■ REVIEW

Perisynaptic Schwann Cells at the Neuromuscular Junction: Nerve- and Activity-Dependent Contributions to Synaptic Efficacy, Plasticity, and Reinnervation

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Glial cells are increasingly recognized for their important contributions to CNS and PNS synaptic function. Perisynaptic Schwann cells, which are glial cells at the neuromuscular junction, have proven to be an exceptionally useful model for studying these roles. Recent studies have shown that they detect and reciprocally modulate synaptic efficacy in an activity-dependent manner in the short term. In addition, perisynaptic Schwann cells guide reinnervating nerve sprouts after deinnervation, and many important parameters of this are dependent on synapse activity. Thus, it is hypothesized that perisynaptic Schwann cells are key integrators in a continuum of synaptic efficacy, stability, and plasticity at the neuromuscular junction, which is important for maintaining and restoring synaptic efficacy. NEUROSCIENTIST 9(2):144–157, 2003. DOI: 10.1177/1073858403252229

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The nervous system is mostly composed of two cell types, neurons and glia, that together determine its function. Traditionally, glial cell research has focused on their important roles in maintaining a proper environment for neuronal function (Kettenmann and Ransom 1995). Complementing these findings, recent experiments have demonstrated that certain glia play more dynamic roles at synapses (Araque and others 2001). Some of these data have led to the hypothesis that the synapse is a tripartite unit consisting of glial cells that actively modulate communication between presynaptic and postsynaptic elements (Araque and others 1999). Moreover, nontraditional, executive roles for glia are being recognized in relation to recovery after nerve injury (Balice-Gordon 1996; Son and others 1996).

We will discuss evidence for the existence of a synapse-glia-synapse regulatory loop that helps to maintain and restore synaptic efficacy at the neuromuscular junction (NMJ). We will address two general themes: The first presents glia as active partners in acute synaptic transmission, and the second concerns their contribution to reinnervation and synapse recovery after injury. Based on the evidence presented, we propose that glial cells play a prominent role in a continuum of synaptic efficacy, stability, and plasticity at the NMJ, which is important for maintaining and restoring synaptic efficacy.

Perisynaptic Schwann Cells Acutely Interact with Neurotransmission at the NMJ

Perisynaptic Schwann cells (PSCs; also known as terminal Schwann cells) are glial cells found at the NMJ that express proteins in common with other glial cells, such as S-100 (Son and Thompson 1995b; Rochon and others 2001). As with glial cells in the CNS, PSCs were traditionally believed to play passive roles. By contrast, it has become apparent that the roles of PSCs at the NMJ are more dynamic than originally appreciated (Table 1).

At mammalian and amphibian NMJs, there are typically 3 to 5 PSCs. Using various markers, it can be shown that PSC processes run in close opposition atop the presynaptic nerve terminal (Fig. 1). In amphibians, PSC processes envelop the nerve terminal and occasion-

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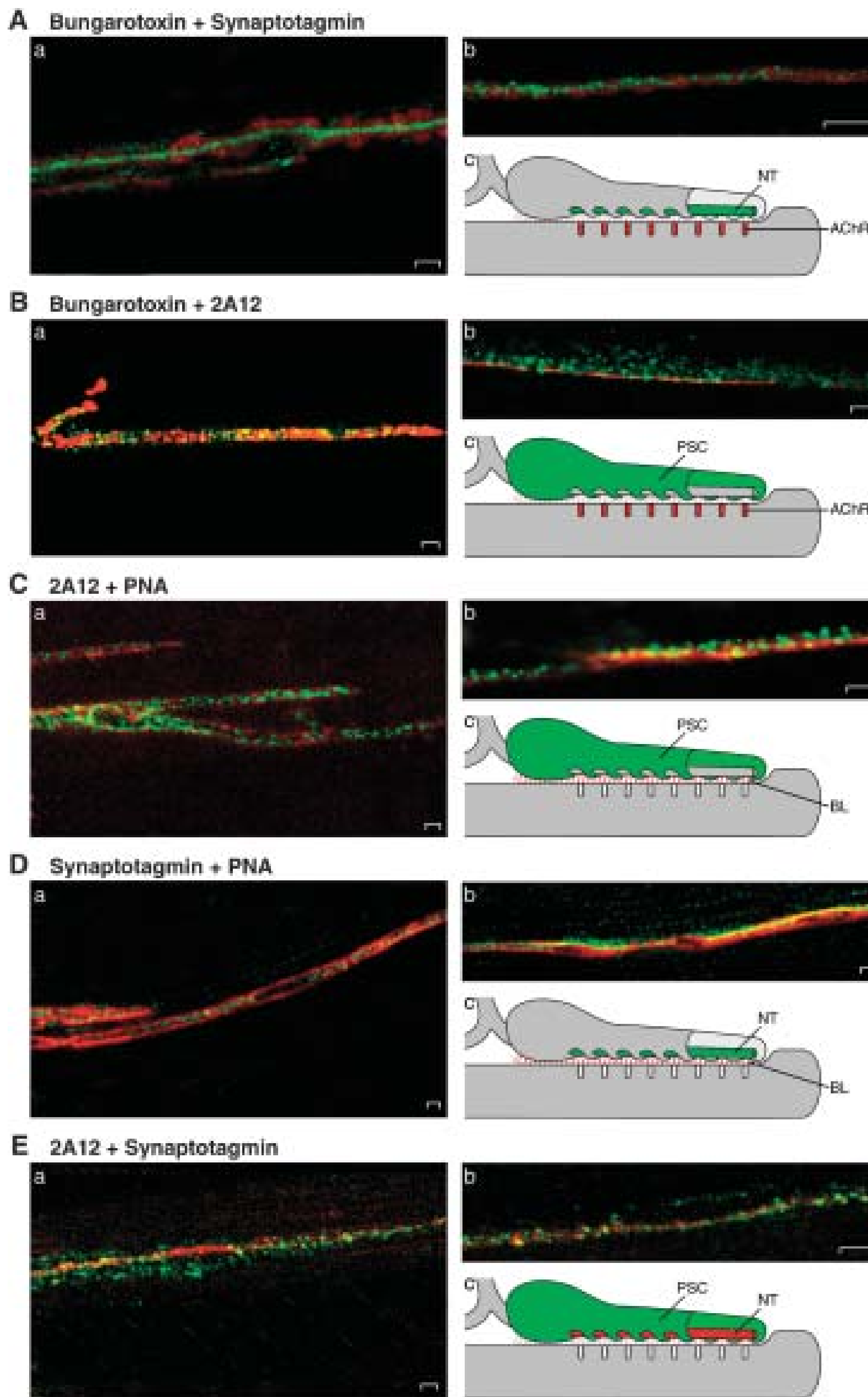

Fig. 1. Major components of the neuromuscular junction are indicated in the frog (*Rana pipiens*) cutaneus pectoris. *A*, En face view. *b*, Side view (muscle fiber on the lower portion of the image). *c*, The colors in the schematic diagram represent the same structures as in *a* and *b*. Scale bar = 5 µm. *A*, Bungarotoxin labeling (red) indicates nicotinic acetylcholine receptors (nAChRs) at the endplate region of the muscle fiber. Immunostaining of the vesicular protein synaptotagmin (green) labels synaptic vesicles in the presynaptic nerve terminal (NT). As can be visualised in *b* and *c*, the NT sits above and directly opposed to the AChR rich endplate region of the muscle fiber. *B*, 2A12 immunostaining (green; antibody kindly provided by Dr. C.-P. Ko) labels perisynaptic Schwann cells (PSCs), and bungarotoxin (red) labels AChRs. As is illustrated in *b* and *c*, PSCs are directly opposed to the endplate region. In *b*, the punctuate 2A12 immunostaining that occasionally impinges upon the red bungarotoxin staining represents PSC arms that wrap the NT. *C*, Peanut agglutin (PNA) (red) labels a proteoglycan located in the basal lamina (BL), and 2A12 (green) labels the PSC. As can be seen in *b* and *c*, the BL is underneath the PSC and is localized adjacent to the endplate region of the muscle fiber. *D*, PNA labeling (red) of the BL and synaptotagmin immunostaining (green) revealing the NT shows in *b* and *c* that the BL is underneath the NT and is adjacent to the endplate region of the muscle fiber. *E*, 2A12 immunostaining (green) revealing PSCs and synaptotagmin immunostaining (red) revealing the NT show the NT wrapped by PSC fingers, as can be seen in *b*. Note the green outline of the PSC somata above the level of the NT in *b*.

