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Glial imaging during synapse remodeling at the neuromuscular junction

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Glia are an indispensable structural and functional component of the synapse. They modulate synaptic transmission and also play important roles in synapse formation and maintenance. The vertebrate neuromuscular junction (NMJ) is a classic model synapse. Due to its large size, simplicity and accessibility, the NMJ has contributed greatly to our understanding of synapse development and organization. In the past decade, the NMJ has also emerged as an effective model for studying glia–synapse interactions, in part due to the development of various labeling techniques that permit NMJs and associated Schwann cells (the glia at NMJs) to be visualized in vitro *and* in vivo. *These approaches have demonstrated that Schwann cells are actively involved in synapse remodeling both during early development and in post-injury reinnervation.* In vivo *imaging has also recently been combined with serial section transmission electron microscopic (ssTEM) reconstruction to directly examine the ultrastructural organization of remodeling NMJs. In this review, we focus on the anatomical studies of Schwann cell dynamics and their roles in formation, maturation and remodeling of vertebrate NMJs using the highest temporal and spatial resolution methods currently available.*

Keywords: Schwann cell, synapse elimination, reinnervation, *in vivo* imaging, ssTEM reconstruction

INTRODUCTION

The nervous system is made up of two classes of cells: neurons and glia. Neurons communicate with each other through specialized gaps called synapses. Neurotransmitters are released from presynaptic axon terminals and act upon postsynaptic receptors to elicit conductance changes in the postsynaptic membrane. Much of our higher brain function, such as learning, memory and consciousness, is based upon the interconnectivity of neurons through the $\sim 10^{14}$ excitatory and inhibitory synapses in the cerebral cortex (Pakkenberg *et al.*, 2003). However, understanding the cellular basis of higher cortical functions becomes difficult considering these synapses not only vary in structure, location and excitability, but are also highly intermingled. Further exacerbating this difficulty is the fact that the connectivity and efficacy of an individual synapse can be modified, particularly during learning and memory formation.

Given the complexity of the neuronal interconnections, a recent strategy to better understand synaptic dynamics has been to literally watch a single synapse over time in living animals. Transgenic mice with fluorescent proteins in the cytoplasm of neuronal subsets (Feng *et al.*, 2000) have made such studies not only possible, but also fairly routine. The recent widespread use of these mice in time lapse imaging studies has revealed a seeming paradox: dendritic spines and axon boutons in the superficial cortex of living mice are both stable and dynamic (Grutzendler *et al.*, 2002; Trachtenberg *et al.*, 2002; Portera-Cailliau *et al.*, 2005). Despite the debate over animal lines, quantification criteria and imaging techniques (Pan and Gan, 2008), the general consensus is that dynamics vary among synapses. While the majority of synapses appear to be stable over long periods of time, some hotspots of dynamism remain (Holtmaat *et al.*, 2005; Zuo *et al.*, 2005). Considering glia are closely associated with synapses and actively participate in synaptic transmission and remodeling, understanding synaptic dynamism in the cortex will be difficult without fully understanding the role of the glial component of the synapse.

As with the understanding of pre and postsynaptic components of the synapse, the role of glia during synaptic modifications *in vivo* is just now beginning to be understood at a peripheral synapse, the neuromuscular junction (NMJ). Despite many attempts to examine glial dynamics in the living brain (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005), there are several advantages for using the NMJ as a model system to study glial and synaptic interactions *in vivo*. In particular, (1) NMJs are approximately 1000 times larger than synapses in the brain; (2) neuronal and glial components can be monitored simultaneously at the same NMJ; (3) NMJs are easily accessible for imaging and electrophysiological examination; (4) the structural organization and molecular components are well characterized in the vertebrate NMJ; (5) the NMJ is amenable for both molecular and ultrastructural studies that can ultimately reveal the underlying cellular and molecular mechanisms of observed structural changes. Accordingly, this review will focus on the role of Schwann cells in anatomical organization of NMJs, with particular emphasis on mammalian and amphibian *in vivo* studies. This is to distinguish it from many other general reviews that focus on either glial functions in general (Barres and Barde, 2000; Haydon, 2001; Ransom *et al.*, 2003; Allen and Barres, 2005; Volterra and Meldolesi, 2005; Halassa *et al.*, 2007), or other aspects of Schwann cells (Scherer, 1997; Feng *et al.*, 2005; Rousse and Robitaille, 2006).

