Dual pools of actin at presynaptic terminals

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Abstract

We investigated actin’s function in vesicle recycling and exocytosis at lamprey synapses, and show that FM1-43 puncta and phalloidin-labeled filamentous actin (F-actin) structures are colocalized, yet recycling vesicles are not contained within F-actin clusters. Additionally, phalloidin also labels a plasma membrane-associated cortical actin. Injection of fluorescent G-actin revealed activity-independent dynamic actin incorporation into presynaptic synaptic vesicle clusters but not into cortical actin. Latrunculin-A, which sequesters G-actin, dispersed vesicle associated actin structures and prevented subsequent labeled G-actin and phalloidin accumulation at presynaptic puncta, yet cortical phalloidin labeling persisted. Dispersal of presynaptic F-actin structures by Latrunculin-A did not disrupt vesicle clustering or recycling, nor alter the amplitude or kinetics of EPSCs. However, it slightly enhanced release during repetitive stimulation. While dispersal of presynaptic actin puncta using Latrunculin-A failed to disperse synaptic vesicles or inhibit synaptic transmission, presynaptic phalloidin injection blocked exocytosis and reduced endocytosis measured by action potential-evoked FM1-43 staining. Furthermore, phalloidin stabilization of only cortical actin following pretreatment with Latrunculin-A was sufficient to inhibit synaptic transmission. Conversely, treatment of axons with jasplakinolide, which induces F-actin accumulation but disrupts F-actin structures in vivo, resulted in increased synaptic transmission accompanied by a loss of phalloidin labeling of cortical actin but no loss of actin labeling within vesicle clusters.

Marked synaptic deficits seen with phalloidin stabilization of cortical F-actin, in contrast to the minimal effects of disruption of a synaptic vesicle associated F-actin, led us to conclude that two structurally and functionally distinct pools of actin exist at presynaptic sites.

Keywords: synaptic vesicle release, cytoskeleton, exocytosis, endocytosis
Introduction

Actin appears ubiquitously at presynaptic terminals (Hirokawa et al. 1989; Landis et al. 1988; Wagner and Kelly 1979), however, its presynaptic functions remain elusive (Cingolani and Goda 2008). Actin exists in two states, monomeric G-actin, which is evenly distributed throughout axons at synaptic and extrasynaptic sites (Zhang and Benson 2002), and filamentous F-actin, which may surround vesicle pools (Kuromi and Kidokoro 1998; Richards et al. 2004; Sankaranarayanan et al. 2003; Shupliakov et al. 2002) with lower density within the pool (Bloom et al. 2003; Morales et al. 2000). Actin’s association with presynaptic vesicle clusters has led to its proposed structural role. Actin filaments have been proposed to associate with synaptic vesicles via Synapsin (Bahler and Greengard 1987; Li et al. 1995), which tethers vesicles (Landis et al. 1988; Li et al. 1995; Pieribone et al. 1995). However, F-actin disruption fails to disperse vesicle clusters (Bourne et al. 2006; Gaffield et al. 2006; Kuromi and Kidokoro 1998; Richards et al. 2004; Sankaranarayanan et al. 2003; Shupliakov et al. 2002) while disruption of Synapsin results in a decrease in synaptic vesicle cluster size (Pieribone et al. 1995; Siksou et al. 2007).

Actin has also been proposed to play a dynamic role. Reorganization of F-actin can be induced by stimulation in many preparations (Bernstein and Bamburg 1989; Bloom et al. 2003; Sankaranarayanan et al. 2003; Shupliakov et al. 2002). However, G-actin has also been shown to incorporate into presynaptic F-actin clusters at rest, suggesting additional activity-independent reorganization (Bourne et al. 2006). Indeed, actin turnover is necessary for synaptic transmission because stabilization of F-actin by phalloidin (Vandekerckhove et al. 1985), inhibits neurotransmitter release (Bernstein and Bamburg 1989; Photowala et al. 2005), while agents that prevent actin polymerization or induce depolymerization result in only minimal enhancing effects on exocytosis (Kuromi and Kidokoro 1998; Sankaranarayanan et al. 2003) and synaptic transmission (Cole et al. 2000; Kuromi and Kidokoro 1998; Morales et al. 2000;
Richards et al. 2004; Wang et al. 1996). Actin may also act as a barrier to exocytosis (Dillon and Goda 2005), for block of actin polymerization can enhance evoked release (Morales et al. 2000; Wang et al. 1996), However, this may not be true for all synapses (Sakaba and Neher 2003).

Lastly, actin has been implicated in endocytosis. Actin has been visualized in association with clathrin-coated vesicles (Kaksonen et al. 2003; Merrifield et al. 1999) and as filaments attached to endocytosed vesicles in fibroblasts (Merrifield et al. 1999; Merrifield et al. 2005) and lamprey axons (Shupliakov et al. 2002). F-actin disruption causes deficits in vesicle recycling determined ultrastructurally, manifested as increases in clathrin coated intermediaries, reduced vesicle recycling, vesicle pool depletion, and the appearance of recycling vesicles associated with filaments emanating from endocytic zones (Bloom et al. 2003; Shupliakov et al. 2002). However, actin polymerization inhibitors show little effect on vesicle recycling (Sankaranarayanan et al. 2003).

While the roles of actin at presynaptic sites remain contested, these discrepancies may be compounded by differences in synapse type (Dillon and Goda 2005) and synaptic activity (Cingolani and Goda 2008). However, discrepancies may also have arisen because of different approaches used and different access to the presynaptic terminal, which limit comparative analyses. In most synapses it is impossible to access the terminal directly. Thus, we have utilized the unique accessability of lamprey giant axon terminals to determine the effects of modification of actin polymerization and depolymerization on synaptic transmission and vesicle cycling. We present evidence that in lamprey giant axons, destabilization of a dynamic actin pool associated with synaptic vesicle clusters does not modify synaptic transmission and leaves the entire synaptic vesicle cycle intact. However, alterations to the stability of a membrane associated cortical F-actin pool (cortical actin) dramatically effects exocytosis and synaptic transmission.
**Materials and Methods**

Experiments were performed on isolated spinal cords of 90 larval and young adult lampreys (*Petromyzon marinus*). All procedures on animals conformed to institutional guidelines (University of Illinois at Chicago, Animal Care Committee). This committee adheres to guidelines set out by the National Institutes of Health (USA) and of the Association for Assessment and Accreditation of Laboratory Animal Care. The animals were anesthetized with tricaine methanesulfonate (MS-222; 100 mg l$^{-1}$; Sigma, St Louis, MO, USA) applied in the aquarium water. They were then sacrificed by decapitation, and dissected in a cold saline solution (Ringer) of the following composition (mM): 100 NaCl, 2.1 KCl, 2.6 CaCl$_2$, 1.8 MgCl$_2$, 4 glucose, 5 HEPES, adjusted to a pH of 7.60.

