Brief Communications

Bidirectional Modulation of Transmitter Release by Calcium Channel/Syntaxin Interactions *In Vivo*

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Protein interactions within the active zone of the nerve terminal are critical for regulation of transmitter release. The SNARE protein syntaxin 1A, primarily known for important interactions that control vesicle fusion, also interacts with presynaptic voltage-gated calcium channels. Based on recordings of calcium channel function *in vitro*, it has been hypothesized that syntaxin 1A– calcium channel interactions could alter calcium channel function at synapses. However, results at synapses *in vitro* suggest two potentially opposing roles: enhancement of neurotransmitter release by positioning docked vesicles near calcium channels and inhibition of calcium channel function by interaction with SNARE proteins. We have examined the possibility that these two effects of syntaxin can occur at synapses by studying the effects on transmitter release of manipulating syntaxin 1A– calcium channel interactions at *Xenopus* tadpole tail neuromuscular synapses *in vivo*. Introduction of synprint peptides, which competitively perturb syntaxin 1A– calcium channel interactions, decreased quantal content at these synapses and increased paired-pulse and tetanic facilitation. In contrast, injecting mRNA for mutant (A240V, V244A) syntaxin 1A, which reduces calcium channel modulation but not binding *in vitro*, increased quantal content and decreased paired-pulse and tetanic facilitation. Injection of wild-type syntaxin 1A mRNA had no effect. The opposing effects of synprint peptides and mutant syntaxin 1A provide *in vivo* support for the hypothesis that these interactions serve both to colocalize calcium channels with the release machinery and to modulate the functional state of the calcium channel. As such, these two effects of syntaxin on calcium channels modulate transmitter release in a bidirectional manner.

Key words: **calcium; syntaxin; synprint; neuromuscular junction; *Xenopus*; facilitation**

Introduction

When an action potential depolarizes the presynaptic plasma membrane, voltage-gated calcium (Ca^{2+}) channels are activated and the resultant Ca^{2+} influx triggers synaptic vesicle fusion (Fatt and Katz, 1951; Katz and Miledi, 1967; Llinas, 1982). SNARE proteins required for exocytosis (Sollner et al., 1993; Kee et al., 1995; Sutton et al., 1998; Wu et al., 1999) interact with voltage-gated Ca^{2+} channels (Bennett et al., 1992; Yoshida et al., 1992; Sheng et al., 1994), but the physiological significance of this interaction *in vivo* is unknown. Syntaxin 1A is an integral plasma membrane SNARE protein that binds SNAP25, synaptobrevin/VAMP (vesicle-associated membrane protein), and Ca^{2+} channels (Trimble et al., 1988; Oyler et al., 1989; Bennett et al., 1992; Sollner et al., 1993; Hodel et al., 1994; Sheng et al., 1994; Yokoyama et al., 1997). The H3 domain of syntaxin 1A binds to the II–III intracellular loop of the α1B subunit of the N-type Ca^{2+} channel (the "synprint" site; amino acids 718–963) (Sheng et al., 1994, 1998; Mochida et al., 1996; Rettig et al., 1996; Kim and Catterall, 1997). This interaction between Ca^{2+} channels and the release machinery has been hypothesized to be important in colocalizing these channels with proteins involved in Ca^{2+}-sensitive vesicle fusion (Mochida et al., 1996; Rettig et al., 1997).

Synprint peptides perturb the interaction between Ca^{2+} channels and syntaxin 1A and reduce transmitter release (Mochida et al., 1996; Rettig et al., 1997). The decrease in transmitter release was interpreted to be attributable to dissociation of the Ca^{2+} channel from the Ca^{2+} sensor for transmitter release. Other experiments suggest that the interaction between syntaxin 1A and the synprint site enhances calcium channel inactivation (Bezprozvanny et al., 1995; Wiser et al., 1996; Degtiar et al., 2000; Bergsman and Tsien, 2000; Jarvis et al., 2000, 2002; Smirnova et al., 1995; Sutton et al., 1999; Zhong et al., 1999; Zamponi, 2003). In particular, coexpression of syntaxin 1A with N-type Ca^{2+} channels in *Xenopus* oocytes increases inactivation, but a mutant form of syntaxin 1A (A240V, V244A) binds to the channel without functional effect (Bezprozvanny et al., 2000).