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Structural organization of NMJs

The vertebrate NMJ is a typical 'tripartite' synapse, which is made up of a presynaptic motor axon terminal, a postsynaptic muscle fiber and several terminal Schwann cells (TSCs). TSCs at NMJs are the counterparts to astrocytes in the central nervous system (CNS). Despite the same embryologic origin, TSCs are morphologically and functionally distinct from the myelinating Schwann cells (MSCs) that wrap the motor axons of peripheral nerves. MSCs are comparable to oligodendrocytes in the CNS, but typically associate with only one axon in motor nerves. In adulthood, several TSCs partition and tile one NMJ with little overlap (Livet *et al.*, 2007) (Zuo and Misgeld, unpublished data). Their total number is actively regulated and correlates with the size of the NMJ (Lubischer and Bebinger, 1999). As shown by both electron and light microscopic studies, adult TSCs cap axon terminals, but largely avoid the synaptic cleft (see below) (Birks *et al.*, 1960a; Heuser *et al.*, 1976; Desaki and Uehara, 1981). A basal lamina surrounds the muscle fiber and becomes continuous with the TSC basal lamina. The portion of the basal lamina that is directly opposed to the nerve terminals is referred to as the synaptic basal lamina. A schematic drawing of a mammalian NMJ is illustrated in Fig. 1, showing the anatomical arrangement between TSCs, MSCs, presynaptic axon terminals, postsynaptic muscle cell and the synaptic basal lamina. TSCs also closely monitor synaptic activity and influence transmitter release by the nerve terminal. Work by Robitaille *et al.* (1997) demonstrates that Schwann cells possess muscarinic ACh receptors, and respond to neurotransmission with elevated cytosolic Ca^{2+} concentration (Jahromi *et al.*, 1992; Reist and Smith, 1992). This activation leads to a profound depression in transmitter release through a G-protein-coupled receptor pathway (Robitaille, 1998; Castonguay and Robitaille, 2001).

Schwann cells at mature NMJs

The structural stability of the vertebrate NMJ has been studied for many years by visualizing presynaptic axon terminals of motor neurons with vital dyes or genetically coded fluorescence, and postsynaptic acetylcholine receptors (AChRs) on muscle fibers with fluorescent α-bungarotoxin. While adult mammalian NMJs exhibit minimal changes over months to years (Balice-Gordon and Lichtman, 1990), adult amphibian NMJs show some retraction and extension of axon terminal sprouts (Chen *et al.*, 1991). This difference in NMJ dynamics may simply be related to the subtle evolutionary diversity between these two vertebrates, but another more intriguing possibility is suggested by a slight ultrastructural difference in Schwann cell and synapse apposition between mammalian and amphibian NMJs. Mammalian TSCs are completely excluded from the synaptic cleft (Fig. 2A) whereas in amphibians, perisynaptic Schwann cells (PSCs, the amphibian analog of TSCs), interdigitate small processes between the axon terminal and the synaptic basal lamina (Fig. 2B; Heuser and Reese, 1973).