**Electrophysiology**

Lamprey reticulospinal axons were impaled and recorded from under current clamp conditions with conventional sharp microelectrodes containing 1-3M KCl. For those experiments in which phalloidin was injected into the presynaptic terminal, the phalloidin (labeled with Alexa Fluor 568 or 488; Invitrogen, Eugene, OR, USA) was previously dissolved in deionized water at a concentration of 3 units µl$^{-1}$ (final concentration 100 µM) and stored at -20°C for no longer than 4 weeks. Prior to the experiment, the stock solution was added to an equal volume of 3M KCl, and the electrode was filled with this combination. Electrode impedances ranged from 20 to 50 MΩ. Phalloidin was applied to the interior of the recorded axon by pressure injection from the microelectrode. For experiments in which G-actin was injected into the presynaptic terminal, the G-actin (actin from rabbit muscle labeled with Alexa Fluor 488; Invitrogen, Eugene, OR, USA) was stored at -20°C in buffer at a concentration of 11 mg ml$^{-1}$ for no longer that 4 weeks. Prior to the experiment the stock solution was further diluted in G-actin buffer (2 mM HEPES, 0.2 mM CaCl$_2$, 0.2 mM ATP, pH 8.0 (final concentration 1.1 mg ml$^{-1}$)) and the electrode was filled with...
this combination. Patch electrodes for whole-cell postsynaptic recordings contained (mM): K
methane sulfonate 102.5, NaCl 1, MgCl₂ 1, EGTA 5, HEPES 5. Osmolarity and pH were
adjusted to physiological levels (240 mosmol l⁻¹ and 7.2, respectively).

**Imaging**

Confocal imaging was performed using a modified Biorad MRC 600 confocal
microscope. Two excitation wavelengths were used (488 nm argon ion laser and 568 nm
krypton-argon laser) through the AOTF-coupled fibre optic launch (Prairie Technologies,
Madison, WI, USA). Excitation was applied through a custom dichroic mirror with sharp
excitation bands matching the two laser wavelengths (Omega Optical). Two detectors were
placed after a second dichroic, with a transmission band from 500 to 560 nm and a long pass
reflection from 580 nm. Emission filters were band pass (500-560 nm) and long pass (above
580 nm). The photomultiplier outputs were amplified with low-noise current amplifiers (Stanford
Instruments, CA, USA) and digitized to 12 bits with a National Instruments board and custom
software written under Matlab (Mathworks). The scan head mirrors were driven through the
MRC 600 scan head amplifiers with the same custom software. This software is available on our
website (http://alford.bios.uic.edu/Research/software.html).

Volume data (Fig. 1, Fig. 2) was reconstructed using Voxblast software (Vaytech, Iowa
City, Iowa). It is not possible to display volume data linearly, therefore, we have graphically
represented voxel intensities, color and opacity data (Fig. 1D) to demonstrate how different
regions of axon labeling with phalloidin was displayed. Voxel intensity histograms from the
datasets shown (Fig. 1B, 1E) are displayed, overlaid with voxel opacity curves used to display
data, to demonstrate that this cortical actin labeling was distinct from background fluorescence
sampled outside of the labeled axons (Fig. 1D) (blue curve). A distinct population of low-
intensity voxels (gray) are separable from this background (light blue). The opacity curve shown
in this graph represent opacities applied to the 3D voxel reconstructions (Fig. 1Bi, Bii; low intensity fluorescence; black, and high intensity fluorescence; red). The colors used in these histograms demonstrate the color coding of voxels in the images.

**Application of FM dye and Latrunculin-A**

FM1-43 (5 µM) was applied as a stream from a small pipette placed over the surface of the spinal cord (Fig. 1B). Constant flow was ensured with use of a syringe pump. Two thousand stimuli were applied to a microelectrode-recorded axon during the dye application, while the presence of the dye in the tissue surrounding the axons was confirmed with imaging. Dye application was subsequently terminated. During the staining protocol, synaptic activity was blocked with glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and D-aminophosphonovalerate (AP5) (10 and 100 µM respectively). Excess dye was removed with Advasep 7 (Kay et al. 1999; 1 mM 5 min; Cydex, Inc., Lenexa, KS, USA) to reveal areas of stimulus-dependent staining. For experiments when axons were treated with Latrunculin-A (Invitrogen, Eugene, OR, USA; Sigma, St Louis, MO, USA), the Latrunculin-A was previously dissolved in DMSO to a concentration of 24 mM and stored at -20ºC for no longer than 4 weeks. Prior to the experiment, the stock solution was added to 4 ml (final concentration 12 µM) of Ringer solution and superfused over the tissue in the same manner as FM application. Saturation of the tissue was monitored by adding Fast green to the solution.

All means of data are expressed as means ± standard error of the mean. All statistical tests of significance used Students T test for difference in mean or mean of means.
Results

Colocalization of actin and synaptic vesicles labeled with FM 1-43

Presynaptic actin has been proposed to surround a pool of vesicles in lamprey axons (Bourne et al. 2006; Shupliakov et al. 2002). Although the localization of the active recycling pool of vesicles and presynaptic actin has not been shown in vivo in this preparation. Therefore, we determined the spatial relationship between the recycling synaptic vesicle pool and F-actin in the presynaptic terminal.

We investigated the location of F-actin in axons and presynaptic terminals using phalloidin. Axons were recorded from with electrodes containing KCl (1.5 M) and Alexa 598 labeled phalloidin (50 µM). Phalloidin was pressure ejected into axons from the microelectrode (Fig. 1A, red). Phalloidin has been shown to label ring-like structures associated with presynaptic peri-active zones (Bourne et al. 2006; Shupliakov et al. 2002). However, we found that following phalloidin injection more complex bar-like structures were seen (Fig. 1Bii, Cii) in addition to structures that sometimes resembled rings (arrow and arrowhead in Fig. 1Bii, respectively). In addition we found that at lower fluorescence intensities, a second distribution of phalloidin labeling is detectable above background signal (Fig. 1D) that labels continuously the plasma membrane. Thus, in the three dimensional reconstruction (Fig. 1B) diffuse cortical staining is observed at the plasma membrane (gray), punctuated by distinct points of phalloidin staining (red). The diffuse sub-membrane staining (Fig. 1B, gray), that was not previously reported is consistent with cortical actin (Trifaro et al. 1992), while the more intense structures (Fig. 1B and E, red) we now further show (see below) to be associated with recycling synaptic vesicle clusters.

To quantify the relative locations and intensity of the cortical and vesicle cluster associated fluorescence, phalloidin staining in two optical sections (from dashed lines in Fig. 1Bi) is shown separately (Fig. C). Absolute intensities in a cross section at each of these optical sections are plotted from lines that passed through phalloidin labeling associated with a synaptic...
vesicle cluster (white dashed line) and through a region with no synaptic vesicle cluster (gray
dashed line). Labeling associated with the synaptic vesicle clusters is clearly distinct from the
axon cortical actin signal, which is in turn distinct from background fluorescence.

To confirm that F-actin clusters and recycled vesicles colocalize, we labeled recycling
vesicles with the styryl dye FM1-43 followed by injection of phalloidin into the axons (n=8
axons). To label synaptic vesicles with FM1-43, we recorded intracellularly from reticulospinal
axons and stimulated (2000 action potentials, 5 Hz) while applying FM1-43 to the spinal cord
through a perfusion pipette held immediately above the ventral surface of the spinal cord (Fig.

1A, green) (Photowala et al. 2005). Excess dye was cleared from the tissue with Advasep-7
(Kay et al. 1999) to reveal fluorescently labeled synaptic vesicle clusters (Fig. 1E, green). We
then injected Alexa Fluor 598 labeled phalloidin into the same axon through the recording
microelectrode. Using confocal microscopy we imaged serial optical sections of the axons to
reveal the location of F-actin relative to FM1-43 labeled vesicle clusters.