The potential physiological significance of this interaction of syntaxin with the presynaptic Ca^{2+} channel *in vivo* is not understood. Some have argued that this interaction aids in the colocalization of the vesicle release apparatus with presynaptic Ca^{2+} channels (Mochida et al., 1996; Rettig et al., 1997), whereas others have hypothesized that these interactions modulate channel function and may relay information about the vesicle docking state to Ca^{2+} channels (Bezprozvanny et al., 1995; Bergsman and

Received April 13, 2006; revised Nov. 21, 2006; accepted Nov. 22, 2006.

This work was supported by National Institutes of Health Grants R01 NS043396 (S.D.M.) and R01 NS22625 (W.A.C.). We thank S. Cho, K. Cosgrove, J. D. King, and F. J. Luo for many helpful discussions.

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DOI:10.1523/JNEUROSCI.4213-06.2007

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Tsien, 2000). Here we have studied the functional impact of modifying presynaptic Ca^{2+} channel-syntaxin 1A interactions *in vivo* using extracellular recordings of excitatory postsynaptic potentials (EPPs) at developing *Xenopus* tadpole tail neuromuscular synapses. Our results support the hypothesis that Ca^{2+} channel–syntaxin 1A interactions serve both to colocalize proteins involved in Ca^{2+}-sensitive vesicle fusion with presynaptic calcium channels and to functionally modulate Ca^{2+} channels at neuromuscular synapses *in vivo*.

Materials and Methods

Peptide/mRNA injections. Synprint peptide was prepared as described previously (Sheng et al., 1994). Purified synprint peptide (9.2 nl total volume; mixed 1:1 with 200 μM fluorescent dextran) was injected into both blastomeres of two-cell stage *Xenopus* embryos treated with 2% cysteine in Normal Frog Ringer (NFR) (in mM: 116 NaCl, 10 HEPES, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$) at pH 8.5 and bathed in 10% NFR plus 4% Ficoll. Successful injections were confirmed by immunoblots against the His tag (Rettig et al., 1997). Rat syntaxin 1A mRNA was transcribed from cDNA (kindly provided by Dr. Richard Scheller, Stanford University, Stanford, CA) using a T7 transcription kit and verified by gel electrophoresis. Mutant (A240V and V244A) syntaxin 1A cDNA was produced by Stratagene Quick Change Site-Directed Mutagenesis and verified by sequencing. Syntaxin 1A mRNA was diluted 1:750 with DDH_2O, mixed 1:1 with GFP mRNA, and the mixture was injected. As a control, rat GST (1 mg/ml in 150 mM potassium acetate; Sigma, St. Louis, MO) was injected into both blastomeres of two-cell stage embryos. Following ~80 h of development (stage 42–46) (Nieuwkoop and Faber, 1967), tadpoles showing dextran or GFP fluorescence were placed in NFR, pH 7.4, plus 0.2 mg/ml tricaine anesthetic (Sigma). The tadpole tail was dissected, skinned, and fixed with histoacryl glue (Indermil; U.S. Surgical, Norwalk, CT) to a recording dish in NFR.

Electrophysiological recordings. Patch electrode-shaped glass pipettes were used as extracellular stimulating and recording electrodes. Recordings were made from stage 42–46 (Nieuwkoop and Faber, 1967) *Xenopus* tadpole tail neuromuscular synapses in NFR with 3 μM curare (Bachem, King of Prussia, PA) to prevent contractions. Stimulating and recording electrodes were placed in the same chevron cleft. Paired-pulse recordings of EPPs were done at 10, 20, 40, 60, and 100 ms interstimulus intervals (repeated 30 times) and averaged for analysis. In the same animals, 10 stimulus pulses at 100 Hz were delivered to examine tetanic facilitation, and 10–15 trials were averaged for analysis. To quantify the strength of synapses, quantal content was calculated using the failure method (m = ln [trials/failures]) (del Castillo and Katz, 1954) after 150 evoked-response trials. Acquisition and analysis were performed using pClamp software (Molecular Devices, Sunnyvale, CA). Paired-pulse ratios and tetanic facilitation were plotted as EPP amplitude of each response divided by the amplitude of the first response. Significance was determined via two-population t test or ANOVA.