So are Schwann cells processes in the synaptic cleft responsible for dynamism in amphibian NMJs? Although we currently lack temporal and spatial resolution to unequivocally answer this question, there is strong evidence that Schwann cell processes guide axon terminal sprouting in normal adult amphibian NMJs. Using fluorescent peanut agglutinin (PNA), which binds to the extracellular matrix around glial cells (Ko, 1987), Ko and colleagues repeatedly imaged frog

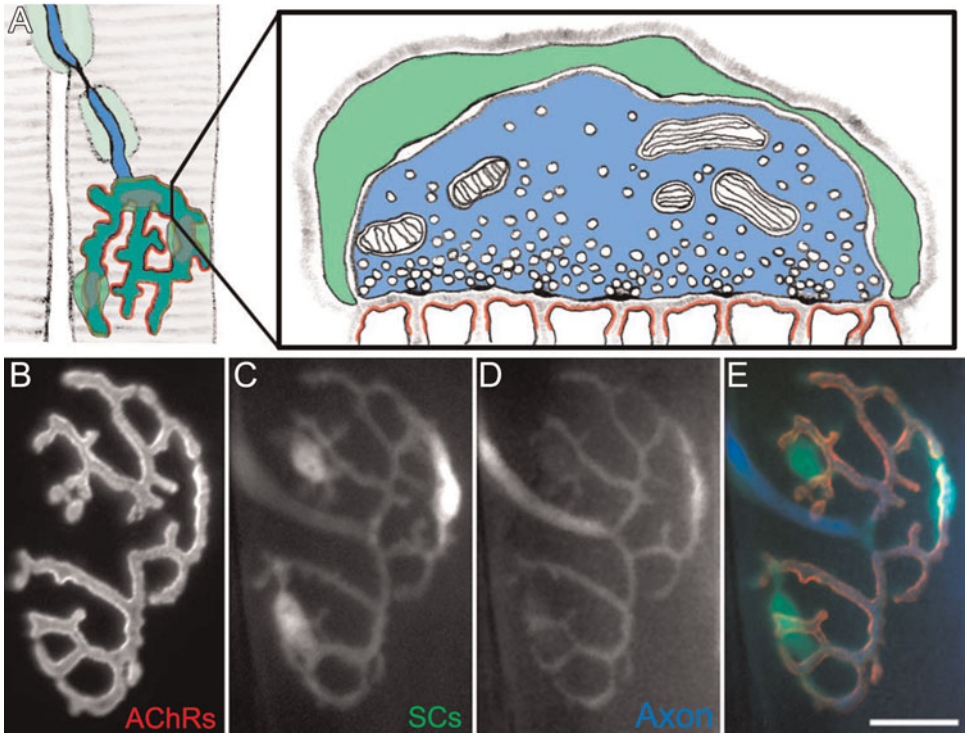

Fig. 1. Structure of mouse NMJ. (A) Drawing illustrates the relationships between the axon (blue), Schwann cells (green) and AChRs (red). An illustration of an electron micrograph shows the ultrastructural arrangement of TSC processes (green), axon terminal (blue), AChRs (red) and basal lamina (gray). (B) AChRs, (C) Schwann cells, (D) axon terminals and a (E) composite image from *in vivo* epifluorescent image. Scale bar: 10 μm. Panels B–E, original data.

