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Stability of the frog motor nerve terminal: roles of perisynaptic Schwann cells and muscle fibers

Ling Xin
University of Massachusetts Amherst

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STABILITY OF THE FROG MOTOR NERVE TERMINAL: 
ROLES OF PERISYNAPTIC SCHWANN CELLS AND MUSCLE FIBERS

A Thesis Presented

by

LING XIN

Submitted to the Graduate School of the 
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STABILITY OF THE FROG MOTOR NERVE TERMINAL: 
ROLES OF PERISYNAPTIC SCHWANN CELLS AND MUSCLE FIBERS

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Approved as to style and content by:

__________________________________________
Elizabeth A. Connor, Chair

__________________________________________
John R. Nambu, Member

__________________________________________
Patricia Wadsworth, Member

__________________________________________
Patricia Wadsworth, Director
Molecular and Cellular Biology Program
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CHAPTER 1

INTRODUCTION

Although synapse stability and maintenance are very important for the maturation and continuous function of a synapse, a thorough understanding of the underlying mechanisms and contributing factors still remains unclear. The neuromuscular junction is a highly specialized synaptic structure for the transmission of an electrical impulse from the motor neuron to a skeletal muscle fiber (Figure 1A). Motor neurons, whose cell bodies are located in the ventral horn of the spinal cord, extend myelinated axons that arborize into fine unmyelinated branches when they reach target muscle fibers. Each motor neuron can innervate multiple muscle fibers (Dale et al., 2005), but each muscle fiber receives inputs from only one motor neuron resulting in only one neuromuscular junction for each muscle fiber. A neuromuscular junction consists of four physical components: the presynaptic nerve terminal with capping perisynaptic Schwann cells (PSCs), the synaptic basal lamina occupying the synaptic cleft, and the postsynaptic muscle fiber (Hughes et al., 2006).

The frog neuromuscular junction is a useful system for studying synapse stability and function. It has highly specialized cellular and molecular structure (Figure 1B). At the adult frog neuromuscular junction, release sites of the motor nerve terminal are characterized by clusters of synaptic vesicles, active zones, and the molecular machinery for neurotransmitter release. These release sites are separated by nonrelease domains and are distributed at 1μm intervals along the length of motor nerve terminal. Presynaptic release sites are precisely apposed by junctional folds in the muscle membrane where concentrations of acetylcholine receptors (AChR) are found (Kuffler
and Yoshikami, 1975). The presynaptic motor nerve terminal is capped by 1-12 nonmyelinating PSCs (Frietas and Connor, unpublished results). PSCs occasionally project thin cytoplasmic extensions that encircle nerve terminals in nonrelease domains. The synaptic basal lamina, a network of extracellular matrix molecules including Type IV collagen, laminin and heparan sulfate proteoglycan, lies in the synaptic cleft and is an extension of the basal laminae of PSCs and the muscle fiber. The coordinated maintenance of all synaptic components is required for stable and effective synaptic transmission. Here, I will focus on the mechanisms of the maintenance of the nerve terminal organization.

Studies from mammalian neuromuscular junctions have suggested that retrograde factors from muscle fibers are vital for the stability of motor nerve terminals. Segments of nerve terminal branches retracted rapidly after blockade of AChRs or removal of muscle fibers (Balice-Gordon and Lichtman, 1994; Rich and Lichtman, 1989). Our lab has previously assayed the role of the target muscle fiber in the stabilization and maintenance of frog motor nerve terminals by analyzing target-deprived nerve terminals using repeated in vivo imaging. The results showed that the nerve terminals were well maintained functionally and structurally during the first two months after target loss, suggesting that nonmuscle factors play a role in the stabilization of nerve terminals (Dunaevsky and Connor, 1998).

Several lines of evidence point to a dynamic role of the PSCs in the function of the neuromuscular junction including the stability of motor nerve terminal. PSCs possess neurotransmitter receptors and ion channels, allowing them to sense synaptic activity and respond by increasing the intracellular concentration of calcium (Verkhratsky and

2
Steinhause, 2000; Rochon et al., 2001). This Ca\(^{2+}\) increase can reciprocally effect
synaptic transmission; blocking Ca\(^{2+}\) increase in PSCs prevented the potentiation of
transmitter release (Castonguay and Robitaille, 2001). These results demonstrated that
PSCs contribute to short-term regulation of neurotransmission. PSCs can also respond
to nerve injury by sprouting. These sprouts precede and guide nerve growth (Koriala et
al., 2000; Kang et al., 2003; Hayworth et al., 2006). In knockout mice that lack
Schwann cells, neuromuscular junctions could be transiently formed but retracted
within a few days (Lin et al., 2000; Wolpowitz et al., 2000). These results suggested
that PSCs are dispensable for initial synapse formation but essential for subsequent
synapse growth and maintenance. Further, Schwann cell processes lead sprouting motor
axons to reinnervate neuromuscular junctions (Son et al., 1996).