To demonstrate the association between vesicles and phalloidin labeling, the cortical,
low intensity phalloidin staining was thresholded out (voxels above a value of 120 are shown in
red; see Fig. 1D for opacity settings and color look-up tables) to reveal presynaptic F-actin
structures. The FM1-43 labeling appears as fluorescent puncta associated with the intensely
labeled presynaptic F-actin. Phalloidin labeling of F-actin has been previously shown to form
ring-like structures in lamprey giant axons that have been suggested to surround the
presynaptic vesicle pool (Bourne et al. 2006; Shupliakov et al. 2002). Here we now show optical
sections through phalloidin labeled F-actin associated with the synaptic vesicle puncta revealed
complex structures that colocalized with FM labeled vesicles but do not surround the pool of
vesicles. In contrast, the F-actin structures were found both around and contained within the
labeled vesicle pools (Fig. 1Eiv, yellow), and others where labeled vesicle clusters spanned
across two independent F-actin structures (Fig. 1Eiii, arrowheads). Taken together our results
suggest that the full component of active vesicle pools is not contained within filamentous rings
of F-actin, and furthermore that two distinct pools of actin appear presynaptically.

Visualization of actin incorporation in axons

In quiescent axons, monomeric G-actin has been shown to incorporate into clusters at
presynaptic active sites (Bourne et al. 2006). To confirm the rate of G-actin incorporation into
synaptic clusters and to determine whether this actin incorporates into the cortical actin pool, we
microinjected Alexa 488 Fluor labeled monomeric G-actin into 6 axons in 6 preparations. The
large electrodes used for injection were immediately removed and the health of the axon was
confirmed by re-impalement of the axons with a high impedance KCl (1M) containing
micropipette. Using confocal microscopy we confirmed that monomeric G-actin clustered at
synaptic sites (Fig. 2). Fluorescently labeled actin clusters appeared within minutes of injection
(Fig. 2A). Their structure was visualized following reconstruction of confocal serial sections and
revealed complex actin congregates. While some of these appeared as rings in optical sections
(Fig. 2B), similar to phalloidin labeling, more common were bar-like structures consistent with
the phalloidin labeling (Fig. 1). We imaged these structures for up to one hour after G-actin
injection (Fig. 2C). Fluorescence intensity of the initially labeled structures reached its peak
within 20 to 30 mins (Fig. 2D,E). However, the size of the structures measured as cross-
sectional area continued to grow for up to one hour post-injection (Fig. 2D,E), indicating
continuous turnover of endogenous non-fluorescent actin with injected labeled G-actin.

However, in a marked difference from the images obtained following phalloidin injection,
no cortical actin signal was observed during this period. Profile plots calculated from regions
between labeled actin clusters in a single optical section (between vertical white lines in Fig. 2B)
show simply uniform fluorescence in the axon volume. Comparison with those obtained
following phalloidin labeling (Fig. 1B and Ci) demonstrates an absence of fluorescent G-actin
labeled cortical actin, even though considerable diffuse actin fluorescence is visible throughout
the cytosol (Fig. 2A and C, gray). It appears that during steady state conditions, actin associated
with presynaptic vesicle clusters is dynamic with respect to a more stable cortical actin.

Disruption of the dynamic actin cytoskeleton

Presynaptic actin is believed to play a role in endocytosis (Merrifield et al. 2002) and
recycling of vesicles to the vesicle cluster (Bloom et al. 2003; Shupliakov et al. 2002); (Bourne
et al. 2006; Richards et al. 2004). However, in cell culture, block of actin polymerization does
not prevent vesicle recycling (Sankaranarayanan et al. 2003). We sought to determine whether
block of actin polymerization in the lamprey presynaptic terminal alters synaptic vesicle cycling.

It was first necessary to demonstrate that F-actin structures can be disrupted by
inhibiting actin polymerization. We used fluorescent G-actin labeling to measure the efficacy of
the monomeric actin sequestering drug, Latrunculin-A. We injected axons with labeled G-actin
followed by application of Latrunculin-A (12 µM) by perfusion over the spinal cord (see
methods), and imaged the intensity of synaptic vesicle associated fluorescent actin labeling
(Fig. 3A). In control experiments, punctate fluorescent labeling appeared rapidly, and increased
in intensity over 40 to 50 mins (Fig. 3Ai). During application of Latrunculin-A, the labeled G-actin
structures dispersed within 40 minutes of the start of treatment (to 30±9% of control
fluorescence measured at the same time post G-actin injection; Fig. 3Aii, B). Furthermore, in
axons pretreated with Latrunculin-A for 30 minutes prior to labeled G-actin injection into the
axons, labeled G-actin did not cluster into these punctate structures; the fluorescently labeled
actin remained dispersed throughout the axon (Fig. 3C; n=3). This inhibition of actin clustering
was sustained for up to one hour after removal of Latrunculin-A treatment (data not shown). Our
observation of labeled G-actin incorporation into synapse associated structures within minutes
of injection and their continued growth in size and intensity over 30 mins, and the disruption of
these structures within 30 mins of Latrunculin-A application, confirms that presynaptic vesicles
are colocalized with a dynamic actin structure that is continuously turning over G-actin
monomers (Bourne et al. 2006) and indicates that Latrunculin-A treatment can be used to disperse these actin clusters.

However, while fluorescently labeled phalloidin and G-actin both label a punctate pool of actin at synaptic active sites, only phalloidin labels cortical actin. We wished to determine whether Latrunculin-A also disrupts this actin structure. We, thus, injected phalloidin into axons after 30 minutes of treatment with Latrunculin-A (12 µM). Compared to control axons injected prior to Latrunculin-A treatment (Fig. 4A), after Latrunculin-A pretreatment, phalloidin failed to label synapse associated structures in any of the injected axons. However phalloidin still labeled the cortical actin as in controls (Fig. 4B). This indicates that a stable F-actin cytoskeleton present along the plasma membrane of the axons is not dispersed after Latrunculin-A treatment for 30 minutes under quiescent conditions, a time period sufficient to disperse actin clusters at synaptic sites. This conclusion is also supported by our finding that fluorescent G-actin fails to label the cortical actin structure (Fig. 2). Thus treatment with Latrunculin-A can allow us to assess the role of the dynamic vesicle associated actin in synaptic transmission and vesicle cycling.

Synaptic transmission is not blocked by Latrunculin-A treatment

It has been suggested both that preventing F-actin formation with Latrunculin-A has little effect on synaptic vesicle cycling (Sankaranarayanan et al. 2003) but, alternatively, also that disruption of actin polymerization increases release probability (Morales et al. 2000). To determine the physiological effect of blocking actin polymerization on synaptic transmission at the lamprey presynaptic terminal, we conducted paired cell recordings between reticulospinal axons and their postsynaptic partners during and after treatment with Latrunculin-A (30 min, 12 µM). Latrunculin-A treatment had no effect on the amplitude or kinetics of evoked EPSCs (Fig. 5; stimulated at 15s intervals) during or after 30 mins of treatment (mean EPSC amplitude from
20 to 30 mins of Latrunculin-A treatment was 97±9% of control; after wash of Latrunculin-A, responses remained at 92±6% of control; n=6; neither was significantly different from the control EPSC amplitude; Fig. 5A). This is at a time point when actin labeled structures at synapses are dispersed (Fig. 3A, B).