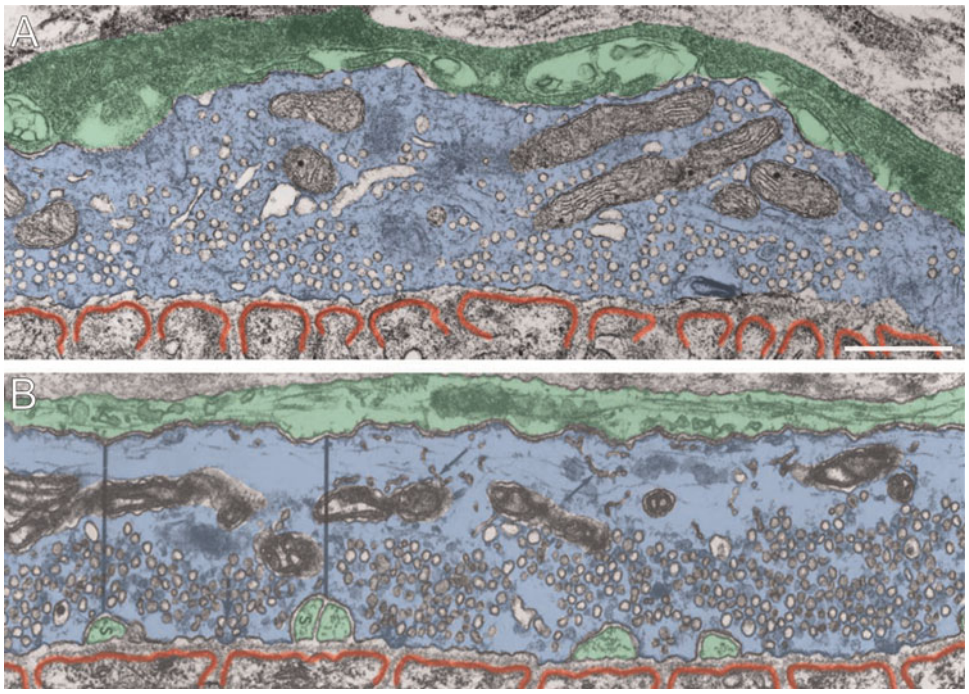

Fig. 2. Comparison of (A) mammalian and (B) amphibian NMJ ultrastructure. Schwann cells (green), axon terminals (blue) and AChRs (red) are arranged similarly between mammalian and amphibian NMJs, with the exception of small TSC processes in the synaptic cleft in the amphibian junction. Scale bar, 500 nm. Panel A, original data. Panel B, adapted from Heuser and Reese (1973) with permission.

NMJs *in vivo* and showed that synaptic extracellular matrix extended distally and preceded axon terminal sprouts (Chen and Ko, 1994). An elegant follow-up experiment using correlated semi-serial section transmission electron microscopy (ssTEM) of identified sprouting NMJs further showed that PSCs covered in extracellular matrix sprouted into extrasynaptic territory, potentially leading nerve terminals into new synaptic territory (Ko and Chen, 1996).

If extension of Schwann cell processes induces axonal sprouting in mature synapses, then do stable Schwann cells confer this stability to axon terminals? To address this question, many studies have attempted to perturb Schwann cell stability and assess the integrity of neuromuscular synapses. In frog, an antibody to PSCs followed by complement-mediated cell lysis was used to ablate Schwann cells from NMJs. In this way PSCs could be removed without damaging the underlying nerve terminals or muscle fibers. One week after PSC ablation, presynaptic function decreased by approximately one-half, while postsynaptic function was unchanged. In addition, retraction of nerve terminals increased over ten-fold at PSC-ablated NMJs (Reddy *et al.*, 2003). A similar dependence of axon terminal stability from MSCs has been observed in various dysmyelinating axon pathologies (Scherer, 1999; Martini, 2001; Yin *et al.*, 2004). The analogous experiment to PSC ablation at the mammalian NMJ requires injection of a subset of anti-disialosyl antibodies to selectively remove TSCs. Acute TSC injury or ablation shows no major deleterious influence on synapse function over the short term (hours) (Halstead *et al.*, 2005). However, whether prolonged TSC loss interferes with NMJ function and structural integrity remains unclear.

While loss of Schwann cells might lead to decreased synaptic function, over-active Schwann cells (i.e., intrusion of TSC processes into synaptic clefts) disrupt mammalian NMJs and cause synapse remodeling. During development, TSC processes are prevented from entering the synaptic cleft by synaptic laminin, which is a heterotrimeric glycoprotein in the synaptic basal lamina and concentrated at the synaptic cleft of mouse NMJs. Mice lacking synaptic laminin subunit β2 exhibit developmental defects in NMJ formation. In these animals, Schwann cells invade into synaptic clefts and separate the adhesion between the synaptic basal lamina and nerve terminals. As a result, the efficacy of neuromuscular transmission is significantly reduced and the synaptic connection is disrupted (Noakes *et al.*, 1995; Patton *et al.*, 1998). In another example, Hayworth *et al.* (2006) used a Tet-On system to induce expression of constitutively active ErbB2 receptors (caErbB2Rs) in adult Schwann cells, and found that TSC sprouting preceded nerve terminal sprouting, resulting in disruption of original synaptic sites. In summary, TSC dynamics must be tightly regulated to maintain normal function of neuromuscular synapses.