ally intrude into the synaptic cleft (Couteaux and Pecot-Dechavassine 1974; Peper and others 1974; Jahromi and others 1992). These extensions occur at 1 to 3 µm intervals between terminal active zones, where neurotransmitter release occurs and where ion channels that regulate release are present (Robitaille and others 1990). Directly opposed to active zones on the muscle fiber are postjunctional folds that contain clusters of nicotinic acetylcholine receptors (AChRs) at their crest. In mammals, PSC processes do not traverse the synaptic cleft

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but are still closely opposed to the terminal and its release sites (Salpeter 1987). Considering the proximity of PSC processes to presynaptic transmitter release sites and postsynaptic receptor sites, these perisynaptic elements are well positioned to detect and modulate synaptic activity.

Although PSCs do not myelinate the nerve terminal, they express proteins typical of myelinating Schwann cells (e.g., P0, myelin associated protein, and galactocerebroside [Georgiou and Charlton 1999]). Nevertheless, adult PSCs are characterized by markers that distinguish them from myelinating Schwann cells, including an antigen that is recognized by the 2A12 monoclonal antibody (Astrow and others 1998). Also, PSCs have many more neurotransmitter receptors and ion channels than myelinating Schwann cells. In this regard, PSCs are more analogous to CNS astrocytes than myelinating Schwann cells. For instance, they have functional L-type voltage-dependent Ca^{2+} channels (Robitaille and others 1996), muscarinic (Jahromi and others 1992; Robitaille and others 1997; Georgiou and others 1999), purinergic (Robitaille 1995), and substance P (SP) receptors (Bourque and Robitaille 1998). PSCs express receptors for neurotransmitters that have been identified at the NMJ, such as acetylcholine (ACh) and ATP. Moreover, they also contain and/or can synthesize numerous potential neuromodulatory substances, including nitric oxide (NO) (Descarries and others 1998), prostaglandins (Pappas and others 1999), and glutamate (Lévesque and Robitaille 2000).

The anatomical relationship between PSCs and the presynaptic and postsynaptic elements of the NMJ and the presence of receptors capable of detecting neurotransmission and of substances able to modulate neurotransmission suggest that synaptic activity is likely to provoke PSC responses that could modulate subsequent synapse activity. Related to this, three criteria should be fulfilled for PSCs to be considered active partners in synaptic transmission:

1. PSCs should detect synaptic activity.
2. PSC activity should be changed by synaptic activity.
3. The PSC response to synaptic activity should subsequently change synaptic output.

Evidence indicating that PSCs satisfy these three criteria will be discussed below. Together, these data show that PSCs are dynamic elements involved in the acute regulation of neurotransmission at the NMJ.

Perisynaptic Schwann Cells Respond to Neurotransmission

Using in situ amphibian muscle preparations (where NMJ anatomy is identical to in vivo), the capacity of PSCs to detect and respond to synaptic activity has been demonstrated with Ca^{2+} imaging and confocal microscopy. High-frequency stimulation of the motor nerve was observed to be associated with rapid elevation of PSC Ca^{2+} (Jahromi and others 1992; Reist and Smith 1992). PSC Ca^{2+} responses were eliminated when neurotransmitter release associated with high-frequency firing was blocked (Jahromi and others 1992). This suggests that the Ca^{2+} response was related to neurotransmitter release, not changes in extracellular ion concentration caused by repetitive neuronal depolarization or other factors. Moreover, PSCs responded to exogenous ACh and ATP with Ca^{2+} elevation, and both of these neurotransmitters are released by motor neurones during activity (Jahromi and others 1992). Mammalian PSCs respond similarly to synapse activity (Rochon and others 2001), indicating that synapse-glia interactions are a fundamental, evolutionarily conserved feature of the NMJ.

PSC Ca^{2+} responses to local applications of ATP or ACh were not blocked in the absence of extracellular Ca^{2+} (Jahromi and others 1992), implying that intracellular Ca^{2+} stores are responsible for this response. ACh activates the postsynaptic muscle fiber via nicotinic receptors. By contrast, PSCs do not respond to nicotinic stimulation (Jahromi and others 1992; Reist and Smith 1992), but they respond with Ca^{2+} elevation after muscarinic stimulation. The pharmacology of this response is such that muscarine and other muscarinic agonists readily induce the release of Ca^{2+} (Robitaille and others 1997). Only gallamine, a subtype nonspecific muscarinic antagonist, was able to block the effect of muscarine. Thus, a receptor with unique pharmacology mediates the PSC response to muscarine at the normally innervated amphibian NMJ (Robitaille and others 1997; Georgiou and others 1999). At mammalian NMJs, ACh also elicits a large PSC Ca^{2+} response that is derived from intracellular stores (Rochon and others 2001). As in the amphibian, this depends on muscarinic, not nicotinic, receptors. Unlike the amphibian, the muscarine response is blocked by atropine, thus conforming to a more typical pharmacology.

PSCs also respond to purinergic agonists. At amphibian NMJs, adenosine induced Ca^{2+} responses via A1 receptors and ATP acted through P2X and P2Y receptors (Robitaille 1995). Following nerve-evoked transmitter release, adenosine receptor blockade did not prevent amphibian PSC Ca^{2+} responses. By contrast, antagonism of ATP receptors reduced responses, showing that these cells are activated by endogenous ATP released during synaptic transmission (Robitaille 1995). Similar to the amphibian NMJ, adenosine induced Ca^{2+} responses in mammalian PSCs, with this being dependent on A1 receptor activation (Rochon and others 2001). Ca^{2+} responses to endogenous activity were partially blocked by A1 antagonism. Thus, unlike the amphibian, endogenous adenosine plays a role in activating mammalian PSCs (Rochon and others 2001). Similar to amphibians, ATP induced a large Ca^{2+} response in mammalian PSCs. Here, however, Ca^{2+} elevations were not dependent on P2 receptors. Moreover, although enzymes exist at the NMJ that dephosphorylate ATP, the capacity of ATP to induce PSC Ca^{2+} was not impaired by A1 receptor antagonists

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(Rochon and others 2001). Thus, an ATP metabolite other than adenosine contributes to this effect.

It is clear from these experiments that there have been evolutionary modifications in the manner that PSCs respond to neurotransmission at the NMJ. These are typified by the slightly more prominent role played by adenosine in the response of mammalian versus amphibian PSCs following nerve stimulation. Such differences are likely related—and contribute to—the different physiology of amphibian and mammalian NMJs. However, the conservation of function between these synapses is reflected by the strong similarities between mammal and amphibian PSC responses (e.g., sensitive to ACh via muscarinic versus nicotinic receptors).