Thus Latrunculin-A treatment leaves low frequency evoked EPSCs unaffected. However, disruption of actin polymerization has been shown to alter release probability (Morales et al. 2000) and to slightly enhance FM1-43 destaining (Sankaranarayanan et al. 2003). Consequently, forty minutes after Latrunculin-A treatment and stable recording of paired cell responses with no alteration in amplitude of synaptic response, the stimulation frequency was increased to 5Hz for 2000 stimulations to ensure that the readily releasable pool of primed vesicles was exhausted (Gerachshenko et al. 2005). High frequency stimulation decreased evoked EPSC peak amplitudes in control (to 48 ± 10% of amplitude at low frequency, n=6; Fig. 5B) and Latrunculin-A treated axons (to 71 ± 8% of amplitude at low frequency, n=6; Fig. 5C). Frequency-dependent inhibition during 5Hz stimulation in control axons was significantly greater (p<0.05) than in Latrunculin-A treated axons. In both conditions inhibition was released upon returning to low frequency stimulation (to 81±6% and 96±14% in control and Latrunculin-A conditions respectively, not significantly different). Thus, Latrunculin-A pretreatment reduces high-frequency-mediated short-term depression of synaptic responses, but does not cause a loss of synaptic transmission following exhaustion of the readily releaseable pool.

Vesicle recycling is possible following Latrunculin-A treatment

Based on electron microscopic evidence, it has been proposed that actin polymerization is required for endocytosis and recycling of endocytosed vesicles to the synaptic vesicle pool (Bourne et al. 2006; Richards et al. 2004; Shupliakov et al. 2002) although this may not be true for all central synapses (Morales et al. 2000; Sankaranarayanan et al. 2003). We therefore tested if Latrunculin-A treatment would diminish vesicle exocytosis, endocytosis or recycling.
Initially, we determined whether vesicle clusters pre-labeled with FM1-43 could be destained after Latrunculin-A treatment. Axons were first labeled with FM1-43 (Fig. 6A). The spinal cord was then treated with Latrunculin-A (12 µM, 30 mins) to disperse presynaptic actin clusters. During Latrunculin-A treatment the fluorescence of FM 1-43 labeled puncta was monitored and showed a slow decay not significantly different from controls (Fig. 6Ai). The axon was then stimulated to evoke destaining. After 16000 stimuli, fluorescence was reduced to 33±2% (Fig. 6Aii, open circles, n=5) of fluorescence after labeling (Fig. 6Ai, bottom image. This was not significantly different from control destaining with no Latrunculin-A (Fig. 6Aii; Destaining in control was to 37±3%; n=6, closed circles).

Actin has been proposed to have a role in the correct recycling of vesicles to synaptic pools (Brodin et al. 2000; Shupliakov et al. 2002) and vesicle mobility and transportation of vesicles between synaptic pools (Cole et al. 2000; Kuromi and Kidokoro 1998; Sakaba and Neher 2003). Thus, we sought to determine whether vesicle clusters could be labeled after Latrunculin-A treatment and subsequently destained. Axons were treated with Latrunculin-A for 30 minutes and then stimulated in FM1-43. FM1-43 labeling was then measured after clearing the tissue with Advasep-7. It was clear that labeled vesicles were endocytosed (Fig. 6Bi) after Latrunculin-A treatment. We then monitored vesicle cluster stability over 40 minutes following Latrunculin-A treatment; vesicle clusters did not disperse and remained stable at active sites as seen in controls (Fig. 6Bii). A further period of stimulation of these labeled axons again caused destaining (to 19±4% of initial fluorescence, Fig. 6Bii open circles; n=5; Fig. 6Bi bottom image). Indeed, this destaining was significantly more complete than control destaining (Fig. 6Bii, closed circles; p<0.01; to 37±3%, n=6). This is consistent with our electrophysiological data showing a reduced synaptic depression during high frequency stimulation and earlier published work in neuronal culture (Sankaranarayanan et al. 2003). We confirmed that longer treatment with Latrunculin-A for durations equivalent to our staining and destaining protocol failed to disrupt the
pool of vesicles (Fig. 6C, fluorescence after 60 mins was reduced to 73±4% in control (n=14) and to 72±11% after Latrunculin-A treatment (n=4).

Thus, block of actin polymerization does not prevent endocytosis or exocytosis of synaptic vesicles, and consistent with results from hippocampal culture (Sankaranarayanan et al. 2003) slightly increases the pool of vesicles that can be mobilized. Because the intensity of FM1-43 loading between individual axons varies, it was not possible to compare directly control endocytosis with that recorded following Latrunculin-A treatment in the above experiments. To overcome this limitation, in a further 3 preparations, vesicle labeling with FM1-43 was tested at the same synapses in the same axons, before and after treatment with Latrunculin-A. Axons were recorded with microelectrodes and labeled with FM1-43 under control conditions as above (Fig. 6Di). These axons were then destained with 16000 stimuli (20Hz, Fig. 6Di bottom image) to provide a control loading fluorescence intensity and a control rate of destaining. Data for the axon shown is plotted as a raw bit value (Fig. 6Dii) and for all axons is normalized to the initial staining intensity (Fig. 6E). A single exponential fit to the destaining curve reveals a time constant of 5.8 ± 0.8 min (6960 ± 960 stimuli, Fig. 6E). The axon was then treated with Latrunculin-A (12 µM; 30 mins). The tissue was again superfused with FM1-43 and the axon stimulated to induce recycling. Post-dye-loading fluorescence was not significantly different from control loading (Mean puncta intensity increased to 114±23% of control loading after subtraction of the residual fluorescence from the first destaining stimulus). The axon was destained once more. Destaining rates were more rapid than control. A single exponential fit to this destaining data reveals a time constant of 3.2 ± 0.4 min (3840 ± 480 stimuli; significantly different from control; p<0.05, Fig. 6E). It is clear from these experiments that neither endocytosis nor exocytosis is prevented by block of actin polymerization. Indeed, these experiments demonstrate for the first time that the entire vesicle cycle from endocytosis to exocytosis remains intact after block of actin polymerization and dispersal of synaptic F-actin structures within the presynaptic vesicle pool. However, consistent with earlier work (Sankaranarayanan et
and our electrophysiological data Latrunculin-A may slightly enhance release during periods of high frequency stimulation.

Stabilization of polymerized actin disrupts vesicle recycling and synaptic transmission

We have previously shown that presynaptic injection of phalloidin inhibits chemical evoked EPSCs but not presynaptic action potentials, evoked Ca\(^{2+}\) transients or the electrical component of synaptic transmission (Photowala et al. 2005). This inhibition occurs rapidly (in fewer than 30 stimuli and less than 5 min), and acts faster than depletion of the readily releasable pool of vesicles in lamprey giant synapses, which requires between 170 and 400 presynaptic action potentials (Gerachshenko et al. 2005). Phaloidin might act to prevent exocytosis directly, or to disrupt the synaptic vesicle pool. To determine the effect of phalloidin on the stability of the FM1-43 labeled vesicle pool, presynaptic terminals were loaded with FM1-43, and the tissue was subsequently cleared of excess dye with Advasep-7 (1 mM). This revealed FM1-43 labeled vesicle clusters (Fig. 7A). In control conditions this fluorescence showed a slow decay in intensity to 75±7% of initial fluorescence over 50 mins (n=5; Fig. 7Ai,ii, Fig. 7C open squares). In a further 12 axons, after FM1-43 loading and an initial measurement of FM1-43 fluorescence intensity (Fig. 7Bi), fluorescently labeled phalloidin was injected into the axon. FM1-43 fluorescence intensity was then measured as phalloidin incorporated into presynaptic F-actin to reveal punctate actin at synapses. Phalloidin incorporation was measured as an increase in fluorescence intensity at each synaptic site (Fig. 7Biii, iv; Fig. 7C, red circles). This had no significant effect on the location or fluorescence intensity of FM1-43-labeled synaptic puncta (Fig. 7C black closed circles, mean from 12 axons with phalloidin, green trace taken from axon in Fig. 7B). FM1-43 puncta fluorescence after 50 mins was 68±8% of initial fluorescence (not significantly different from control axons).