For further examination of the role of PSCs in synaptic function, PSCs at frog
neuromuscular junctions can be selectively ablated \textit{in vivo} by complement-mediated
cell lysis. In PSC ablation studies by Ko and colleagues, about 13\% of the PSC-ablated
neuromuscular junctions showed either partial retraction or complete loss of the nerve
terminal as well as a decrease in presynaptic release 1 week after PSC ablation.
However, within 1 day of the PSC ablation, defined as acute ablation, little alteration in
either the structure or function of the nerve terminals was observed (Reddy et al., 2003).
Therefore, neither muscle fiber removal for less than 2 months nor PSC ablation alone
induced significant change in the structure and function of motor nerve terminals. One
possibility is that sufficient stabilization cues are provided to nerve terminals as long as
one synaptic partner remains, either the muscle fiber or the PSC. Alternatively, the basal
lamina may be the important factor. To test these possibilities, one can ask how nerve
terminal stability is affected by loss of both target muscle fiber and associated perisynaptic Schwann cells.

The goal of my thesis is to investigate the morphology of nerve terminals deprived of both their target muscle fiber and associated PSCs. We combined the methods of microsurgery and complement-mediated PSC ablation to kill both muscle fibers and PSCs at frog neuromuscular junctions. Nerve terminal stability was assayed by immunohistochemistry using markers of the synaptic site and nerve terminal. My hypothesis is that both muscle fiber and PSCs contribute to the stability of motor nerve terminals. In their absence, I predict that the distribution of the nerve terminal marker will be altered compared to that observed when nerve terminals are deprived of only one of the synaptic members, either PSCs or muscle fibers. Data rejecting this hypothesis will suggest a role of synaptic basal lamina in the maintenance of motor nerve terminals.
CHAPTER 2
METHODS

Selective removal of muscle fibers

Cutaneous pectoris (CP) muscles of adult frogs (*Rana pipiens*) are located just beneath the skin of the thorax and are accessible for manipulation and surgical intervention. The muscle fibers of cutaneous pectoris muscles were damaged as described previously in order to cause selective muscle fiber degeneration while preserving the innervating axons and synaptic sites (Yao, 1988; Dunaevsky and Connor, 1998). Briefly, careful cuts were made in nonsynaptic muscle regions of fibers to induce their degeneration and phagocytosis. Following muscle damage, the skin was sutured and the frogs recovered from anesthesia. Regeneration of muscle fibers was prevented by X-irradiation of the thorax for three consecutive days at the time of microsurgery (Dunaevsky and Connor, 1998).

In vivo ablation of perisynaptic Schwann cells

Complement-mediated cell lysis was used to ablate PSCs as described by Reddy et al., 2003. Briefly, this method uses an antibody specific for PSCs, an IgM monoclonal antibody, 2A12 (mAb 2A12), that recognizes the surface membrane of PSCs (Astrow et al., 1998). Reddy and his colleagues have demonstrated that exposure to mAb 2A12, followed by guinea pig complement serum, selectively kills PSCs at neuromuscular junctions of cutaneous pectoris muscles (Reddy et al. 2003). The 2A12 hybridoma cell line was provided by our collaborator, Dr. Chien-Ping Ko at the University of Southern California. I raised the hybridoma cells, tested the supernatant for its ability to stain
PSCs, and produced 2A12 ascites (see Results). Control frogs in PSC ablation experiments were treated with normal frog Ringer’s solution (NFR: 160mM NaCl, 4mM KCl, 1.8mM CaCl₂, 1mM NaH₂PO₄, pH 7.2) instead of mAb 2A12, followed by guinea pig complement. The ablation process was as follows: Adult frogs (*Rana pipiens*) were anaesthetized in 0.1% MS-222 (Sigma-Aldrich, St. Louis, MO) and the skin over the chest of these frogs was opened. These frogs were then injected in the space beneath each CP muscle with ~150µl NFR solution containing mAb 2A12 ascites (1:7.5) (PSC-ablated frogs) or with ~150µl NFR solution alone (control frogs). After a three hour incubation at room temperature, frogs were then injected with guinea pig complement serum (2:1) diluted with distilled water and incubated for 15 minutes warmed to 34°C. Undiluted guinea pig complement serum was then injected and the frogs were incubated for an additional 45 minutes at 34°C. and then 1 hour on ice. 