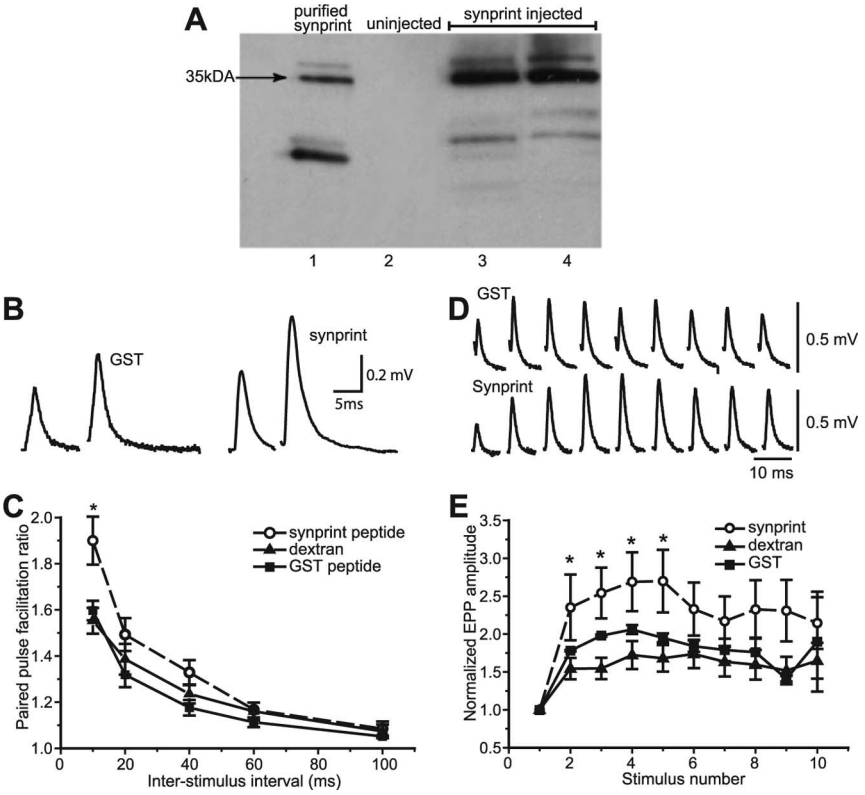

Figure 1. Effects of synprint injection on paired-pulse and tetanic facilitation. ***A***, Western blot of synprint-injected animals. Lane 1, His-tagged purified syntaxin protein. Lane 2, Homogenate from a control, uninjected *Xenopus* tadpole. Lanes 3 and 4, Homogenate from synprint-injected animals that were brightly fluorescent (indicating injection success). Lanes 2–4 each contain the homogenate from a single *Xenopus* tadpole. All tadpoles were staged 42–46. ***B***, Representative paired-pulse-evoked EPPs recorded from a control (GST-injected) animal (left panel) and a synprint-injected animal (right panel). ***C***, In the summary plot of paired-pulse responses (mean ± SEM), synprint-injected animals (open circles, dotted line; $n = 9$) had synapses with significantly greater paired-pulse facilitation at the 10 ms interstimulus interval than control animals injected with either GST (filled squares, solid line; $n = 4$) or fluorescent dextran (filled triangles, solid line; $n = 15$). ***D***, Representative tetanic facilitation (at 100 Hz) of EPP responses recorded from a control (GST-injected) animal (top trace) and a synprint-injected animal (bottom trace). ***E***, Summary plot of tetanic facilitation (mean ± SEM) recorded from synprint-injected animals (open circles, dotted line; $n = 9$) compared with control animals injected with GST (filled squares, solid line; $n = 4$) or fluorescent dextran (filled triangles, solid line; $n = 5$). In ***A*** and ***C***, the stimulus artifact has been omitted for clarity. *Significantly different, $p < 0.05$, one-way ANOVA.