We then determined whether phaloidin altered presynaptic loading with FM1-43 or subsequent stimulus-evoked unloading of the dye. Axons were labeled with FM1-43 in 3
preparations as above to establish control fluorescence labeling (Fig. 8Aii). After clearing excess dye with Advasep-7 (1 mM), the axon was stimulated at 5Hz to destain the axon and measure control rates of destaining. After 16000 stimuli, fluorescence was reduced to 31±8% of prestimulus intensity (Fig. 8B,D). Following this period of action potential-evoked destaining, the axon was labeled with fluorescently tagged phalloidin by microinjection through the recording electrode (Fig. 8Ci). Phalloidin labeled the same structures that had previously been stained with FM1-43. After 20 mins, to allow stabilization of phalloidin labeling of the presynaptic terminals (see Fig. 7C, red), the tissue was again superfused with FM1-43, stimulated as before to evoke FM1-43 dye labeling and cleared once more with Advasep-7. After phalloidin labeling, the subsequent endocytosis and labeling of presynaptic vesicles was substantially reduced compared with control (Staining was 41±9% of initial stain, representing a relative fluorescence increase of only 21% of the control stain prior to phalloidin, after subtraction of the residual fluorescence; Fig. 8B, Cii, D, E, this compares with control restaining intensities of 124 ± 8% of initial staining in control axons – which after subtraction of the residual labeling represents 93 ± 8% of initial staining, Fig. 8E). Additionally, a further period of stimulation to evoke exocytosis failed to destain the terminal fluorescence (Staining reduced to 90±9% of the fluorescence labeling after phalloidin, Fig. 8E, compared to a reduction to 35 ± 9% in control axons that had been stained, destained, restained and destained once more).

From these data showing a stronger effect of phalloidin on exocytosis than endocytosis, and from our previous results demonstrating a very rapid loss of evoked synaptic transmission following presynaptic phalloidin injection (Photowala et al. 2005), phalloidin appears to directly inhibit exocytosis. FM1-43 restaining after phalloidin application is reduced but not eliminated. This is consistent with reduced compensatory endocytosis leading from inhibition of exocytosis. One might expect a proportionate loss of endocytosis following block of exocytosis, however these process can be uncoupled (Neale et al. 1999; Smith and Neher 1997; Yao et al.) though we cannot rule out an effect of phalloidin stabilization of F-actin on vesicle endocytosis.
Phalloidin inhibition of synaptic transmission does not act at dynamic F-actin puncta

Previous studies have indicated that perisynaptic actin seen at the electron microscopic level is necessary for vesicle recycling (Bloom et al. 2003; Bourne et al. 2006; Shupliakov et al. 2002). However, we have now demonstrated that in common with cultured hippocampal neurons (Morales et al. 2000), dispersal of this actin leaves synaptic transmission and vesicle cycling intact. Because phalloidin injection is possible in lamprey giant axons, we have also been able to demonstrate that block of actin depolymerization blocks synaptic transmission and arrests vesicle recycling. We therefore hypothesize this effect of phalloidin acts at a different F-actin pool, and because the cortical actin remains after treatment with Latrunculin-A including at synaptic active sites, it or a component of this at the synaptic active zone is a possible candidate.

We therefore determined what effect stabilization of only the cortical actin had on synaptic transmission. We pre-treated spinal cords with Latrunculin-A (12µM, 30 min) to disperse synaptic actin clusters, but leave cortical actin intact. Paired cell recordings were then performed (Fig. 9). EPSCs were readily recording after pre-treatment with Latrunculin-A (Fig. 9Ai upper trace). After recording evoked EPSCs, labeled phalloidin was injected into the axon. Confirming that pretreatment with Latrunculin-A dispersed punctate F-actin, the phalloidin did not label any presynaptic active sites (Fig. 9B), yet it still accumulated immediately under the axon membrane to label cortical actin (Fig. 9B, D). During this time, EPSCs were inhibited corresponding to an increase in phalloidin intensity at the axonal edge (Fig. 9C, D; 15 mins after phalloidin injection EPSCs were reduced to 7.3 ± 4.2% of control amplitudes, n=4 preparations). Phalloidin clearly inhibits synaptic transmission in the absence of any labeled presynaptic F-actin clusters.
Jasplakinolide disruption of actin enhances neurotransmission and blocks cortical actin stabilization with phalloidin

While phalloidin stabilization of cortical actin alone was sufficient to block synaptic transmission, we wished to rule out a possible non-specific result of blockade of actin depolymerization. Thus, we performed paired cell recordings and treated the spinal cord with jasplakinolide, a known blocker of actin depolymerization, which can also enhance polymerization and disrupt F-actin structures \textit{in vivo} by favoring F-actin nucleation (Bubb et al. 2000). Evoked EPSCs were recorded for 5 minutes (at 30s intervals) after which jasplakinolide (2µm) was applied to the superfusate. During jasplakinolide application EPSC amplitudes increased (to 328 ± 109 % of control) over 40 mins of recording in jasplakinolide (Fig. 10A,B; n = 4). This result was in marked contrast to that observed following phalloidin injection (Fig. 9). We thus determined the effect of jasplakinolide treatment on presynaptic F-actin by pretreating axons with jasplakinolide and subsequently visualizing F-actin by injecting labeled phalloidin. Jasplakinolide was superfused over the spinal cord for a minimum of 20_min after which, we pressure injected labeled phalloidin into single axons (Fig. 10C,D). While phalloidin was still found to cluster at presynaptic sites, we were not able to detect any cortical labeling (Fig. 10E). We do not believe that this is soley the result of competitive binding of actin by phalloidin and jasplakinolide (Bubb et al. 1994), because jasplakinolide did not prevent phalloidin labeling of synaptic vesicle associated actin clusters and because its application after phalloidin treatment did not reduce cortical actin labeling by phalloidin (data not shown). Thus, a jasplakinolide induced disruption of cortical actin appears to correlate well with an enhancement in evoked neurotransmitter release.
Discussion

Actin is found throughout presynaptic terminals, however, its role in the physiology of synaptic transmission remains unclear (Doussau and Augustine 2000). Indeed, published works on presynaptic actin propose contradictory roles for its physiological functions. While there is evidence for its active role in endocytosis and vesicle recycling (Bloom et al. 2003; Bourne et al. 2006; Brodin and Shupliakov 2006; Pieribone et al. 1995; Rosahl et al. 1995; Shupliakov et al. 2002) and in exocytosis (Lang et al. 2000; Vitale et al. 1995), others have proposed a structural function with no strong role in vesicle cycling (Sankaranarayanan et al. 2003). Beyond synapses, a role for actin has been proposed during clathrin-mediated endocytosis (Kaksonen et al. 2003; Kaksonen et al. 2006; Merrifield et al. 2002; Merrifield et al. 2005), though this may not be true of all cells (Fujimoto et al. 2000). However, these conclusions are drawn using a wide variety of preparations and conditions, and thus it is possible that actin subserves many of these roles (Cingolani and Goda 2008).