Immediately following the completion of the ablation process, the efficiency of the PSC ablation was verified by staining the muscles *in situ* with peanut agglutinin (PNA) PNA-Tritc (1:50; Sigma-Aldrich, St. Louis, MO), a marker of the synaptic and Schwann cell basal lamina (Ko 1987), and a membrane impermeable nuclear dye, ethidium homodimer (EthD; 1:5000; Invitrogen-Molecular Probes, Carlsbad, CA). In some experiments, the CP muscles were dissected immediately after verification of ablation. In other experiments, the frogs were sutured and allowed to recover after verification.

Since EthD only labels nuclei of membrane-damaged cells (Reddy et al. 2003), only the nuclei of successfully ablated PSCs are positively stained by EthD. In control preparations, the PSCs should not be damaged and the PSC nuclear space should not be
labeled with EthD. One week after PSC ablation process, as the ablated PSCs are phagocytosed, their nuclei are removed and the EthD stain is lost from ablated sites. As a result, in preparations dissected 1 week after the ablation process, Hoechst33342 nuclear stain (1:5000; Invitrogen-Molecular Probes, Carlsbad, CA) was used to stain nuclei of all cells. If a synaptic site continues to be devoid of living PSCs, the PSC nuclear space, delineated by PNA stain, should be Hoechst33342-negative.

**Immunohistochemistry**

Preparations were dissected in NFR solution in Sylgard-lined dishes, fixed with 4% paraformaldehyde for 30 minutes, then rinsed in a phosphate buffered solution (PBS, Huang et al. 1998) with 1% Triton X-100 (PBST) and incubated in blocking solution (BKS: 5% normal goat serum and 0.5% Triton X-100 in PBS) before incubation with antibodies. Presynaptic nerve terminals were identified by staining with mAb SV2 (Developmental Hybridoma Bank, Iowa City, Iowa) that recognizes an integral membrane protein in synaptic vesicles (Dong et al., 2006). It is often used to stain nerve terminals because of its robust staining signal (Panzer, 2005). However, while MAbSV2 stain is a marker of nerve terminal differentiation, anti-SV2 immunostaining alone is not sufficient to determine whether a nerve terminal has retracted or not. For example, loss of SV2 labeling might result from dispersion of synaptic vesicles rather than nerve terminal retraction. Therefore, an antibody directed against acetylated tubulin (A-tub; 1:200, Sigma-Aldrich, St. Louis MO) was used to stain neuronal microtubules within the nerve terminal. Combining SV2 and A-tub stain provides information as to the differentiation state of the nerve terminal as well as its presence at a synaptic site.
Following ablation and/or surgery, synaptic sites were visualized with peanut agglutinin
(PNA; Sigma-Aldrich, St. Louis, MO) that stains Schwann cell-associated basal lamina
(Ko 1987). Postsynaptic acetylcholine receptors (AChRs) were labeled with rhodamine-
conjugated $\alpha$-bungarotoxin ($\alpha$-BTX; Sigma-Aldrich, St. Louis, MO).

In some control experiments, muscles or target-deprived preparations were first
incubated with 2A12 supernatant or NFR solution prior to fixation with 4%
paraformaldehyde for 30 minutes. MAb 2A12 only stains living synaptic sites (Astrow
et al., 1998). Preparations were then rinsed and incubated with CY3-conjugated
secondary antibodies (Jackson Immuno Labs).

After staining, preparations were mounted on glass slides with Vectashield
mounting medium (Vector labs) and stored at -20°C until imaging.

**Imaging**

Images were captured by a Zeiss meta 510 confocal microscope, and processed with
the Zeiss LSM software and Adobe photoshop 7.0. As Hoechst33342 stain could not be
seen in the confocal microscope. So I confirmed the ablation in the fluorescent scope
when selecting a synaptic site for imaging. Imaging was performed at the UMASS
Central Microscopy Facility.