Results

Enhanced paired-pulse facilitation by injection of synprint peptide

To perturb Ca^{2+} channel interactions with SNARE proteins at synapses *in vivo*, we injected synprint peptides and evaluated transmitter release at *Xenopus* tadpole tail neuromuscular synapses. At stage 42–46, ~3 d after injection, the animals were brightly fluorescent and synprint peptides were still present (Fig. 1*A*) (Rettig et al., 1997). At these ages, synapses are positioned at the ends of individual muscle cells within the clefts between chevron borders (Kullberg et al., 1977). To assay transmitter release *in vivo*, extracellular recordings of EPPs were made in the cleft between chevrons, and measurements of paired-pulse and tetanic facilitation were made to evaluate short-term synaptic plasticity. Synprint-injected animals (Fig. 1*B*,*C*) showed significantly greater paired-pulse facilitation than controls. When examined with 10 stimuli at 100 Hz (Fig. 1*D*,*E*), synprint-injected animals also showed significantly greater tetanic facilitation. These data suggest that neuromuscular synapses from synprint peptide-injected animals are weaker initially and therefore are more able to exhibit facilitation.

Reduced paired-pulse facilitation by overexpression of mutant syntaxin

To contrast with synprint peptide effects, the syntaxin 1A mutant (A240V, V244A) that prevents syntaxin-mediated modulation of Ca^{2+} channel inactivation without affecting Ca^{2+} channel–syntaxin binding (Bezprozvanny et al., 2000) was used as an

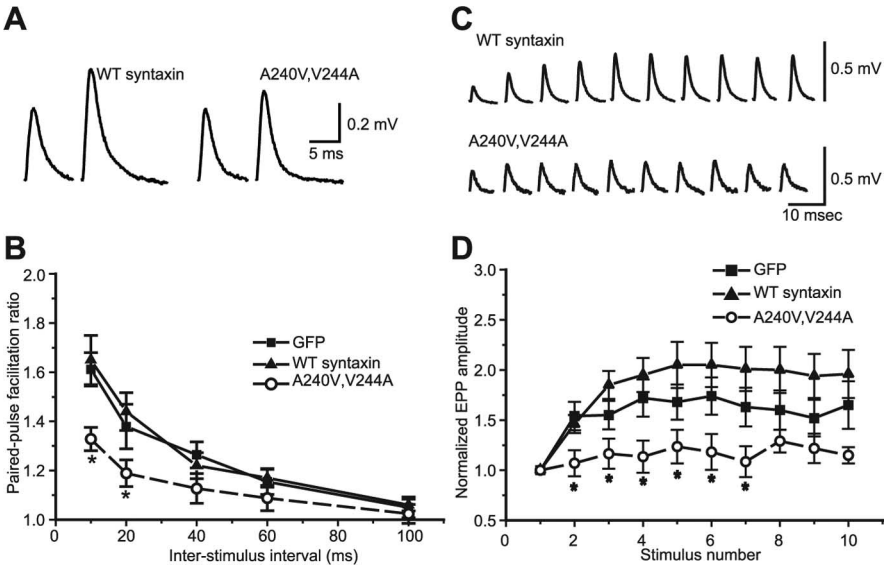

Figure 2. Effects of wild-type (WT) and mutant (A240V, V244A) syntaxin mRNA injection on paired-pulse and tetanic facilitation. ***A***, Representative paired-pulse-evoked EPPs recorded from a control (wild-type syntaxin mRNA-injected) animal (left) and a mutant (A240V, V244A) syntaxin mRNA-injected animal (right). ***B***, In the summary plot of paired-pulse responses (mean ± SEM), mutant (A240V, V244A) syntaxin mRNA-injected animals (open circles, dotted line; $n = 8$) had synapses with significantly reduced paired-pulse facilitation at the 10 and 20 ms interstimulus intervals compared with control animals injected with either GFP mRNA (filled squares, solid line; $n = 9$) or wild-type syntaxin mRNA (filled triangles, solid line; $n = 12$). ***C***, Representative tetanic facilitation (at 100 Hz) of EPP responses recorded from a control (wild-type syntaxin mRNA-injected) animal (top trace) and a mutant (A240V, V244A) syntaxin mRNA-injected animal (bottom trace). ***D***, Summary plot of tetanic facilitation (mean ± SEM) recorded from mutant (A240V, V244A) syntaxin mRNA-injected animals (open circles, dotted line; $n = 7$) compared with control animals injected with GFP mRNA (filled squares, solid line; $n = 6$) or wild-type syntaxin mRNA (filled triangles, solid line; $n = 4$). In ***A*** and ***C***, the stimulus artifact has been omitted for clarity. *Significantly different, $p < 0.05$, one-way ANOVA.