The presynaptic actin cytoskeleton has previously been envisaged as a ring-like structure in lamprey (Bourne et al. 2006), surrounding a vesicle pool labeled with anti-Synapsin antibodies (Shupliakov et al. 2002). Similarly, an association of actin with synaptic vesicles has been observed in cultured hippocampal neurons (Sankaranarayanan et al. 2003) and the frog neuromuscular junction (Richards et al. 2004). We demonstrated that vesicle clusters loaded with FM1-43 are colocalized with phalloidin decorated F-actin. However, the phalloidin labeled actin at synapses does not surround vesicle clusters as previously described but is contained within them. Additionally, our injections of phalloidin or G-actin into axons often revealed complex actin conglomerates in addition to rings. Furthermore vesicle clusters were often associated with more than one phalloidin labeled presynaptic site. This localization of single vesicle clusters with multiple active sites has also been observed in EM reconstructions in lamprey (Gustafsson et al. 2002) and is suggestive of the dynamic sharing of vesicles between active zones (Cingolani and Goda, 2008). The discrepancy between our findings, and previous
results in which F-actin was found to surround the vesicle pool could be a result of our stimulation protocol which may label a larger proportion of the vesicle cluster with FM dye than inferred from anti-Synapsin antibody labeling. It is known that anti-Synapsin antibodies disrupt the distal portion of the vesicle pool (Pieribone et al. 1995) causing a loss of reserve pool vesicles. In addition, previous ultrastructural EM studies investigating presynaptic actin localization have either identified filaments as comet tails associated with recycling vesicles, or as gold particle labeled anti-actin antibodies (Shupliakov et al. 2002). It is possible that smaller actin filaments are not detectable by these methods (Siksou et al 2011). Clearly though, the actin clusters labeled by either phalloidin or G-actin are unnecessary to retain synaptic vesicle clusters, as has been shown previously (Bourne et al. 2006; Gaffield et al. 2006; Kuromi and Kidokoro 1998; Richards et al. 2004; Sankaranarayanan et al. 2003; Shupliakov et al. 2002).

Actin’s association with the periphery of presynaptic active sites, its colocalization with endocytic proteins (Bloom et al. 2003; Morgan et al. 2004) and the disruption of clathrin-mediated endocytosis upon disruption of actin polymerization (Bourne et al. 2006) has led to an hypothesis that actin is necessary for synaptic vesicle recycling from the endocytic region during stimulation (Brodin and Shupliakov 2006). Stimulation has been shown to alter actin polymerization within synaptosomes (Bernstein and Bamburg 1989), its distribution in presynaptic terminals of hippocampal neurons in culture (Sankaranarayanan et al. 2003) and in lamprey presynaptic terminals (Bloom et al. 2003; Shupliakov et al. 2002). However, in lamprey, activity-independent actin turnover at this region was also seen in axons in both depleted and physiological extracellular [Ca$^{2+}$] (Bourne et al. 2006). We also show that actin dynamically clusters at presynaptic sites under quiescent conditions (Figs. 2,3), yet does not incorporate into cortical actin. Furthermore, we have now shown that disruption of this more dynamic actin by sequestration of G-actin monomers with Latrunculin-A does not inhibit neurotransmission or vesicle cycling. We also observed, a slight enhancement in neurotransmitter release during high frequency stimulation. While this latter effect is not profound, similar effects have also been
recorded in other systems (Bernstein and Bamburg 1989; Cole et al. 2000; Richards et al. 2004; Wang et al. 1996), although under conditions of prolonged high frequency stimulation block of actin polymerization may inhibit synaptic transmission (Kuromi and Kidokoro 1998; Richards et al. 2004) or prolong recovery of synaptic transmission (Cole et al. 2000). It is difficult to reconcile our results with those seen in previous studies utilizing Latrunculin and other stronger actin depolymerizing agents. Though the greater effects seen with depolymerizing agents known to sever actin filaments may have additional effects on cortical actin not previously
examined (see below).

Within presynaptic terminals, actin filaments are closely associated with synaptic vesicles (Gotow et al. 1991), but do not contact vesicles directly, rather it is thought to associate with Synapsin filaments which might tether vesicles in a phosphorylation-dependent manner (Shupliakov et al. 2011). Indeed, disruption of Synapsin depletes synaptic vesicles at synapses, and inhibits vesicle cycling during high frequency stimulation (Pieribone et al. 1995). Because of actin’s association with Synapsin (Bloom et al. 2003) and the physiological deficits in vesicle cycling seen with actin disruption (Cole et al. 2000; Morales et al. 2000; Wang et al. 1996) it has been proposed that actin may also help to anchor vesicles at the terminal and play a role in exocytosis.

In the lamprey reticulospinal axon, preventing actin depolymerization with phalloidin rapidly blocks synaptic transmission (Photowala et al. 2005) and we now show this treatment, also disrupts vesicle recycling. Intriguingly, phalloidin labeling of cortical actin specifically blocks synaptic transmission. Indeed, EPSCs are inhibited much faster than can be accounted for by loss of the primed vesicle pool determined using botulinum B toxin (Gerachshenko et al. 2005). Similarly phalloidin markedly reduced vesicle labeling by FM1-43 and completely blocked destaining. However, as it does so, it does not alter EPSC kinetics – neither synaptic delay, nor rise-time nor decay. Phalloidin, like Latrunculin-A, though, does not disperse vesicle clusters. Thus, it seems unlikely that phalloidin alters synaptic structure. There is limited information on...
such treatment in other synapses because it is difficult to apply phalloidin to presynaptic
terminals. Although, in other preparations the membrane permeable agent, jasplakinolide, a
known stabilizer and active promoter of actin filaments (Bubb et al 2000), has been used to
determine the effects of inhibiting actin depolymerization on synaptic transmission and vesicle
cycling. However, in hippocampal cultures, application of jasplakinolide had no effect on
spontaneous synaptic transmission (Morales et al 2000) nor did it effect the rate of destaining of
FM labeled synaptic vesicles (Sankaranarayanan et al. 2003). We observed a marked increase
in EPSC amplitude during evoked synaptic transmission, which was accompanied by a loss of
the ability of phalloidin to label cortical actin. We do not believe that this is merely a competition
between jasplakinolide and phalloidin as has been demonstrated (Bubb et al. 1994) because
phalloidin was still able to bind to presynaptic clusters of actin, and furthermore injection of
phalloidin prior to jasplakinolide treatment still labeled cortical actin. A possible explanation for
our results is that in contrast to in vitro conditions, jasplakinolide in vivo may disrupt existing F-
actin filaments and induce anomalous accumulations of actin clusters (Bubb et al. 2000). None-
the-less in jasplakinolide or Latrunculin-A combined with phalloidin treatments, we have
observed that alterations to the cortical actin result in more pronounced deficits in synaptic
transmission and vesicle cycling.

We propose that this cortical actin, or a subcomponent at the active zone which
unfortunately cannot be separately resolved using these techniques, is involved in vesicle
cycling, because its labeling with phalloidin and its stabilization alone is associated with a block
of synaptic transmission even after the disruption of synaptic vesicle cluster associated actin by
pretreatment with Latrunculin-A. The location of this actin is reminiscent of cortical actin, which
has been proposed to play a necessary role in various forms of non-neuronal exocytosis (Eitzen
2003; Vitale et al. 1995) and has been implicated in large dense core vesicle docking (de Wit
2010). It is also possible that a more stable pool of actin provides a framework for myoglobin,
which may also play an important role in vesicle recycling (Mochida 1995; Srinivasan et al.
2008; Takagishi et al. 2005). However, our proposal would also agree with the recent concept of
a matrix of filaments within the active zone that might alter vesicular release and motility
(Pechstein and Shupliakov 2010). We conclude that there exist, at minimum, dual pools of actin
at the presynaptic terminal. These pools have different rates of turnover from G- to F-actin, and
the two pools have unique roles during vesicle recycling with the stable pool that is resistant to
treatment with Latrunculin-A may play a direct role in exocytosis, such that its further
stabilization with phalloidin entirely blocks synaptic transmission.