TSCs extend processes and guide motor nerves during reinnervation

Despite the overall stability at normal adult NMJs, Schwann cells become 'reactive' following axon injury and participate in the removal of nerve terminal debris (Miledi and Slater, 1968). During this process, Schwann cells invade between the synaptic basal lamina and the degenerating nerve terminal (Miledi and Slater, 1970; Jirmanová, 1975). As Schwann cells begin to occupy the position of degenerating nerve terminals at synaptic sites, they also synthesize and release quanta of acetylcholine after denervation (Birks *et al.*, 1960b; Miledi and Slater, 1968; Bevan *et al.*, 1973). Such release is more common at frog terminals than in mammals. Despite the temptation to attribute some maintenance of the nerve-vacated synaptic site to this transmitter release, we

still do not understand what functional significance, if any, it has.

Besides their phagocytic role during axon degeneration and withdrawal, Schwann cells also play active roles in reestablishing synaptic connections during reinnervation. Cell culture studies have shown that Schwann cells provide preferred surfaces for axonal extension (Fallon, 1985; Bixby *et al.*, 1988). In mice, regenerating axons grow along old Schwann cell tubes, following them back to the original synaptic site (Nguyen *et al.*, 2002). Axons also form 'escaped fibers', following Schwann cell processes extending away from the junction (Son and Thompson, 1995; Ko and Chen, 1996; O'Malley *et al.*, 1999; Macleod *et al.*, 2001). These TSC processes are often longer than the escaped fibers that appear to be growing along them, suggesting they guide the axon rather than follow. New synapses are formed along the new pathway of TSC processes and terminal extension (Kang *et al.*, 2003). In partially denervated muscles, TSCs extend their processes from denervated synaptic sites to nearby intact synapses forming cellular bridges. These Schwann cell bridges guide nerve sprouts from intact junctions to reach denervated synaptic sites (Son and Thompson, 1995; Love *et al.*, 2003). Thus, Schwann cells determine structural changes at NMJs including how motor neurons distribute their terminal processes and how synaptic connections are reestablished during reinnervation.

TSCs participate in early synaptic formation and remodeling

In contrast to the stable adult NMJ, *in vivo* imaging of the developing neuromuscular synapse shows high dynamics during both initial synapse formation and subsequent competitive rearrangements. In embryonic development, Schwann cell precursor cells follow motor axons through the periphery to reach muscles. During this journey, axons provide both migratory guidance and mitogenic stimulation for Schwann cells (Jessen and Mirsky, 2005). Despite co-migration of axons and Schwann cells, Schwann cells are not required for the initial formation of NMJs. For example, in mutant mice lacking Schwann cells, axons still navigate to their targets and form initial synaptic contacts (Morris *et al.*, 1999; Woldeyesus *et al.*, 1999; Lin *et al.*, 2000; Wolpowitz *et al.*, 2000). At these newly formed synapses, Schwann cells continue to depend on motor neurons to survive. Neonatal denervation leads to the rapid, apoptotic death of Schwann cells at rat NMJs, and injection of glial growth factor prevents this apoptosis *in vivo* (Trachtenberg and Thompson, 1996). A recent study has further shown that induction of caErbB2Rs expression selectively in neonatal Schwann cells is sufficient to rescue them from denervation-induced apoptosis (Hayworth *et al.*, 2006).