inhibitor of the syntaxin modulation of Ca^{2+} channel gating. Overexpression of wild-type syntaxin had no effect on paired-pulse facilitation compared with GFP alone (Fig. 2*A*,*B*). In contrast, neuromuscular synapses from mutant (A240V, V244A) syntaxin-injected animals had significantly reduced paired-pulse facilitation compared with wild-type syntaxin or GFP alone injected (Fig. 2*A*,*B*). When examined with 10 stimuli at 100 Hz (Fig. 2*C*,*D*), mutant (A240V, V244A) syntaxin-injected animals also showed significantly reduced tetanic facilitation. These data suggest that neuromuscular synapses from mutant (A240V, V244A) syntaxin-injected animals are initially stronger and therefore facilitate less.

Comparison of effects of synprint peptides and mutant syntaxin

Comparison of mean results (Fig. 3*A*) shows that the four control conditions (wild-type syntaxin, GFP, GST, or fluorescent dextran) are not significantly different, whereas mutant (A240V, V244A) syntaxin reduces paired-pulse facilitation, and synprint increases paired-pulse facilitation. To test the hypothesis that synprint-injected synapses were weaker than mutant (A240V, V244A) syntaxin-overexpressed synapses, we used a failure analysis to measure quantal content. As expected, synprint-injected animals had synapses with significantly smaller quantal content than mutant (A240V, V244A) syntaxin-overexpressing animals (2.1 ± 0.15 vs 2.9 ± 0.20; $p < 0.05$), whereas the analysis of control synapses fell between these two experimental manipulations (2.4 ± 0.27) (Fig. 3*B*).

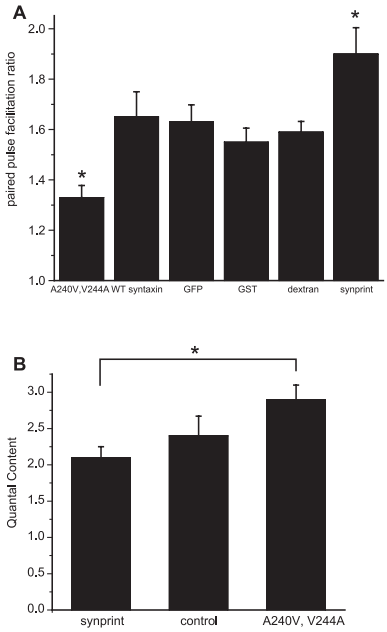

Figure 3. ***A***, Summary plot of the effects of all experimental manipulations on paired-pulse facilitation at the 10 ms interstimulus interval (mean ± SEM). Synprint-injected animals showed significantly greater paired-pulse facilitation, whereas mutant (A240V, V244A) syntaxin mRNA-injected animals showed significantly reduced paired-pulse facilitation. *Significantly different, $p < 0.05$, one-way ANOVA. ***B***, Effects of experimental manipulations on quantal content as determined by failure analysis (mean ± SEM). Synprint-injected animals had significantly smaller quantal content than mutant (A240V, V244A) syntaxin mRNA-injected animals. Quantal content estimates in control conditions (1.8 mM Ca^{2+}) fell between the two experimental conditions. *Significantly different, $p < 0.05$, one-way ANOVA.