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Figure 1, Distribution of phalloidin labeling and FM labeled vesicle clusters in axons

A. Recording schematic. FM1-43 was superfused over the ventral spinal cord (green). Axons impaled with microelectrodes containing KCl (3M) were loaded with FM1-43 by intracellular stimulation and the tissue cleared of dye with Advasep-7. Axons were re-impaled (electrode contained Alexa Fluor 568 phalloidin, red), for injection into the axon. Confocal z-sections were taken of live axons.

B. Phalloidin labels both synaptic structures and cortical actin.

Bi. 3D reconstruction viewed along length of the axon (caudal - rostral) showing ventral half of axon and phalloidin labeling (ventral-left). Red – intense labeling at puncta, grey – dimmer, diffuse labeling under plasma membrane – cortical actin (see D for color look-up-table (LUT)).

Bii. View of same axon from the spinal ventral surface.

Ci. Optical section from dashed line (a) in (Bi). Graph shows intensity profile through the section at dashed lines (white – through active zone; graph color from same LUT as (B). (gray - outside synaptic regions). This graph is in gray.

Cii. Optical section (b) in (Bi). Graph of intensity profile along dashed line in image. Colors from LUT as for (Ci).

D. Voxel intensity histogram (top) from dataset in (B). Background noise sampled from outside axons (blue line). This matches background noise under the image (light blue bars). Other histogram bar colors represent the LUT used to generate 3d reconstructions. Black to red curve represents opacity values used in (B). Voxel intensity histogram (bottom) for FM1-43 labeling – opacity curve to display FM1-43 in (E) in green.

Ei. 3d overlay of phalloidin structures (red), and FM1-43 labeling (green). (Line - section in Eiii).

Eii. Axon from spinal ventral surface (view from left of Ei) (line - position of section in Eiv).
Eiii. View as (Ei) but top sections removed (cut at dashed line in Ei) to reveal phalloidin and FM1-43 colocalization (arrows – phalloidin, red, FM loaded vesicles bright green) at the cut at dashed line in (Ei). Yellow - colocalization at cut.

Eiv. Sagittal cut of FM1-43 and phalloidin seen from lateral spinal cord with lower half below dashed line in (Eii) removed.

Vesicle clusters are associated with phalloidin, sometimes spanning more than one phalloidin structure (arrowheads in Eii, Eiii). FM/phalloidin colocalized (yellow) can be contained within an FM1-43 puncta (arrowhead in Eiv).
Figure 2, G-actin incorporates into presynaptic puncta but not cortical actin

Reticulospinal axon impaled with a microelectrode containing Alexa 488 G-actin (1.1 mg ml\(^{-1}\)) and buffer solution (2 mM HEPES, 0.2 mM CaCl\(_2\), 0.2 mM ATP, pH 8.0). This was pressure injected into the axon. Fluorescent actin structures formed within minutes of injection, and increased in fluorescence over 80 minutes.

A. 3D reconstruction of actin fluorescence close to the axon perimeter 5 mins after injection. Hemi-axon is shown in transverse section (left) and from the ventral spinal surface (right). Red structures are bright actin labeling consistent with synaptic vesicle cluster colocalization, gray is the diffuse labeled G-actin within the axon.

B. Single optical section shown in linear grayscale taken from the dashed line in (A). The graph is a profile plot of intensity taken from between the vertical white lines. There is no cortical actin signal.

C. Same views as (A) but after an hour of recording.

D. Mean intensity (black) and cross-sectional area (red) of all clusters in (A) plotted with time after injection.

E. Mean area and intensity (6 preparations) normalized to value 80 mins after injection.
Figure 3, Visualization of actin dynamics and disruption with Latrunculin-A

Axons were labeled with fluorescent G-actin by pressure injection (as Fig. 2). After injection, axons were re-impaled with an electrode containing 3M KCl to monitor membrane potential.

**A. Examples of G-actin labeling at synaptic vesicle clusters over time**

(Ai) In control axon, G-actin incorporated into presynaptic clusters, which increased in fluorescence and then stabilized within 40 minutes.

(Aii) To test Latrunculin-A effects, G-actin was injected into presynaptic terminals. After 20 minutes Latrunculin-A was perfused onto the axon. G-actin clusters dispersed.

**B. Graph of normalized mean fluorescence of puncta from control – open circles (n=7) and Latrunculin-A treated axons – closed circles (n=7).**

**C. Comparison of control G-actin staining, and staining after pretreatment with Latrunculin-A.**

Top panel; puncta form in control axons. Bottom panel; axons were pretreated with Latrunculin-A and subsequently microinjected with G-actin. No G-actin clustering was observed after Latrunculin-A, although diffuse fluorescence was observed.
Figure 4, Latrunculin-A prevents incorporation of phalloidin at presynaptic terminals but not the cortical actin.

Axons were recorded from with a microelectrode and injected with phalloidin (Alexa 488)

Ai. Control axon injected with phalloidin reveals vesicle cluster associated fluorescent structures. Top image - optical section through the ventral axon surface. Bottom image - section through the center of the same axon.

Aii. To quantify this a profile of fluorescence intensity was measured across the longitudinal sections from between the horizontal white lines in (Ai). Profile was placed to avoid presynaptic puncta. A fluorescence peak is seen at the plasma membrane.

Bi. Axon injected with phalloidin after Latrunculin-A treatment (30 min, 12 µM) displays fluorescence with no clustering of phalloidin. Top image is an optical section at the ventral surface, bottom image a section through the center. Although no presynaptic structures are seen, plasma membrane fluorescence is visible.

Bii) Profile of fluorescence intensity from between lines in phalloidin labeled axon (Bi) after pretreatment with Latrunculin-A. Fluorescence peaks are seen at the membrane.
Figure 5. Depolymerization of actin enhances synaptic responses to high frequency stimulation

A. At low frequencies of stimulation (1/15 Hz), Latrunculin-A had no effect on EPSC amplitude.
Aii. Histogram of mean normalized amplitudes of EPSCs during control, Latrunculin-A treatment and 30 minutes after Latrunculin-A (6 preparations).

B. Periods of high frequency stimulation (5Hz) reduced the mean EPSC amplitude.
Bi. Representative traces (10 sequential EPSCs) before and during 5Hz stimulation and
Bii. Means of these EPSCs before, during and after 5Hz stimulation.
Biii. Histogram of mean normalized amplitudes of EPSCs during 1/15 Hz stimulation, 5 Hz and returned to 1/15 Hz in controls (6 preparations).

C. After Latrunculin-A treatment 5 Hz stimulation had less effect.
Ci. Representative traces (10 sequential EPSCs, top traces) before during and after 5Hz stimulation in Latrunculin-A treated preparations.
Cii Means of these EPSCs before, during and after 5Hz stimulation.
Ciii. Histogram of mean normalized amplitudes of EPSCs during 1/15 Hz stimulation, 5 Hz and returned to 1/15 Hz in Latrunculin-A treated preparations (6 preparations).
Figure 6, Vesicle recycling is preserved in Latrunculin-A


Aii. Graph of normalized puncta fluorescence with time. Axons labeled as in (Ai). Fluorescence measured before and during Latrunculin-A (open circles; n=5), or control recordings (closed circles; n=6) for 40 mins and then destained with 20 Hz stimulation.

Bi. Axons loaded with FM1-43 as in (A) 30 mins after Latrunculin-A (12 µM) treatment. Top image - FM1-43 puncta in single axon. Bottom image - destained axon following 12000 stimuli.

