretion (Lu et al., 1991; Schaeffer et al., 1994). However, unlike synaptophysin, exogenous synapsins caused a substantial increase in the mean quantal size. Thus, in developing synapses, synapsins may also be involved in organizing a population of mature synaptic vesicles near the active zone. This is consistent with the known interaction of synapsins with the actin filaments and synaptic vesicles (Greengard et al., 1993) as well as the finding that synapsin II promotes morphological differentiation of presynaptic structures in transfected cells (Han et al., 1991).

Both synapsin's and synaptophysin's function during synapse development and transmitter secretion may be regulated by phosphorylation. Synapsin's properties are altered by Ca^{2+} /calmodulin-dependent protein kinase II (CamKII) (Greengard et al., 1993). Synaptophysin is also phosphorylated by CamKII in a Ca^{2+} - and depolarization-dependent manner on its C-terminal (Rubenstein et al., 1993) and is the major substrate for tyrosine kinase activity in synaptic vesicles (Pang et al., 1988; Barnekow et al., 1990). It is likely that the protooncogene pp60^{c-src} is responsible for the tyrosine phosphorylation that also occurs on the C-terminal domain. Interestingly, this phosphorylation can be inhibited by anti-synaptophysin antibodies (Barnekow et al., 1990). The function of phosphorylation of synaptophysin is unknown, but it may result in changes in transmitter secretion.

Recent experiments demonstrate that constitutive and regulated secretion pathways share homologous proteins (Bennett and Scheller, 1993). A non-neuronal homolog of synaptophysin has been identified in human erythroleukemia cells that is 54% homologous to synaptophysin at the nucleotide level (Zhong et al., 1992). Thus, synaptophysin may have a general role in exocytosis, and synaptic vesicle-mediated transmitter secretion in neurons may originate from a ubiquitous exocytotic pathway existing in all cells (Kelly, 1993; Popov and Poo, 1993). Our findings provide direct evidence for a role of this protein in transmitter release from intact neurons, and are consistent with the model that synaptophysin functions in fusion pore formation and/or vesicle docking (Monck and Fernandez, 1992). The effectiveness of expression of exogenous synaptophysin mRNA in the early embryo also demonstrates the usefulness of the blastomere injection approach in manipulating the expression

of various synaptic proteins *in vivo* and in elucidating their functions in synaptic transmission.

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