In early postnatal development, initial synapse formation is followed by selective synapse elimination. At the mouse NMJ, multiple axons converge upon a single synaptic site at birth. During the first two postnatal weeks, all but one axon withdraw and the remaining axon occupies the entire junction. This reduction in innervation is not due to naturally occurring cell death that occurs prenatally, but rather due to a postnatal competition between axons to be the sole innervation source to the muscle fiber. Classic histological stains along with more modern fluorescent time lapse imaging reveal that withdrawing axons become atrophic with a large distal bulb (O'Brien *et al.*, 1978; Riley, 1981; Keller-Peck *et al.*, 2001). Small orphaned segments termed axosomes often appear distally to the bulb (Bishop *et al.*, 2004). Both S100 antibody staining (Bishop *et al.*, 2004) and S100-GFP transgenic mice (Song *et al.*, 2008) demonstrate that axosomes are fully embedded within Schwann cells rather than other phagocytic cells like macrophages. Furthermore, ssTEM has revealed these orphaned axosomes are not full of degenerated cellular debris, but rather contain normal organelles (Fig. 3).

This simple observation raised the possibility that perhaps Schwann cells actively participate in synapse elimination by selectively dismantling seemingly healthy axons rather than

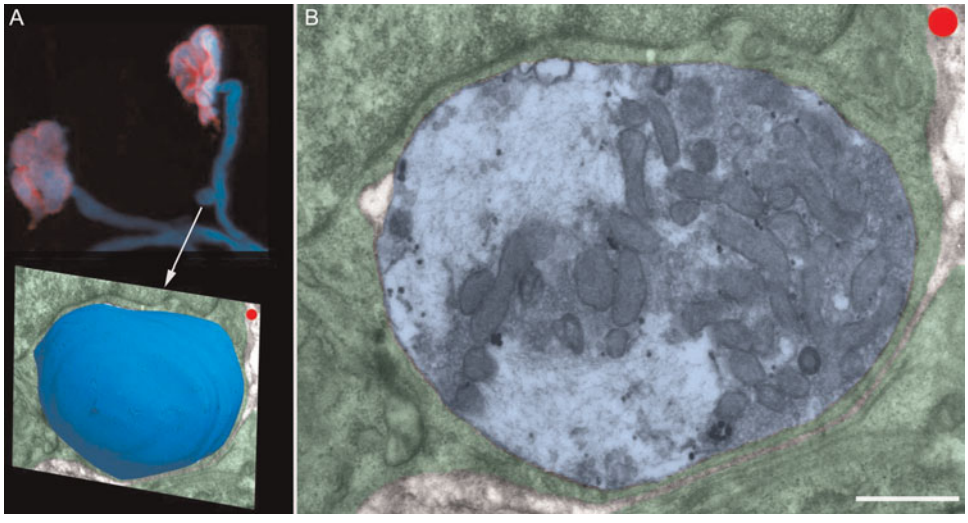

Fig. 3. Correlated confocal and ssTEM of developing NMJs. (A) A confocal projection of P9 NMJs showing axons (blue) and AChRs (red). A small axosome has formed near the preterminal axon of the right NMJ. A surface reconstruction from 52 serial transmission electron micrographs appears below. A single electron micrograph has been inserted into the surface reconstruction at a place corresponding to its three-dimensional location. A red dot has been placed in the upper right corner of the electron micrograph for orientation to panel B. (B) An electron micrograph through the center of the axosome (blue) from panel A shows that it is entirely engulfed by a cell that is surrounded by basal lamina that extends to the NMJ we presume to be a Schwann cell (green). The axosome contains normal appearing vesicles, mitochondria and neurofilaments. Scale bar, 1 μm. Original data.

scavenging shed axonal debris. At this time we cannot confirm or reject this idea, but some insight might be drawn from a recent study aimed at determining the lysosomal contribution to axon removal in both glia and neurons. Song *et al.* (2008) used an *ex vivo* imaging preparation of mouse triangularis sterni muscle (Kerschensteiner *et al.*, 2008) to show that degradative events appear to begin within retracting axons and then are completed through axosome formation and degradation within the surrounding Schwann cells.

Future directions – combining *in vivo* and ultrastructural examination

Despite the accumulating knowledge on the dynamics of synapse remodeling at the NMJ, many questions remain regarding Schwann cells' role in synaptic plasticity. For example, what happens to Schwann cells associated with the losing axon during synapse elimination? How do multiple TSCs partition and maintain the stable adult NMJ? What happens to Schwann cells during synapse loss during aging and pathology? Answers to these questions rely on our ability to selectively label synaptic and glial components *in vivo*, and follow their dynamics with high spatial and temporal resolution.