In addition to classic neurotransmitters, peptides are released during repetitive synaptic activity at the NMJ. For instance, SP was shown to induce Ca^{2+} responses in amphibian PSCs that were dependent on intracellular stores and were blocked by an NK-1 receptor antagonist (Bourque and Robitaille 1998). Despite inducing Ca^{2+} responses itself, exposure to SP reduced Ca^{2+} responses associated with synaptic activity, muscarine, or ATP exposure (Bourque and Robitaille 1998). Such reduced PSC Ca^{2+} responses associated with SP are similar to the response run-down that is normally associated with repetitive nerve stimulation. In accord with this hypothesis, the run-down associated with repetitive nerve stimulation was attenuated in the presence of an NK-1 antagonist, indicating an important contribution of SP to this phenomenon (Bourque and Robitaille 1998). Thus, endogenous SP controls the capacity of PSCs to respond to classic neurotransmitters at the NMJ, suggesting that PSC responses are closely regulated by changing signals from the nerve terminal. Depending on the duration and intensity of nerve stimulation—resulting in secretion of different neurotransmitters, peptides, and so forth—PSC reaction to neurotransmitter exposure is likely to be changed by an alteration in the balance of intracellular signaling.

Together, the aforementioned studies confirm that PSCs satisfy the first two criteria for being active partners in synaptic transmission at the NMJ, namely, that they detect synaptic activity and that they respond to that activity.

Perisynaptic Schwann Cells Reciprocally Regulate Neurotransmission: Depression

The final criterion for being an active partner in synaptic transmission holds that activation of PSCs by neurotransmission should reciprocally alter synaptic activity at the NMJ. The hypothesis was proposed that G-protein activation and Ca^{2+} elevation, resulting from muscarinic and purinergic receptor activation associated with synaptic activity, would provoke these nonneuronal cells to modulate synaptic efficacy and regulate neurotransmitter release. Such a function would indicate that PSCs contribute to the flow of information at this synapse.

To evaluate the contribution of PSCs to establishing synaptic efficacy in the short term, a technique was developed to enable selective modulation of PSC properties without perturbing the nerve terminal or muscle fiber. As the neurotransmitters known to activate PSCs act through G-protein-coupled receptors, PSCs at identified NMJs were selectively injected with GTP analogues to activate or inhibit G-protein-dependent pathways (Robitaille 1998). PSC activity was potentiated by injection of GTPγS, a nonhydrolizable GTP analogue. As a consequence of this PSC activation, neurotransmitter release was reduced during low-frequency stimulation (0.2 Hz), and quantal analysis indicated that this was related to reduced probability of release (Robitaille 1998). By contrast, under these conditions of low-frequency nerve stimulation, inhibition of G-proteins by GDPβS injection had no effect on neurotransmitter release (Robitaille 1998). This is consistent with experiments showing that low-frequency nerve stimulation is not associated with detectable PSC activation, as indicated by Ca^{2+} elevation (Jahromi and others 1992), although it is sufficient to regulate PSC gene expression in the long term (see below) (Georgiou and others 1994, 1999). Hence, PSCs do not modulate neurotransmitter release in a tonic manner. Rather, their acute influence on synaptic activity is dependent on G-protein activation, presumably in a manner directly related to synapse activity.

The activity-dependent hypothesis was further investigated using injections of GDPβS into PSC under conditions of high-frequency nerve firing (10 Hz, 80 sec) that are associated with synaptic depression (Fig. 2) (Robitaille 1998). Blockade of G-protein signaling, which isolates PSCs from important neurotransmitter messengers, caused reduced synaptic depression (i.e., increased transmitter release) (Robitaille 1998). Thus, high-frequency nerve stimulation activates PSC G-proteins, which leads to the production of neuromodulatory substances that reduce transmitter release. To qualify these conclusions, it is important to note that depression is an intrinsic neuronal function that occurs in the absence of PSCs. However, these data show that synaptic depression can be modulated not only by neuronal elements but also by nonneuronal, glial elements.

These results fulfill the third criterion for PSCs to be an active partner in synaptic transmission, namely, that they provide feedback regulation to the synapse in response to synaptic activity. In effect, PSCs can act like brakes to slow potentially overworked neurons or muscle fibers. These observations led to the proposal that glial cells should be considered important elements of chemical synapses (Ullian and Barres 1998) and helped give rise to the concept of the tripartite synapse (Araque and others 1999), which suggests that synapses are not only composed of presynaptic and postsynaptic elements but also of perisynaptic glial cells.

There are several mechanisms by which PSCs could depress neurotransmission. For instance, PSCs contain neuronal nitric oxide synthase (nNOS) (Descarries and others 1998), and NO is known to reduce neurotransmitter release at NMJs (Lindgren and Laird 1994; Thomas and Robitaille 2001). Moreover, NO is responsible for some of the depression observed during high-frequency

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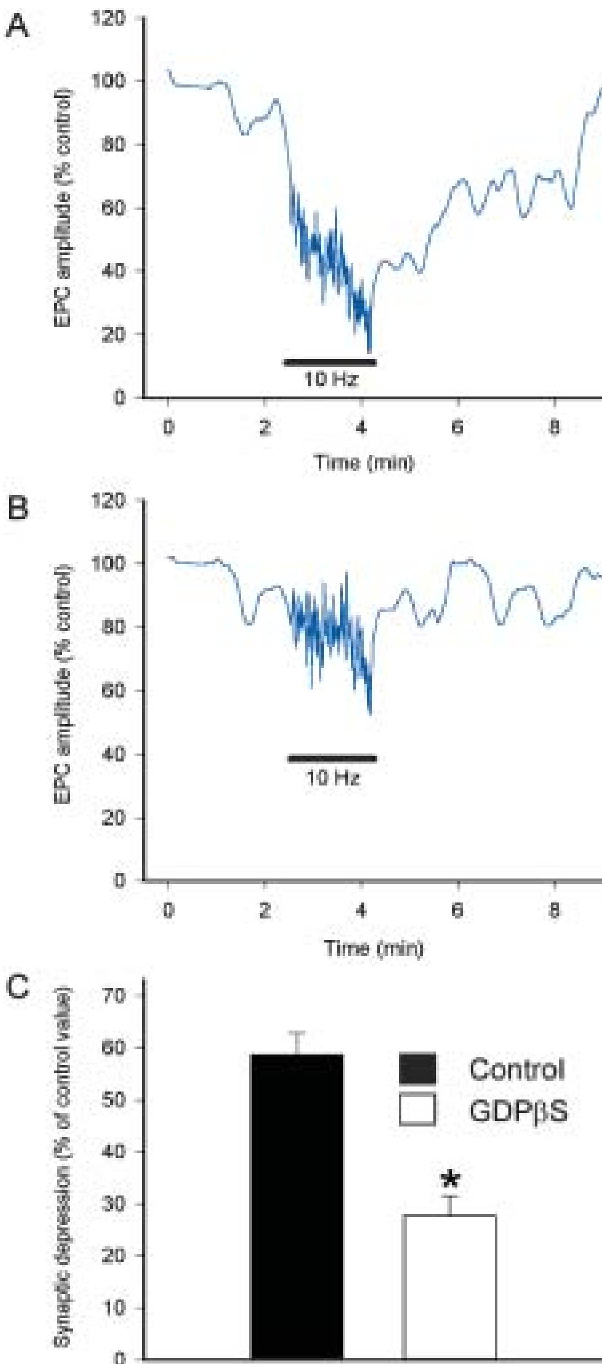

Fig. 2. Perisynaptic Schwann cell (PSC) G-proteins modulate endogenous high-frequency depression. *A*, Endplate current (EPC) amplitude expressed as a percentage of control value before, during, and after a train of stimuli (10 Hz, 80 sec) recorded before the injection of the PSC with GDPβS. EPCs were evoked at a frequency of 0.2 Hz before and after the train of stimuli. *B*, EPC amplitude expressed as a percentage of control value from the same neuromuscular junction as in *A* before, during, and after a train of stimuli (10 Hz, 80 sec) applied 25 min following GDPβS injection in the PSC covering the nerve terminal branch recorded by the focal electrode. EPCs were evoked at a frequency of 0.2 Hz before and after the train of stimuli. *C*, Average depression in control (closed bar) and following GDPβS injection in PSCs (open bar) (n = 5). Note that the amount of synaptic depression was significantly reduced (asterisk means $P < 0.05$, Student's *t*-test). Reprinted with permission from Robitaille (1998). Copyright 1998 by Cell Press.

stimulation (Thomas and Robitaille 2001). It is possible that NO production is associated with activity-dependent GTPase activity, and G-proteins can rapidly activate NOS and/or NO production in neuronal and nonneuronal cells (e.g., Okada 1992; Murthy and others 1998; Ye and others 1999; Manivet and others 2000). This relationship could be relevant to the role of GTP activity in PSC modulation of synaptic depression (see above; Robitaille 1998).