**Quantitation**

Preparation were dissected immediately or 7 days after ablation, processed for
immunohistochemistry, and viewed with an epifluorescence Zeiss Axioskop 20. PNA
stain delineated the boundaries of the synaptic site, widening at the location of PSC
nuclei (Ko, 1987). Synaptic sites were selected for quantitation that could be viewed in total. Each selected synaptic site was scored for: 1) presence of a muscle fiber; 2) presence of PSCs; 3) presence of a nerve terminal and 4) alignment of SV2 and PNA stains. The absence of muscle fiber striations in bright field microscope indicated that the synaptic site was target deprived. The ablation state of each synaptic site was scored by the presence or absence of intact PSC nuclei: PSC-ablated synaptic sites were either EthD positive or Hoechst negative. Only those synaptic sites where the majority of the PSC nuclei were EthD-positive were counted. The presence of a nerve terminal at a PNA-stained synaptic site was determined by anti-A-tub immunostaining and a differentiated nerve terminal was stained by mAb SV2. To assess nerve terminal stability, I compared the overlap between PNA and SV2/A-tub labeling. Absence of SV2/A-tub stain at a synaptic site indicated loss of the nerve terminal.
CHAPTER 3

RESULTS

SV2 as a marker of target-deprived nerve terminals

It has been previously observed that nerve terminals, labeled with FM1-43, a dye incorporated into recycled synaptic vesicles, were well retained for up to 2 months after muscle fiber removal (Dunaevsky and Connor, 1998). I first confirmed that SV2 is a reliable marker of nerve terminals at target-deprived synaptic sites. In my experiments, I compared SV2 staining of intact neuromuscular junctions to that of synaptic sites lacking muscle fibers. One to two months after muscle damage and X-irradiation, cutaneous pectoris muscles were dissected and triply stained with FITC-tagged PNA to mark synaptic sites, rhodamine-conjugated α-BTX for postsynaptic acetylcholine receptors (AChRs), and SV2 for presynaptic nerve terminals. As expected, at control synapses, PNA, SV2 and α-BTX staining were closely aligned (Figure 2, a-d). Of the 203 synaptic sites examined, none showed mismatch between SV2 and PNA stain; the PNA and SV2 stains were colocalized at each synaptic site.

Target-deprived synaptic sites lacked muscle fiber striations and the α-BTX staining was mostly gone, indicating the absence of target muscle fibers (Figure 2, e-h). About 5% of synaptic sites showed mismatch or complete absence of SV2 staining. These data suggest that SV2 staining is a good marker of target-deprived motor nerve terminals; SV2 stain occupied the space delineated by PNA stain as in controls. The nerve terminals were well maintained in the target-deprived synaptic sites, which is consistent with previous reports (Dunaevsky and Connor, 1998). The observed mismatch between
SV2 and PNA staining at the 5% of synaptic sites may be due to damage of axons during the microsurgery to remove muscle fibers (Dunaevsky and Connor, 1998).

Ablation of PSCs using complement-mediated cell lysis

For complement-mediated cell lysis, a cell-specific IgM antibody is required that marks the surface of the cells to be lysed; 2A12 is such a marker for perisynaptic Schwann cells (Reddy et al. 2003). I have confirmed that mAb 2A12 labels PSCs at the frog neuromuscular junction and can be used to successfully ablate them. Normal frog neuromuscular junctions have from 2-12 PSCs associated with the motor nerve terminal at a single synaptic site (Frietas and Connor, unpublished observations). To test the ability of mAb2A12 to stain PSCs, muscles were incubated with mAb 2A12 supernatant, ascites, or normal frog Ringer’s solution followed by CY3-conjugated secondary antibodies. The mAb 2A12 supernatant solutions (Figure 3) and mAb 2A12 ascites (data not shown) stained synaptic sites in a similar manner to PNA (Figure 3).