Many approaches have been used to visualize Schwann cells *in vivo*. Fluorescent lectins (such as PNA) that bind to components of glial extracellular matrix have been used successfully to label Schwann cells at frog NMJs (Astrow *et al.*, 1998). In mice, one of the early attempts to label Schwann cells utilized a blue fluorescent dye – calcein blue. When linked to an acetoxymethyl (AM) ester, calcein blue provides selective labeling of Schwann cells at NMJs *in vivo* (O'Malley *et al.*, 1999). However, these dye labeling methods have several drawbacks, such as unreliable cell labeling and the need for repeated dye application. To overcome these problems, we have generated transgenic mice that express fluorescent proteins under the control of a glial-specific promoter. The protein S100B is a commonly used marker for glial cells (Ludwin *et al.*, 1976; Stefansson *et al.*, 1982) and elements of its promoter have been characterized and shown to drive expression in stably transfected glial cells *in vitro* (Allore *et al.*, 1990; Jiang *et al.*, 1993; Castets *et al.*, 1997). One line of these S100-green fluorescent protein (GFP) mice exhibits bright and selective GFP expression in all Schwann cells in the neuromuscular system and has been used for *in vivo* imaging (Zuo *et al.*, 2004). This line has been further crossed with transgenic mice expressing cyan fluorescent protein (CFP) under *Thy-1* promoter in all motor neurons (Feng *et al.*, 2000). By labeling AChRs with a non-blocking dose of rhodamine-conjugated α-bungarotoxin in these mice, we are able to observe all three components (presynaptic terminals, postsynaptic receptors and Schwann cells) of the NMJ in living mice. Following changes in TSCs at individual junctions over time (days to months), our previous study has shown that TSCs, like the nerve terminals they cover, are mostly stable in adult mice (Zuo *et al.*, 2004) (Fig. 4). Furthermore, we have found enhanced TSC dynamics (movement, addition or loss) at aging NMJs, parallel with the postsynaptic AChR remodeling and axon branch re-organization. These studies represent one of the first studies tracing the dynamic nature of all three structural components of individual synapses in living mammals.

In vivo imaging has provided valuable temporal information about the dynamics and sequence of morphological