Perisynaptic Schwann Cells Reciprocally Regulate Neurotransmission: Potentiation

The functional consequence of Ca^{2+} elevation in PSCs has been unknown, despite being a well-documented response to neurotransmitter release. A series of experiments using manipulations of intracellular Ca^{2+} was initiated to investigate this question (Castonguay and Robitaille 2001). PSC internal stores of Ca^{2+} were depleted by blocking the Ca^{2+}-ATPase pump with thapsigargin. This was associated with a delayed and slow elevation of intracellular Ca^{2+}, after which neurotransmitter agonists could not elicit a Ca^{2+} rise (Castonguay and Robitaille 2001). These data indicate that PSC Ca^{2+} stores are loaded at rest and are ready to respond to physiological input (i.e., an increase in synaptic activity). In addition, thapsigargin exposure rapidly, stably, and irreversibly prolonged nerve terminal Ca^{2+} transients associated with action potentials (Castonguay and Robitaille 2001).

Based strictly on the effect of thapsigargin on nerve terminal Ca^{2+}, a rapid and stable increase in transmitter release was expected. Indeed, thapsigargin caused an initial rapid and irreversible increase of transmitter release associated with low-frequency nerve stimulation (0.2 Hz) (Castonguay and Robitaille 2001). However, this was followed by a slower, transient increase in release that returned to ~25% above control. The slow increase in release could be due to the slow increase of PSC Ca^{2+} observed in response to thapsigargin. To test this, BAPTA was specifically injected into PSCs to chelate Ca^{2+}. This manipulation abolished the delayed and slow increase in neurotransmitter release but did not alter the rapid and sustained increase (Castonguay and Robitaille 2001). Thus, increases in PSC Ca^{2+} are accompanied by slow, positive regulation of nerve terminal transmitter release. The hypothesis that PSC Ca^{2+} responses could positively regulate neurotransmission was supported by injections of IP_3 into PSCs, which induced Ca^{2+} release and enhanced low-frequency neurotransmitter release (Castonguay and Robitaille 2001).

It has been well established that high-frequency nerve activity results in PSC Ca^{2+} responses. However, the physiological consequences of these responses were unknown. Given that pharmacological induction of PSC Ca^{2+} was accompanied by enhanced neurotransmission (Castonguay and Robitaille 2001), it was hypothesized that the PSC Ca^{2+} responses associated with high-frequency nerve stimulation had similar effects. In support of this, injections of BAPTA into PSCs resulted in a more pronounced depression accompanying high-frequency stimulation. These data suggest that a glial potentiation of

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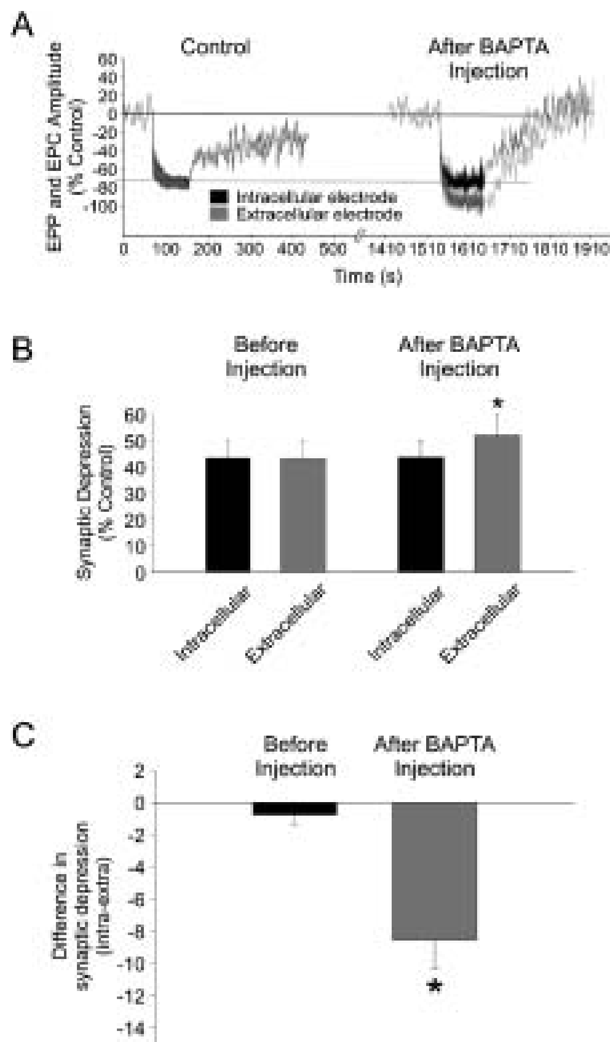

Fig. 3. Ca^{2+} in perisynaptic Schwann cells (PSCs) modulates synaptic depression. *A*, Changes in endplate current (EPC) (gray trace, focal recording) amplitude and endplate potential (EPP) (black trace, intracellular recording) amplitude expressed as a percentage of control before, during, and after the induction of high-frequency depression (10 Hz/80 sec) at the frog neuromuscular junction. Note that the focal and intracellular recordings show the same amplitude of depression. After the injection of BAPTA into a PSC (break in *x*-axis), the focal electrode (gray trace), which records synaptic transmission under the injected PSC, shows a bigger depression than the intracellular one, which records the activity of the whole terminal. This suggests that the presence of BAPTA in PSC blocked a potentiating effect on synaptic transmission in the local region of the PSC. *B*, Mean ± SEM of synaptic depression relative to control level for intracellular and extracellular recordings before and after BAPTA injection into PSCs. Note that after BAPTA injection, depression detected by the focal electrode was significantly greater. *C*, Mean ± SEM of the difference between intracellular and extracellular depression in control and after BAPTA injection into PSCs. Note that after BAPTA injection, the difference between the amplitude of depression recorded by the intracellular and focal electrodes was significantly greater. Reprinted with permission from Castonguay and Robitaille (2001). Copyright 2001 by the Society for Neuroscience.

neurotransmitter release was dependent on the Ca^{2+} response (Fig. 3) (Castonguay and Robitaille 2001). One messenger possibly released by PSCs and involved in this positive feedback may be prostaglandin E2. Indeed, cyclooxygenase is located to PSCs, and prostaglandin E2 enhances neurotransmission at the NMJ (Pappas and others 1999).

As discussed previously, PSC G-protein activity in response to high-frequency nerve stimulation leads to reduced neurotransmission (Robitaille 1998). Although release of Ca^{2+} from internal stores is downstream of G-protein-coupled receptors, these receptors likely activate numerous signaling pathways with diverse consequences. Indeed, receptor-coupled G-proteins are likely to activate pathways in PSCs that enhance (effected by Ca^{2+}) and reduce (unidentified effector) neurotransmission.