Varying extracellular Ca^{2+} resulted in mild synaptic depression at 10 mM $[Ca^{2+}]_o$ and progressively stronger paired-pulse and tetanic facilitation at lower $[Ca^{2+}]_o$ (Fig. 4*A*,*B*). Paired-pulse facilitation in synprint-injected animals recorded in 1.8 mM Ca^{2+} was very similar to paired-pulse facilitation recorded in controls in 1.0 mM Ca^{2+}, and paired-pulse facilitation in mutant (A240V, V244A) syntaxin-injected animals recorded in 1.8 mM Ca^{2+} was comparable with that in controls using 7.5 mM Ca^{2+} (Fig. 4*C*). A similar overlap of synprint and mutant syntaxin data with control data at 1.0 mM and 7.5 mM Ca^{2+}, respectively, was observed in tetanic stimulation experiments (Fig. 4*D*). These results support the conclusion that the effects of synprint peptides and mutant syntaxin are caused by changes in calcium dynamics, reflecting alterations in calcium entry through calcium channels and calcium access to locally docked vesicles.

Discussion

Biphasic regulation of synaptic transmission by Ca^{2+} channel/syntaxin interactions *in vivo*

Previous work suggests that interactions of syntaxin with calcium channels are functionally important in active zones (Catterall, 1999; Mochida, 2000; Teng et al., 2001; Atlas, 2001). Presynaptic Ca^{2+} channels bind syntaxin, and this interaction may serve to localize the source for Ca^{2+} ions near the Ca^{2+} sensor, increasing the efficiency of transmitter release (Mochida et al., 1996; Rettig et al., 1997). Syntaxin also increases calcium channel inactiva-

tion, potentially reducing transmitter release, and this inhibitory effect is relieved by other SNARE proteins and synaptotagmin (Bezprozvanny et al., 1995; Smirnova et al., 1995; Wiser et al., 1996; Sutton et al., 1999; Zhong et al., 1999; Bergsman and Tsien, 2000; Degtiar et al., 2000; Jarvis et al., 2000, 2002; Zamponi, 2003). How would these apparently opposing actions affect synaptic transmission *in vivo*? At control synapses, some Ca^{2+} channels in the active zone are associated with a docked vesicle. At sites at which vesicles are docked, we hypothesize that interaction of syntaxin with synaptobrevin and synaptotagmin relieves inhibitory modulation of Ca^{2+} channels. At sites at which docked vesicles are not present, syntaxin-mediated inhibitory modulation may inhibit Ca^{2+} channels, but these channels cannot efficiently initiate transmitter release; thus, disinhibition of them may have little effect on synaptic transmission.

We propose that introduction of synprint peptides perturbs syntaxin–Ca^{2+} channel interactions, disrupts localization of docked synaptic vesicles near calcium channels, and reverses syntaxin-mediated inhibition of calcium channels without docked vesicles. In this condition, we find that synprint peptide reduces quantal content and increases both paired-pulse and tetanic facilitation, consistent with impaired efficiency of transmitter release. Evidently, the effect of the synprint peptide to dissociate calcium channels from docked vesicles is predominant and reduces the efficiency of transmitter release, whereas the effect of synprint peptide to relieve calcium channel inhibition affects predominantly channels that are not associated with docked vesicles and therefore cannot effectively initiate transmitter release. Accordingly, the main effect of inhibition of calcium channels by syntaxin is to reduce unproductive calcium entry at sites with no nearby docked vesicles.