I next tested the effectiveness of the mAb 2A12 supernatant and ascites in ablating PSCs. Following complement-mediated cell lysis using mAb 2A12, synaptic sites were visualized with PNA stain and compromised nuclei were identified with EthD. The mAb 2A12 ascites was superior to the supernatant in ablating PSCs; more synaptic sites were positively stained with EthD homodimer following ablation with ascites when compared to ablation using supernatant. MAb 2A12 ascites successfully and selectively damaged PSCs in vivo. Nuclei of PSC were positively stained with EthD, indicating that the PSCs were compromised or dead (Figure 4). All subsequent ablations were performed using mAb2A12 ascites.
The stability of nerve terminals was first assessed at neuromuscular junctions where PSCs were ablated in the presence of muscle fibers. Immediately following PSC ablation, cutaneous pectoris muscles were dissected and the nerve terminals were marked with SV2 stain. Nerve terminals stained with SV2 had normal morphology at acute PSC-ablated neuromuscular junctions, compared with controls (Figure 5). PSC nuclei in control muscles, exposed to the same protocol with the exception that mAb 2A12 ascites was replaced with NFR, were not stained by EthD. There was no mismatch between SV2 and PNA at any control neuromuscular junctions while 5% of the acute PSC-ablated neuromuscular junctions showed partial or complete loss of SV2 stain (Table 1).

I next examined intact neuromuscular junctions one week after PSC ablation. One week after PSC ablation, muscles were dissected and stained with SV2, PNA, and the membrane-permeable nuclear dye, Hoechst33342. At ablated synaptic sites, no Hoechst-stained nuclei were associated with the widenings in the PNA stain. At control neuromuscular junctions, PSC nuclei were positively stained by Hoechst stain. At 1 week, the frequency of mismatch between SV2 and PNA stains at PSC-ablated synaptic sites was increased. Mismatch or total loss of SV2 staining was seen in about 18% of PSC-ablated synaptic sites (Table 2). As shown in Figure 6, there was a clear mismatch between SV2 and PNA staining at some synaptic sites. Synaptic sites devoid of SV2 stain frequently also were negative for A-tub stain, indicating the total retraction of the nerve terminal from the synaptic site. In contrast, only 3.5% of synaptic sites showed any mismatch in the control group (n=141 neuromuscular junctions, 2 muscles; Table 2).
Stability of motor nerve terminals deprived of both PSCs and target muscle

To determine the factors responsible for nerve terminal stability, I assayed the stability of target-deprived nerve terminals immediately or one week after PSC ablation. The choice of one week for analysis is directed by the observation that PSCs reoccupied synaptic sites in about eleven days (Reddy et al. 2003). One-two months after muscle fiber damage and X-irradiation, PSCs were ablated using 2A12 complement-mediated cell lysis. As in other experiments, the success of the ablation was confirmed by in vivo inspection of EthD staining of PSC nuclei immediately after ablation.

Nerve terminals were well maintained when PSCs were acutely removed from neuromuscular junctions without muscle fibers (Figure 7). In control PSC-ablated muscles, 95% of the target-deprived synaptic sites showed normal SV2 staining. In acute PSC-ablated muscles, only 10% of the synaptic sites without muscle fibers displayed mismatch between PNA and SV2 stain (Table 3).

One week after PSC ablation, PNA staining was weak or hardly visible at target-removed synaptic sites, making it difficult to identify synaptic sites. Of the synaptic sites identified one week after PSC ablation, 6% of the synaptic sites without muscle fibers had mismatch between SV2 and PNA staining (n=68 neuromuscular junctions, 3 muscles). Due to the reduced level of PNA stain, nearly all of the scored target-deprived and ablated synaptic sites were identified by the presence of SV2 stain; 64 of 68 synaptic sites only had SV2 stain without visible PNA stain (Table 4 and Figure 8). It is possible that nerve terminals that had retracted from ablated target-deprived synaptic sites were not scored due to the severe reduction in PNA stain at ablated sites. Therefore,
it is difficult to accurately assess the degree of nerve terminal stability in the absence of both PSC and target muscle fibers.
CHAPTER 4
DISCUSSION

To the best of our knowledge, this is the first analysis of nerve terminal structure in the absence of both glial and target cells. This Master’s thesis investigates motor nerve terminals deprived of either a target muscle fiber or PSCs or both. Using microsurgery followed by X-irradiation, muscle fibers were permanently killed. I have determined that SV2 staining of motor nerve terminals is normal 1-2 months after muscle fiber deprivation. I also found that nerve terminals are unaltered after acute PSCs ablation. About 18% of the synaptic sites displayed mismatch between SV2 and PNA stain after one week-PSC ablation. These results were consistent with previous reports (Dunaevsky and Connor, 1998; Reddy et al., 2003). Ninety percent of nerve terminals were well maintained when PSCs are acutely removed from neuromuscular junctions without muscle fibers. About 6% of the target-deprived nerve terminals showed SV2 and PNA stain mismatch after one week after PSC ablation.