Perspective: Perisynaptic Schwann Cells and Synaptic Plasticity

In response to synaptic activity, a balance of positive and negative reciprocal modulation of synaptic efficacy by PSCs likely contributes to the various plastic events appropriate for a given level of activity (Fig. 4). It may seem counterproductive for the PSC to issue positive and negative neuromodulatory signals within a short time. However, it is known that decreased transmitter release during high-frequency stimulation results from the summation of several plastic events, including depression, facilitation, augmentation, and potentiation (Regehr and Stevens 2001). Accordingly, the counterbalancing tendencies of PSCs are in keeping with synaptic plasticity, and perisynaptic glial cells may play important roles in these different processes.

Because patterns of neurotransmission are important for the normal maintenance of the NMJ, PSCs may make contributions to changes in long-term synaptic efficacy associated with various physiological or pathological states. Any alterations in PSC condition (i.e., extended processes after denervation; see below) may shift their tendency to enhance or decrease neurotransmission. Although changes in PSC properties may respond to, rather than precede, other changes at the NMJ, it is likely that any changes in PSC modulation of transmitter release make contributions to the observed long-term changes in synaptic efficacy (Table 1).

Perisynaptic Schwann Cells Contribute to Maintenance and Repair of the NMJ

In keeping with the capacity of PSCs to detect synaptic activity, classic studies have shown that these cells respond to long-term changes in other NMJ elements (Miledi and Slater 1969; Bevan and others 1973; Dennis and Miledi 1974; Jirmanova 1975; Ko 1981). Indeed, in addition to the possibility that PSC modulation of neurotransmission might indirectly contribute to synaptic stability, PSCs contribute by other means to establishing or maintaining synaptic efficacy. Three general themes will be discussed in relation to this:

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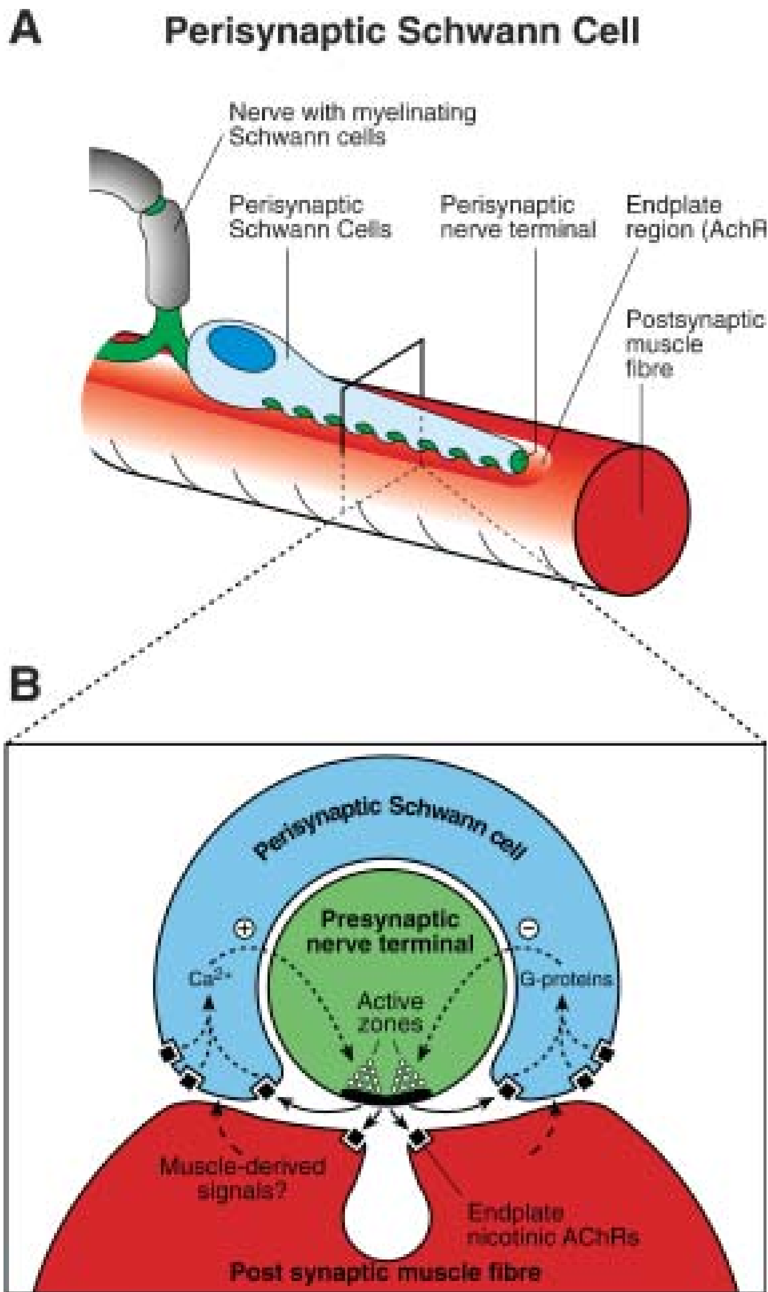

Fig. 4. Synapse-glia interactions at the amphibian neuromuscular junction (NMJ). *A* and *B*, Perisynaptic Schwann cells (PSCs) are closely opposed to nerve terminal branches. PSC processes run atop nerve terminal branches and intermittently extend fingers into the synaptic cleft. In the presynaptic nerve terminal, synaptic vesicles are concentrated in active zones, where neurotransmitter release occurs. On the postsynaptic muscle fiber, directly opposed to active zones, nicotinic acetylcholine receptors (nAChRs) are concentrated at endplate postjunctional folds. *B*, Functional relations between presynaptic, postsynaptic, and perisynaptic elements at the NMJ, illustrated in cross section. Neurotransmitters released by the presynaptic nerve terminal activate the muscle fiber and PSC. Notably, ACh binding endplate nAChRs induces muscle fiber contraction. ACh and ATP bind muscarinic and purinergic G-protein-coupled receptors and activate PSCs during high-frequency stimulation, which is associated with release of Ca^{2+} from internal stores. Subsequently, PSCs issue positive and negative feedback signals that modulate neurotransmission in a reciprocal fashion and contribute to the plasticity (e.g., depression) observed during high-frequency synaptic activity. G-protein activity, through an unidentified mechanism(s), provokes a PSC response that reduces neurotransmitter release. Nitric oxide may be involved in this effect. By contrast, the rise in intracellular PSC Ca^{2+} results in a PSC-derived signal that increases neurotransmitter release. Prostaglandin may be involved in this effect. Muscle fiber-derived signals released in response to presynaptic activity may also contribute to PSC activation. Peptides released at the NMJ during high-frequency stimulation, such as substance P acting through the NK-1 receptor, modulate the normal rundown of the Ca^{2+} response during high-frequency nerve stimulation.

1. PSCs respond to changes in neurotransmitter release and denervation with changes in protein markers.
2. PSCs interact with other elements of the NMJ and help reestablish synapses during reinnervation.
3. PSCs respond to and contain numerous molecules known for tropic, trophic, and synapse organizing properties.

We support the hypothesis that PSCs are important sensors of the condition of the synapse, which contribute to reestablishing NMJ efficacy after disruption and likely help maintain synapse stability under normal conditions.