Bii. Normalized fluorescence with time from all axons pretreated with Latrunculin-A (open squares; n=5) or left as untreated controls (closed circles; n=6) then labeled as in (Bi). Control and Latrunculin-A treated axons were monitored for 40 mins, then destained.

Ci. Vesicle clusters in axons loaded with FM1-43 prior to treatment with Latrunculin-A (Top, - after loading; Bottom - after 1hour and Latrunculin-A treatment)

Cii. Graph of mean FM1-43 labeling during and after treatment with Latrunculin-A (open circles; n=4) we identical to untreated controls (closed circles; n=14) up to 1 hour after treatment.

D. Latrunculin-A neither prevented endocytosis, recycling nor subsequent exocytosis.

Di. Axon showing FM1-43 labeling (top) and after 16000 stimulus-evoked destaining (bottom).

Dii. Same axon after Latrunculin-A treatment (12 µM, 30 mins), then restained (top) and after destaining (with 16000 stimuli) following Latrunculin-A treatment (bottom).

Diii. Data from axon above. Fluorescence measured throughout cycles of staining and destaining in control and post-Latrunculin-A. Letters mark corresponding images.

Div. Pooled data from 4 axons treated as above. Exponentials (gray) fitted to the destaining data. Mean s of these exponentials obtained for these axons are shown. Gray denotes period of stimulation.
Figure 7, Presynaptic phalloidin injection leaves presynaptic vesicle clusters intact.

A. Presynaptic terminals were labeled with FM1-43 as for Figure 1. These puncta remained labeled for over 50 mins (Ai) although fluorescence was reduced to 70% of control after 50 mins (C; open squares).

B. Axons were labeled with FM1-43 with presynaptic recording electrodes containing Alexa 568 labeled phalloidin.

Bi. FM1-43 labeling prior to phalloidin microinjection.

Bii. FM1-43 labeling 40 mins post phalloidin microinjections demonstrates that FM1-43 labeling remains intact.

Biii. No phalloidin fluorescence is visible prior to injection.

Biv. Vesicle cluster associate F-actin visible following phalloidin injection colocalizes with FM1-43 puncta.

C. Graph of FM1-43 and phalloidin fluorescence at puncta over time. Mean fluorescence of all FM1-43 puncta in the imaged axon (B, green) and of FM1-43 in all axons (black closed circles) subsequently labeled with phalloidin. The stability of this fluorescence signal did not differ significantly from control axons not labeled with phalloidin (black open squares). Phalloidin binding to puncta following microinjection increases following injection (red; time of injection marked by black arrow). Images in (B) taken at time points indicated.
Figure 8, Presynaptic phalloidin injection inhibits exocytosis and compensatory endocytosis.

**A.** An axon was first impaled with an electrode containing KCl (Ai: no phalloidin signal) and stimulated (2000 stimuli) in FM1-43 to label presynaptic vesicle clusters (Aii).

**B.** Graph of FM1-43 fluorescence at puncta in (Aii). Axon stimulated intracellularly (16000 stimuli, 20Hz) and intensity of puncta declined. The presynaptic electrode was removed and replaced with one containing phalloidin, which was pressure injected (black arrow). Intracellular phalloidin application was confirmed by imaging its Alexa 568 label (Ci; image at red arrow in (B), which marked the same puncta previously labeled with FM1-43 (Aii). The tissue was again superfused with FM1-43 and the axon stimulated (2000 stimuli). This revealed the same puncta (labeled with FM1-43) but with significantly reduced fluorescence than in (Aii). A further 16000 stimuli failed to destain these puncta.

**Ci.** Phalloidin labels structures associated with vesicle clusters in the same axon as A.

**Cii.** FM1-43 puncta labeled in same axon as (A) but after destaining and restaining following phalloidin injection.

**D.** Graph shows mean normalized data from 3 preparations treated as for (B).

**E.** Summary of staining and destaining data from 3 preparations. Control destaining (green), Control restaining (grey) of the same puncta following the destaining protocol. After phalloidin injection, restaining was minimal (1st red bar). Subsequent destaining (2nd red bar) was also minimal.
Figure 9, Phalloidin blocks synaptic transmission after dispersal of actin at vesicle clusters by Latrunculin-A

A. After pretreatment with Latrunculin-A (12 µM; 30 minutes) a paired cell recording was made between a reticulospinal axon and a postsynaptic neuron. Synaptic transmission was not prevented by Latrunculin-A pretreatment. In these recordings the presynaptic electrode contained phalloidin labeled with Alexa 488.

Ai. (upper traces) 10 sequentially evoked EPSCs following presynaptic action potentials. (lower traces), 10 EPSCs recorded 15 min after phalloidin injection. The remaining response is the electrical component.


B. Images of the Alexa 488 phalloidin labeled axons. (Bi) prior to injection, only the recording microelectrode is visible. (Bii) 1 min after injection, dye is visible in the axon. (Biii) 10 minutes after injection the axon outline is visible. (Biv) 20 minutes after injection. In no cases are presynaptic active zone vesicle clusters labeled by phalloidin, indicating that these F-actin clusters were dispersed by pretreatment with Latrunculin-A.

Graphs to right of each image are intensity profiles from each image from region indicated by white lines in (Biv) Cortical actin is revealed following 10 to 20 mins of phalloidin.

Ci. Graph of peak EPSC amplitude before and after phalloidin injection.

Cii. Intensity of phalloidin alexa 488 fluorescence was measured at the center of the axon (closed circles) and at its edge (open circles) over time. The increase in phalloidin fluorescence at the axon border correlates well with the reduction in EPSC amplitude in (C).

D. Means of EPSCs amplitudes before and after phalloidin injection after treatment with Latrunculin-A (open circles, n = 4), with no pretreatment (closed circles, n = 5) or control recordings after Latrunculin-A but with no phalloidin injection (triangles, n = 5) (NB vertical axis inverted to reflect inward currents recorded).
Figure 10, Jasplakinolide enhances synaptic transmission and prevents phalloidin labeling of cortical actin.

A. Paired cell recordings between reticulospinal axons and their whole cell clamped postsynaptic target neurons. Control responses were recorded to single presynaptic action potentials at 30 s intervals for 5 mins. Jasplakinolide (2µM) was added to the superfusate. Graph shows single EPSC peak amplitudes against time after addition of Jasplakinolide.

B. Examples of 6 sequential EPSCs from control (black) and in Jasplakinolide after 20 mins of application (blue). Bottom traces overlaid - averages of the EPSCs shown above.

C. After treatment with Jasplakinolide (2µM) for a minimum of 20 mins, phalloidin was microinjected into axons through the recording pipette. Fluorescence was imaged confocally to reconstruct labeling in 3D 40 mins after injection. Vesicle cluster associated phalloidin was readily labeled. However, no cortical actin was seen. (Ci) view along the length of the axon, (Cii) view from ventral spinal cord.

D. and E. Single optical sections from positions indicated by the dashed lines in (Ci) through the axon (Di) and at the ventral surface (Ei). Intensity profiles (Dii, Eii) are shown through these optical sections at the dashed lines in (Di) and (Ei) including clusters (red) and regions with no clusters (black). No cortical actin signal is seen at the axon membrane.
A 5 mins

B

Distance across axon
0 20 40 60 80 100 120
0 20 40 60 80 100 120
Intensity (au)

C 60 mins

5 μm

D

Area of puncta (μm²)

Time after actin injection (min)

E

Normalized puncta intensity/area

Time after actin injection (min)
Ai Control puncta
Aii Puncta during Latrunculitin-A

B

Normalized actin puncta fluorescence

Time (mins)

Latrunculin-A

C Actin microinjection

Control

Post Latrunculin-A