Role of PSCs in the maintenance of the neuromuscular junction.

Although PSCs are closely associated with nerve terminals, it was not until the last two decades that they have emerged as active and essential players in the neuromuscular junction. Previous studies have demonstrated that PSCs play multiple roles in the formation, function and repair of neuromuscular junctions (Kang and others 2003; Feng et al., 2005; Feng and Ko, 2007). Using FM4-64 and synapsin-1 to label nerve terminals, Reddy et al. have shown that less than 1% of neuromuscular junctions retracted after acute PSC ablation while ~13% showed partial or total retraction after one week PSC-
ablation. Using SV2, which labels synaptic vesicles, I have found that 5% of neuromuscular junctions showed mismatch between PNA and SV2 stain after acute PSC ablation while ~18% from the one-week PSC ablated group. The variability between the results presented here and Reddy’s is likely due to sample size difference and the different immunostaining markers. When considered with the results of Reddy and colleagues, my results suggest that PSCs are dispensable for nerve terminal stability acutely but play an essential role in the long-term maintenance of neuromuscular junctions. The mechanism by which PSCs stabilize nerve terminals is still unclear. Since the remaining nerve terminals and synaptic size remained normal (Reddy et al., 2003), it appears unlikely that PSCs stabilize nerve terminals simply via a mechanical glue that binds them at synaptic sites. Recently, it has been shown that Schwann cell-conditioned medium (SC-CM) enhanced spontaneous transmitter release at developing neuromuscular junctions in *Xenopus* tissue culture (Cao and KO, 2007). It is possible that Schwann cell-derived functional factor(s) for the potentiation of spontaneous neurotransmission may also participate in the growth and maintenance of developing NMJs (Cao and KO, 2007). All these above-mentioned reports suggest that PSCs might be able to provide tropic factors to help stabilize nerve terminals. It is still necessary to identify these PSC-derived tropic molecules.

**Role of muscle fibers in the maintenance of neuromuscular junction**

In my study, the cutaneous pectoris muscles were dissected one to two months after muscle damage and X-irradiation. Mismatch between nerve terminal and synaptic sites stains was seen at 5.2% synaptic sites. Previous work had demonstrated that 85% of
nerve terminals were well maintained one to two months after target removal (Dunaevsky and Connor, 1998). My results are consistent with that report, further confirming that nonmuscle factors contribute to the short-term stabilization of presynaptic nerve terminals. However, these results could not exclude the possible roles of muscle fiber in the maintenance of nerve terminals. It is possible that muscle-derived stabilizing molecules can persist in the synaptic basal lamina. Therefore, although muscle fibers are removed, these stabilizing substances might be effective for a relatively long period of time (i.e. one or two months) but fail eventually when not replaced. Muscle fibers have been shown to play an important role in the long-term maintenance of synaptic sites by the evidence that about 90% of the nerve terminals showed at least one segment retraction after 6 to 9 months of target removal (Dunaevsky and Connor, 1998).

Removal of both muscle fiber and PSCs from neuromuscular junction

Acute PSC ablation one to two months after muscle fiber removal resulted in partial or complete loss of nerve terminals in 9.9% of the synaptic sites. So the nerve terminals remained stable in the absence of both PSCs and muscle fibers. There are two main possible reasons for this stability. One is that some of the muscle or PSC-derived stabilizing molecules are deposited and stay in the basal lamina which remains at these synaptic sites. The other might be that basal lamina itself can provide some functional signals to stabilize the nerve terminals. The major and most abundant components of basal lamina are laminins and collagens IV (Sanes, 2003). In a recent study, presynaptic defects were observed in the collagen IV chains (α3-6)-deficient knockout mice, but
only after synapses had matured (Fox et al., 2007), demonstrating that synaptic-specific collagen IV chains (α3-6) are necessary for maintenance, rather than for maturation of nerve terminals. This strongly supports that the synaptic basal lamina most probably provides signals to stabilize nerve terminals, especially when PSCs and target muscle fibers are removed. Interestingly, presynaptic spectrin has also been found essential for synapse stabilization at the Drosophila neuromuscular junction (Pielage et al., 2005). Whether presynaptic spectrin also plays a role in the maintenance of frog neuromuscular junction warrants further study.