Perisynaptic Schwann Cells Respond to Long-Term Changes in Neurotransmitter Release: Cytoskeletal Alterations and Process Sprouting

Classic work by Miledi and others established that following denervation, PSCs invade the synaptic cleft (Birks 1960; Bevan and others 1973; Dennis and Miledi 1974; Ko 1981) and can release ACh in a fashion that is dependent on gene transcription (Bevan and others 1973; Dennis and Miledi 1974). More recent work has established that PSCs extend elaborate process networks following denervation (Reynolds and Woolf 1992; Woolf and others 1992; Mehta and others 1993; Chen and Ko 1994; Son and Thompson 1995b, 1995a; Son and others

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Table 1. Summary of Nerve- and Synapse-Activity-Dependent Functions of Perisynaptic Schwann Cells (PSC) at Neuromuscular Junctions (NMJ)

NMJ Condition	Species and Muscle	PSC Function	Reference
Rapid interactions with neurotransmission			
Acute in situ preparation	Frog (*Rana pipiens*), cutaneus pectoris; mouse, levator auris longus	Rapid Ca^{2+} elevation induced by neurotransmitters, notably ACh (muscarinic receptors), adenosine, ATP, and substance P	Jahromi and others (1992); Reist and Smith (1992); Robitaille (1995); Robitaille and others (1997); Bourque and Robitaille (1998); Rochon and others (2001)
Acute in situ preparation	Frog (*Rana pipiens*), cutaneus pectoris	Rapid, activity-dependent negative feedback on neurotransmission by G-protein activation	Robitaille (1998)
Acute in situ preparation	Frog (*Rana pipiens*) cutaneus pectoris	Rapid, activity-dependent positive feedback on neurotransmission by release of Ca^{2+} from intracellular stores	Castonguay and Robitaille (2001)
Nerve- and neurotransmission-dependent protein regulation			
In vivo, nerve cut	Frog (*Rana pipiens*), cutaneus pectoris	Neurotransmission-dependent suppression of GFAP expression	Georgiou and others (1994, 1999)
In vivo, full and partial denervation	Rat, soleus and tibialis anterior	GAP-43 is upregulated	Woolf and others (1992); Mehta and others (1993)
In vivo, full and partial denervation, botulinum toxin	Rat, soleus and tibialis anterior; rat, gastrocnemius	p75 neurotrophin receptor is upregulated	Reynolds and Woolf (1992); Hassan and others (1994)
In vivo, full denervation	Rat, soleus	Increased immunoreactivity to the 4E2 mAb	Astrow and others (1994)
In vivo, full denervation	Mouse, sternomastoid	S-100 immunoreactivity reduced with time after denervation	O'Malley and others (1999)
Characteristics of process extension			
In vivo, full and partial denervation	Rat, mouse, and frog, various muscles	PSCs extend elaborate processes after denervation	Reynolds and Woolf (1992); Woolf and others (1992); Mehta and others (1993); Chen and Ko (1994); Son and Thompson (1995a, 1995b); Son and others (1996); Astrow and others (1998); Love and Thompson (1999); O'Malley and others (1999); Koirala and others (2000)
In vivo, full and partial denervation	Rat, soleus	PSCs extend elaborate processes and connect denervated endplates; these guide reinnervating axons	Son and Thompson (1995a, 1995b)

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Table 1 (continued)

NMJ Condition	Species and Muscle	PSC Function	Reference
In vivo, botulinum toxin	Rat, soleus	PSCs extend processes after blockade of neurotransmission; not as extensive as after denervation	Son and Thompson (1995a)
In vivo, partial denervation	Rat, soleus	PSCs extend processes that preferentially form bridges between denervated and innervated endplates; these guide reinnervating axons	Love and Thompson (1999)
In vivo, partial denervation	Rat, soleus	Bridge formation is neurotransmission dependent; disorganized by botulinum toxin and bungarotoxin	Love and Thompson (1999)
In vivo, full denervation	Mouse, sternomastoid	PSC process extension following denervation; these are maintained/elaborated by reinnervation	O'Malley and others (1999)
In vivo, full denervation	Frog (*Rana pipiens*), cutaneus pectoris and sartorius	Upon reinnervation, PSCs extend processes and connect endplates	Koirala and others (2000)

1996; Astrow and others 1998; Love and Thompson 1999; O'Malley and others 1999; Koirala and others 2000). Moreover, PSCs respond to denervation with functional expression of new neurotransmitter receptors (Robitaille and others 1997). Given that these changes likely involve cytoskeletal alterations, a series of experiments was initiated to investigate the effect of reduced synaptic activity at the amphibian NMJ on glial fibrillary acidic protein (GFAP), a glial intermediate filament component (Georgiou and others 1994, 1999).

In the frog, expression of GFAP is typically very low in PSCs at innervated, active NMJs. By contrast, GFAP immunoreactivity increased markedly following axotomy (Georgiou and others 1994). An increase also occurred when neurotransmission was abolished by blocking Ca^{2+} channels with ω-conotoxin-GVIA or by preventing action potentials with tetrodotoxin. In accord with these findings, low-frequency nerve stimulation (0.5 Hz) prevented GFAP upregulation following axotomy. This result indicates that GFAP expression does not result from the nerve cut but is related to synaptic activity. GFAP expression was directly dependent on reduced presynaptic transmitter release, not secondary changes effected via the muscle fiber, because blocking postsynaptic AChRs with α-bugarotoxin was not associated with these effects (Georgiou and others 1994). It appears that only muscarinic receptors—but not purinergic, SP, or calcitonin gene–related peptide receptors—suppress GFAP expression (Georgiou and others 1999).

The small level of transmitter release (0.5 Hz) was well below that which induces PSC Ca^{2+} responses (e.g., 10 Hz). Thus, PSCs respond distinctly to different levels of synaptic activity, presumably by diverse signaling pathways. In keeping with earlier work showing that other PSC changes following denervation are dependent on transcription (Bevan and others 1973), neurotransmitter release inhibits GFAP upregulation by suppressing gene transcription and protein translation (Georgiou and others 1994). The role of GFAP following reduced transmission has not been precisely defined but could contribute to PSC process formation (see below) and/or help in other compensatory responses to lost neurotransmission, including reorganization of membrane muscarinic receptors (Robitaille and others 1997).

In keeping with PSC process extension following denervation, growth-associated proteins have been reported to be altered following axotomy. Within 24 h of denervation, growth-associated protein 43 (GAP-43) is upregulated in PSCs at the rat NMJ (Woolf and others 1992). Following reinnervation and coinciding with retraction of PSC elaborations, PSC GAP-43 levels drop (Woolf and others 1992). However, unlike the control of GFAP expression, GAP-43 expression was not dependent on neurotransmission and likely resulted from a loss of nerve contact or degeneration products (Hassan and others 1994). Moreover, PSC immunoreactivity for the p75 neurotrophin receptor increases with both denervation (Reynolds and Woolf 1992) and botulinum toxin blockade of neurotransmission (Hassan and others 1994). Other work demonstrated at rat NMJs that an antigen recognized by the 4E2 mAb was increased following denervation (Astrow and others 1994; Son and Thompson 1995a) but not after botulinum toxin blockade of neurotransmission (Son and Thompson 1995a). Conversely, S-100 immunostaining was reduced after denervation of mouse NMJs, suggesting that expression

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of this glial marker is maintained by innervation (O'Malley and others 1999). By contrast, S-100 was still observed at rat PSCs after botulinum toxin treatment (Hassan and others 1994). These differences illustrate the delicate control of PSC markers by different influences associated with innervation, one of which being neurotransmission.

This body of research indicates that disruption of normal innervation results in an extensive array of PSC responses, which include invasion of the synaptic cleft; release of ACh; changes in neurotransmitter receptor properties; expression of growth-associated, cytoskeletal, and other proteins; and extensive process extension. How these responses could be important for denervated endplates and reinnervation is becoming clearer. Indeed, this work complements some of the most exciting recent advances in PSC biology, which demonstrate that PSC processes—in their postdenervation state—guide reinnervating motor axons to endplates.

Perisynaptic Schwann Cells Guide Regenerating Nerve Terminals

Denervation is associated with intense PSC process extension at mammalian (Reynolds and Woolf 1992; Woolf and others 1992; Son and Thompson 1995b, 1995a; Love and Thompson 1999; O'Malley and others 1999) and amphibian (Chen and Ko 1994; Astrow and others 1998; Koirala and others 2000) NMJs. Normally, PSC process are limited to the endplate region. It was first shown in rat muscles that upon nerve injury, processes extend beyond endplate regions (Reynolds and Woolf 1992; Woolf and others 1992). Approximately 2 weeks postinjury, a complex network was observed to cover the muscle surface, whereas upon reinnervation, PSC morphology returned to normal (Reynolds and Woolf 1992; Woolf and others 1992). These original findings showed that mammalian PSCs detect and respond to the state of innervation, but the function of these process extensions was not immediately clear. Subsequent work, much by Thompson and colleagues using the rat soleus muscle, has shown that PSC processes guide reinnervating motor terminals to denervated endplates.