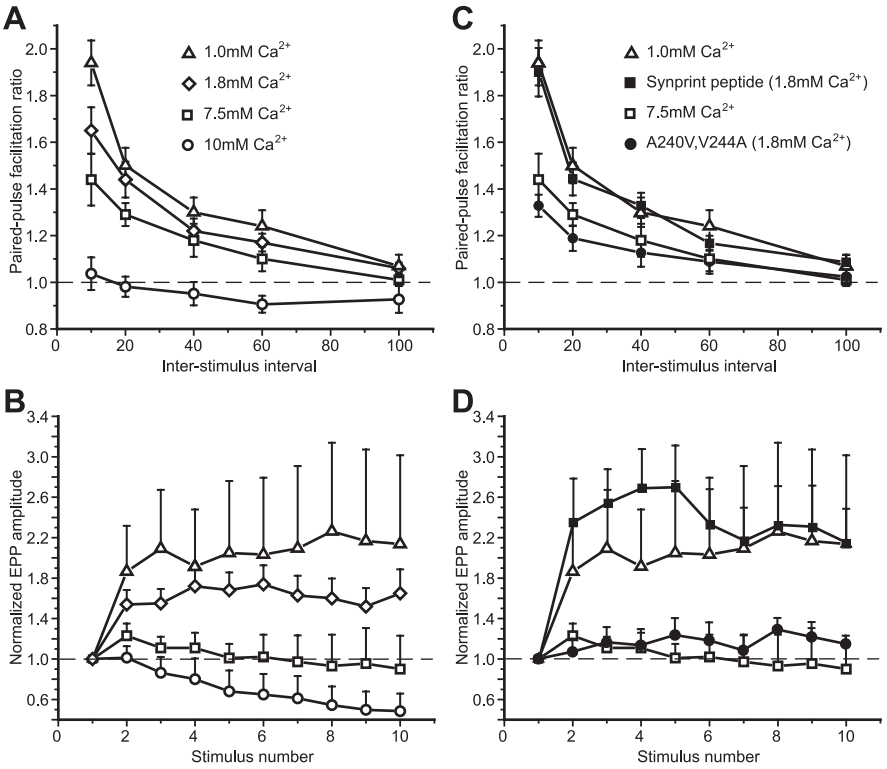

Figure 4. Effects of altering extracellular calcium concentration on paired-pulse and tetanic facilitation. All values plotted represent mean ± SEM. ***A***, The effects of varying extracellular calcium concentration in control uninjected animals on paired-pulse facilitation in 10 mM (open circles), 7.5 mM (open squares), 1.8 mM (open diamonds), and 1.0 mM (open triangles) extracellular calcium. ***B***, The effects of varying extracellular calcium concentration in control animals on tetanic facilitation in 10 mM (open circles), 7.5 mM (open squares), 1.8 mM (open diamonds), and 1.0 mM (open triangles) extracellular calcium. ***C***, The effects on paired-pulse facilitation of synprint peptide introduction (filled squares) and mutant (A240V, V244A) syntaxin (filled circles) recorded in 1.8 mM extracellular calcium overlaid on plots recorded from control terminals in 1.0 and 7.5 mM extracellular calcium. ***D***, The effects on tetanic facilitation of synprint peptide introduction (filled squares) and mutant (A240V, V244A) syntaxin (filled circles) recorded in 1.8 mM extracellular calcium overlaid on plots recorded from control terminals in 1.0 and 7.5 mM extracellular calcium.

Following overexpression of mutant (A240V, V244A) syntaxin, SNARE protein association should be retained, whereas syntaxin-mediated inhibition of Ca^{2+} channels should be reduced (Bezprozvanny et al., 2000). In this case, we would predict a general increase in Ca^{2+} entry into the nerve terminal, which would increase transmitter release (quantal content) and reduce paired-pulse and tetanic facilitation, as we have observed. These results show that there is functionally significant modulation of presynaptic Ca^{2+} entry mediated by interaction of syntaxin with Ca^{2+} channels *in vivo*.

Comparison with invertebrate synapses

A homologous syntaxin mutation in *Drosophila* (Fergestad et al., 2001) causes more substantial loss of neurotransmission. This may reflect complete replacement of wild-type syntaxin by mutant in *Drosophila* versus partial replacement here or may reflect intrinsic differences between invertebrate and vertebrate synapses. Both *Drosophila* and *Lymnaea* Ca^{2+} channels lack a synprint site (Spafford et al., 2003b), but synprint peptides can still inhibit transmitter release in *Lymnaea* (Spafford et al., 2003a). Similar upstream actions of synprint peptides may contribute to their effects in vertebrate synapses, but our ability to mimic effects of synprint peptides by alterations in extracellular calcium supports the conclusion that their main effect is to dissociate docked vesicles from calcium channels.

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