Even one week after PSC-ablation, only 6% of the target-deprived neuromuscular junctions displayed mismatch between the PNA and SV2 stains. For some unknown reason, PNA staining was not stable and hardly visible at most of these synaptic sites. Therefore, it was difficult to identify the synaptic sites and nuclei of PSCs, making it hard to tell whether the PSCs were successfully ablated or not. However, there is some evidence to support that PSCs were ablated in the selected and counted synaptic sites. First, the ablation of PSCs was verified on day 0 and ~95% of the PSCs were PNA- and EthD-positive, indicating they were ablated. Second, one week after the ablation process, there was EthD stain left over in some of the nuclei of PSCs (see Figure 8), suggesting these PSCs were EthD positive staining on day 0 and thus ablated. Finally, one week after ablation, most target-deprived synaptic sites showed reduced or loss of PNA staining.

In practice, I located synaptic sites in the target-deprived, 1 week ablated preparations by the presence of SV2 stain. It is possible that synaptic sites where nerve terminals had retracted accompanied by loss of PNA staining were not detected and thus
not counted. That might be the reason why I didn’t find higher amount of nerve terminal retraction at the synaptic sites deprived of both PSCs and muscle fibers than those deprived of only one of the synaptic members, either PSC or muscle fiber. Since PSCs were ablated, mAb 2A12 is not a useful marker of ablated synaptic sites. Loss of PNA might be accelerated at target-deprived and PSC-ablated synaptic sites because PNA was evident with ablation in the presence of muscle fibers. More accurate data would be obtained if there is another more stable synaptic site maker than PNA.
Figure 1. Frog neuromuscular junction.  A. A motor neuron extends a myelinated axon to contact target skeletal muscle fibers at neuromuscular junctions.  B. In a longitudinal view of a frog neuromuscular junction, release domains, characterized by clusters of synaptic vesicles, are separated by nonrelease domains. Schwann cell cytoplasmic processes are occasionally found in association with nonrelease domains.

(A: [http://labs.anisci.uiuc.edu/meatscience/Library/Copy](http://labs.anisci.uiuc.edu/meatscience/Library/Copy); B: Dunaevsky and Connor, 2000.)
Figure 2. SV2 marks frog motor nerve terminals at target-deprived synaptic sites. Triple labeling of a control (a-d) and one month target-deprived (e-h) synaptic sites stained with PNA (a, e) to mark the boundaries of synaptic sites, α-BTX (b, f) for AChRs, and SV2 (c, g) for nerve terminals. At the control neuromuscular junction, PNA, SV2 and α-BTX staining were colocalized. At the target-deprived synaptic site, α-BTX staining was mostly absent, indicating successful removal of the muscle fiber, while SV2 stain was contained within the boundaries of the PNA-marked synaptic site. Bars, 20µm.
Figure 3. MAb 2A12 selectively stains PSCs at frog neuromuscular junctions. Cutaneous pectoris muscles in whole-mount were double-stained with PNA (a, d) and mAb2A12 supernatant (b, e). MAb2A12 staining colocalized with PNA staining (a-c). In a control muscle treated with NFR and secondary antibody, mAb2A12 staining was not detected (d-f). Widening of mAb2A12 and PNA stains (arrow) marks the location of a PSC nucleus. Bars, 20µm.
Figure 4. Complement-mediated cell lysis with mAb 2A12 ascites results in successful elimination of PSCs. A neuromuscular junction from an adult frog CP was dissected and immunostained 3h after application of mAb 2A12 and complement. FITC-PNA (green) reveals the boundaries of the synaptic site and ethidium homodimer (EthD) labels the nuclei of dying perisynaptic Schwann cells (red). The nuclei of PSCs (arrows) at this synaptic site were localized at widenings in the PNA stain and were EthD positive, indicating the PSCs were dead. Bars, 20µm.
Figure 5. Nerve terminals were well maintained immediately following PSC ablation. A control neuromuscular junction (a-c) and a neuromuscular junction immediately after PSC ablation (d-f) were stained with SV2 (green) for nerve terminals, PNA (red) for PSCs and EthD (red) for dead PSC nuclei. At the control neuromuscular junction, PSC nuclei were EthD negative (arrows), indicating living PSCs. At the PSC-ablated neuromuscular junction, PSC nuclei were EthD positive, indicating the PSCs were ablated (arrow). Nerve terminals stained with SV2 appeared normal in length and morphology at PSC-ablated neuromuscular junctions when compared to controls. Bars, 20μm.
Figure 6. A nerve terminal retracted one week after PSC ablation. Triple labeling of a control neuromuscular junction (a-d) and a neuromuscular junction one week after PSC ablation (e-h) with SV2 (green) for nerve terminals, PNA (red) for PSCs and A-tub (blue) for microtubules. At the control neuromuscular junction, PSC nuclei were Hoechst positive. At the PSC-ablated synapse, Hoechst staining was negative. SV2 staining was normal in the control synaptic site. At the PSC-ablated site, total loss of SV2 and A-tub staining demonstrated the complete disappearance of a branch of the nerve terminal (arrows in e, f) while a neighboring site was maintained. Bars, 20µm.
Figure 7. Nerve terminals were well maintained in the target-deprived synaptic sites immediately after PSCs were ablated. Labeling of a muscle fiber-removed synaptic site after acute-PSC ablation with SV2 (green) for nerve terminals, PNA (red) for PSCs, EthD (red) for dead PSC nuclei and A-tub (blue) for microtubules. PSC nuclei were EthD positive, indicating the PSCs were ablated. The SV2 stain at this target-deprived and PSC ablated synaptic site appeared normal. Bars, 20μm.
Figure 8. Nerve terminals were maintained at a target-deprived synaptic site one week after PSCs were ablated. Labeling of a target-deprived synaptic site one week after PSC ablation with SV2 (green) for nerve terminals, PNA (red) for PSCs, and A-tub (blue) for microtubules. PNA staining was nearly invisible; SV2 and A-tub staining appeared normal. Upper layer: original images; lower layer: images with enhanced contrast. Bars, 20µm.
Table 1. Immediately following PSC ablation, nerve terminals appear normal at synaptic sites in the presence of muscle fibers.