After full denervation, PSC processes were shown to extend between adjacent endplates and often formed fasciculated bundles (Son and Thompson 1995b). During reinnervation, regenerating axons that found endplates after following myelinating Schwann cell tubes to the muscle did not stop. They continued to grow beyond the endplate region, where they followed the PSC process extensions very closely (Son and Thompson 1995b). In this manner, the processes act as bridges between endplates for reinnervating axons. These bridges precede axons, and instances were observed where PSC processes extended well beyond the limit of their associated axon (Son and Thompson 1995b). By contrast, axons were not observed extending past their associated PSC processes. These observations gave the impression that PSC processes guided reinnervating axons to endplates.

Following partial denervation, nerve sprouts from undamaged axons extend from terminals to innervate denervated muscle fibers (Brown and others 1981). In addition to nerve sprouting, PSCs extend numerous long processes after partial denervation (Mehta and others 1993; Son and Thompson 1995a; Love and Thompson 1999). These contact other endplates (Son and Thompson 1995a; Love and Thompson 1999) and the vast majority of connections are formed by processes from denervated endplates contacting innervated endplates (Fig. 5) (Love and Thompson 1999). Interestingly, all nerve sprouts resulting from partial denervation were associated with PSC processes. Moreover, PSC processes always extended beyond their associated nerve sprouts, giving the impression that they were leading the growing sprouts (Son and Thompson 1995a; Love and Thompson 1999). Of the nerve sprouts that had reinnervated endplates, most were associated with PSC processes from denervated endplates (Love and Thompson 1999). The authors indicate that these data suggest that PSC processes from denervated endplates find innervated endplates, where they then induce a terminal sprout and guide it to the denervated endplate. Because most bridges exist between denervated and innervated endplates, PSC processes appear to select innervated endplates, which is clearly an advantage for facilitating reinnervation of their denervated endplate. It is not clear if this is achieved through selective guidance or stabilization by signals from innervated endplates.

In the mouse sternomastoid muscle, shortly after muscle nerve resection (3 days), PSCs extended numerous processes (O'Malley and others 1999). However, the length and number of these processes decreased considerably with continued denervation, and many fewer processes were evident after 1 month. In contrast, if reinnervation took place quickly following denervation (a few days), PSC processes were observed to be abundant (O'Malley and others 1999). Thus, reinnervating axons are involved in inducing and/or maintaining PSC process extensions, which would otherwise be only temporarily sustained. Other nerve- and synaptic-activity-dependent effects on PSC processes have been observed in the rat and frog (see below) (Love and Thompson 1999; Koirala and others 2000). In keeping with findings from rat muscles, in mice, PSCs appeared to guide nerve sprouts during reinnervation (O'Malley and others 1999). Indeed, repeated in vivo observations showed that nerve sprouts grew along preexisting PSC somata and processes and did not precede these glial elements.

Similarities and differences characterize the roles of amphibian and mammalian PSC processes following complete denervation and reinnervation. It is clear that the major raison d'être of frog PSC processes is the same, that is, to guide regenerating nerve sprouts (Koirala and others 2000). Following denervation of the frog cutaneus pectoris and sartorius muscles, PSC

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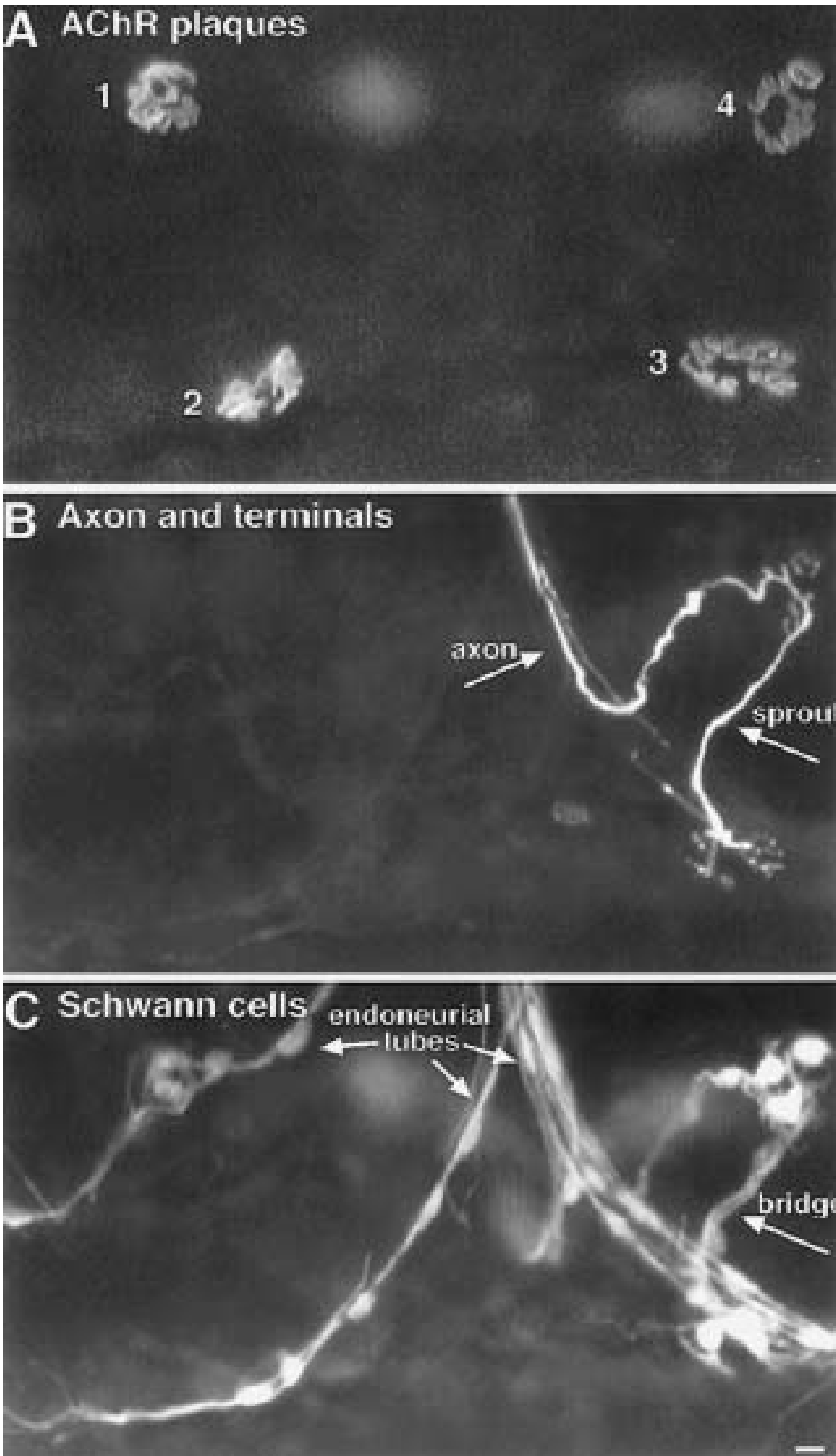