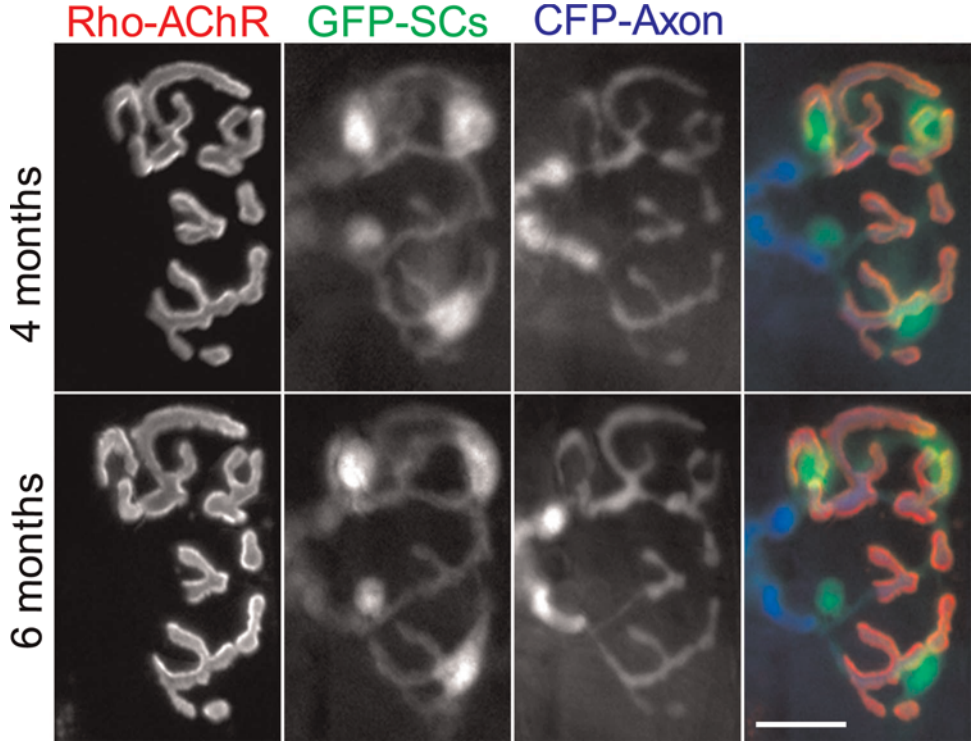

Fig. 4. ***In vivo*** **images of the same NMJ obtained two months apart in the sternomastoid muscle from a living mouse expressing CFP in neurons and GFP in Schwann cells.** AChRs were labeled with a non-blocking concentration of rhodamine-conjugated α-bungarotoxin. Merged images show AChR in red, Schwann cells in green and axons in blue. There are no changes in AChRs, TSCs or axon pattern, suggesting the stability of the adult mammalian MMJ. Scale bar: 10 μm. Original data.

changes in synapses and associated glia. However, it is difficult to infer detailed cellular mechanisms from *in vivo* imaging because the spatial resolution of light microscopy is currently insufficient to elucidate the fine structural interactions within the synapse. The alternative is to use TEM to resolve subcellular organelles, internal structural components and intercellular interactions that light microscopy cannot. Traditional TEM relies upon slicing extremely thin sections (~60 nm) through small areas of tissue. The odds of randomized slices through large tissue samples capturing an event of interest are exceedingly low.

Since the electron microscope does not resolve color in the traditional sense, differentiating cells among a larger homogeneous population requires rendering one cell more electron dense than the others surrounding it. Traditionally, this approach requires using antibodies to a specific cell marker (i.e., anti-GFP to a GFP-labeled cell) followed by a secondary antibody conjugated to horseradish peroxidase (Herrmann *et al.*, 1994; Dunaevsky *et al.*, 2001; Trachtenberg *et al.*, 2002). An alternate approach is to label a cell with a photooxidizing fluorophore and bleach the fluorophore in the presence of diaminobenzidine (Maranto, 1982; Calabrese and Maranto, 1986; Lubke, 1993; Herrmann *et al.*, 1994). Both approaches are useful for relocating axons, but rely on increased electron density within an axon as the identifiable marker. The drawback is that the increased electron density often masks subcellular features and, in the case of antibodies, can require permeabilizing tissue which degrades ultrastructure.

One way we have overcome this problem is to create electron dense fiducial markers (Gan *et al.*, 1999) near the previously imaged NMJ and re-examine the spatial relationship of different synaptic components through ssTEM reconstruction. This approach ultimately permits highly resolved electron micrographs to be interpreted in both a temporal and macroscopic context. Such an approach has already revealed cellular mechanisms for axon pruning at the developing NMJ (Bishop *et al.*, 2004) and axon terminal sprouting (Ko and Chen, 1996).

As time lapse *in vivo* microscopy continues to reveal morphological changes in synapses and glia, finding ultrastructural correlates will become critical for determining the subcellular milieu underpinning these dynamics. Methods that make these correlations more routine at the NMJ will likely translate to CNS synapses.

CONCLUSIONS

Ramón y Cajal posited over a century ago: 'What is the function of glia cells in neural centers? The answer is still not known, and the problem is even more serious because it may remain unsolved for many years to come until physiologists find direct methods to attack it.' Electrically unexcitable, scientists lacked the approach to examine glial cells for almost a century. Equipped with the modern pharmacological and genetic tools, as well as new optical approaches, time may finally come for an understanding of glial function.

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Statement of interest

None.

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