<table>
<thead>
<tr>
<th>Preparation</th>
<th># Muscles</th>
<th># Synaptic sites</th>
<th># Synaptic sites with SV2 and PNA stain mismatch</th>
<th>% Synaptic sites with SV2 and PNA stain mismatch</th>
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<tbody>
<tr>
<td>Ablation control</td>
<td>2</td>
<td>203</td>
<td>0</td>
<td>0%</td>
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<tr>
<td>Acute PSC-ablated</td>
<td>4</td>
<td>121</td>
<td>6</td>
<td>5%</td>
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Table 2. Alteration in the morphology of nerve terminals at control and one week PSC-ablated synaptic sites.

<table>
<thead>
<tr>
<th>Preparation</th>
<th># Muscles</th>
<th># Synaptic sites</th>
<th># Synaptic sites with SV2 and PNA stain mismatch</th>
<th>% Synaptic sites with SV2 and PNA stain mismatch</th>
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</thead>
<tbody>
<tr>
<td>Ablation control</td>
<td>2</td>
<td>141</td>
<td>5</td>
<td>3.5%</td>
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<tr>
<td>One week PSC-ablated</td>
<td>10</td>
<td>285</td>
<td>52</td>
<td>18.2%</td>
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Table 3. Morphology of nerve terminals at target-deprived synaptic sites after control or acute PSC ablation

<table>
<thead>
<tr>
<th>Preparation</th>
<th># Muscles</th>
<th># Synaptic sites</th>
<th># Synaptic sites with SV2 and PNA stain mismatch</th>
<th>% Synaptic sites with SV2 and PNA stain mismatch</th>
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<td>96</td>
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<tr>
<td>Target-deprived Acute PSC ablation</td>
<td>8</td>
<td>141</td>
<td>14</td>
<td>9.9%</td>
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Table 4. Some nerve terminals were maintained at target-deprived synaptic sites one week after PSCs were ablated.

<table>
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<th>Preparation</th>
<th># Muscles</th>
<th># Synaptic sites</th>
<th># Synaptic sites with SV2 and PNA stain mismatch</th>
<th>% Synaptic sites with SV2 and PNA stain mismatch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target-deprived One week PSC-ablated</td>
<td>3</td>
<td>68</td>
<td>4</td>
<td>5.88%</td>
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BIBLIOGRAPHY


Dale Purves (Editor), George J. Augustine (Editor), David Fitzpatrick (Editor), William C. Hall (Editor), Anthony-Samuel Lamantia (Editor), James O. McNamara (Editor), S. Mark Williams (Editor) (2005). Neurosciences, 3, 377.