Fig. 5. Perisynaptic Schwann cell processes extended from denervated endplates preferentially bridge with innervated endplates. Image of a triple-labeled rat soleus muscle 3 days after partial denervation. *A*, Labeling of acetylcholine receptors with Cy5-conjugated α-bungarotoxin. *B*, Labeling of axons and nerve terminals with antibodies to neurofilament and synaptic vesicle protein and an FITC-conjugated secondary antibody. *C*, Labeling of Schwann cells (SC) with anti-S100 antibody and a rhodamine-conjugated secondary antibody. There are four endplates in focus in this region of the muscle (*A*, numbers). Endplates 1 and 2 lack nerve contact and therefore were denervated and have not become reinnervated. Endplate 3 is innervated by a sprout growing from endplate 4, showing that endplate 3 was denervated but has become reinnervated. Endplate 4 is innervated by an axon that was not damaged during the partial denervation. Examination of the SC labeling in *C* shows that perisynaptic Schwann cells form a bridge between endplates 3 and 4. SC labeling is also associated with the endoneurial tubes, that is, the SC wrappings of axons and of the pathways previously occupied by axons. SC processes are extended from the denervated endplates 1, 2, and 3, but only endplate 3 is linked to an adjacent endplate by these processes. The processes from endplates 1 and 2 extended parallel with the muscle fibers (oriented horizontally in this image), not toward each other. Note that the newly established nerve terminal at endplate 3 is extending sprouts that have partially grown up the endoneurial tube. Scale bar, 10 μm. Reprinted with permission from Love and Thompson (1999). Copyright 1999 by the Society for Neuroscience.

processes and reinnervating axons serially observed in vivo were closely opposed to one another. Moreover, the axon never preceded the PSC extension, with the latter extending tens to hundreds of microns beyond the axon terminus (Koirala and others 2000). Like the mammal, PSC process bridges were observed that seemed to facilitate reinnervation. Unlike the mammal, there was little process extension observed following complete denervation before reinnervation had occurred (Koirala and others 2000). Indeed, in the amphibian, the stimulus for PSC process extension appears to be the return of the motor neurons. If the key signal is a return of synapse activity, it is interesting to note that in partially denervated rat muscles, PSC bridges depend on neurotransmission (see below) (Love and Thompson 1999). Despite these differences in timing and regulation, the

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importance of PSC processes in guiding regenerating axons is underscored by its evolutionary conservation in frogs, mice, and rats.

In rat muscles, presynaptic blockade of neurotransmission with botulinum toxin induced both nerve terminal sprouting and PSC sprouting, although to a lesser extent than denervation (Son and Thompson 1995a). As with denervation, all nerve sprouts were associated with PSC processes (Son and Thompson 1995a). Moreover, botulinum toxin completely disorganized the bridge-forming capacity of PSC processes following partial denervation, with PSC processes from denervated endplates not reliably contacting innervated endplates (Love and Thompson 1999). Bridge formation was also impaired by α-bungarotoxin, which blocks postsynaptic AChRs. Together, these data show that 1) PSCs detect reductions in synaptic activity that can contribute to induction of process extension, 2) synaptic activity at innervated endplates is responsible for establishing or maintaining the PSC bridges that direct reinnervating nerve sprouts, and 3) bridge formation is encouraged by activity-dependent signals derived from the muscle fiber.

This suggests that when PSCs do not detect appropriate signals from the nerve, one of which includes neurotransmission, they initiate exploratory process extension. In addition, evidence from the frog and mouse suggest that initiation, intensification, and/or maintenance of PSC process extensions are dependent on reinnervation (Astrow and others 1998; O'Malley and others 1999). Bridge formation is also an activity-dependent process, as has been reported in the rat, with PSCs selectively contacting or maintaining connections with innervated endplates (Love and Thompson 1999). Hence, a coordinated series of distinct PSC responses, which culminate in nerve sprout guidance and restoration of activity at denervated NMJs, are prompted either by reduced activity or by the presence of activity. In addition to the likelihood that PSCs respond directly to nerve-derived signals, extension of their processes is also likely to be influenced by indirect signals derived from muscle fibers in response to nerve activity. In light of this, it is interesting that treatment with the AChR antagonist bungarotoxin results in nerve terminal sprouting (Holland and Brown 1980), and terminal sprouting has since been shown to be closely associated with PSC processes (see above).

Other recent work indicates that PSCs may play accessory or executive roles in neurite extension under normal conditions. Indeed, remodeling of mature amphibian NMJs, involving small extensions and retractions of motor nerve endings, was concurrent with short PSC process extensions and retractions (Macleod and others 2001). Moreover, it has been shown in amphibians that PSC processes lead neurite sprouts during developmental innervation, in a manner akin to that observed in reinnervation following denervation (Herrera and others 2000). These and other avenues of study are sure to reveal any PSC contributions to normal development, stability, and long-term functioning of the NMJ.

It is likely that PSCs' ability to monitor activity—probably by both direct responses to neurotransmitter and indirect effects mediated by muscle fibers—is critical for maintaining the NMJ in its normal physiological state, as well as in coordinating appropriate responses to pathological conditions. These contributions of PSCs, as well as their short-term modulation of transmitter release, show that activity-dependent PSC functions make key contributions to determining the overall function of the NMJ.

Perisynaptic Schwann Cells, Trophic Factors, and Stability of the NMJ

Recent work has exposed several signaling molecules that likely coordinate PSC sprouting and nerve terminal guidance after injury, as well as some that likely maintain the stability of the NMJ under normal conditions. For instance, glial growth factor 2 (GGF2)—a neuregulin that is primarily derived from the motor nerve—promotes the survival of PSCs at immature synapses and induces process extension at NMJs (Trachtenberg and Thompson 1996, 1997). In addition, Schwann cells express agrin, and under some circumstances, notably reinnervation, this may contribute to endplate AChR clustering (Yang and others 2001).

The neurotrophins and their receptors are also found on the cellular elements of the NMJ, including PSCs (Funakoshi and others 1993; Koliatsos and others 1993; Hassan and others 1994; Funakoshi and others 1995; Gonzalez and others 1999; Belluardo and others 2001; Landsman and others 2001; Loeb and others 2002). These include neurotrophin-3 (NT-3), which primarily binds TrkC, as well brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4), which bind TrkB. Neurotrophin availability at the NMJ is related to synapse activity, which is in agreement with the robust changes in synapse structure associated with different activity levels. Neurotransmission promotes expression of muscle BDNF and NT-3 during development (Loeb and others 2002). Each of these factors promotes neuregulin availability, which is critical for AChR expression and normal endplate AChR clusters (Sandrock and others 1997; Loeb and others 2002). At the adult mouse NMJ, TrkB immunoreactivity is localized largely in the postsynaptic fiber (Gonzalez and others 1999). Most interestingly, dominant negative inhibition of muscle TrkB resulted in disassembly of AChR clusters (Gonzalez and others 1999). At adult rat NMJs, reduced activity lowered NT-4 expression (Funakoshi and others 1995), and NT-4 knockout was associated with NMJ disassembly (Belluardo and others 2001). Thus, activity-dependent secretion of TrkB ligands, possibly in an autocrine fashion, may promote normal endplate maintenance.

In addition to neurotrophin effects on endplate organization, recent evidence has implicated the involvement

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of NT-3 and TrkC in PSC process extension. At the NMJ, TrkC immunoreactivity was shown to be specific for PSCs in neonatal and adult mice (Chen and others 2000; Landsman and others 2001). Dominant-negative inhibition of PSC TrkC signaling resulted in process extension reminiscent of denervation, with some PSC processes being associated with nerve sprouts (Gonzalez and others 2000; Landsman and others 2001). Also, denervation-associated PSC process extension was reduced when NT-3 was overexpressed in muscle (Landsman and others 2001). This is particularly intriguing in light of activity-dependent expression of NT-3 at the developing NMJ (Loeb and others 2002). According to these data, normal neurotransmission could promote adequate NT-3 secretion and/or expression, which would then maintain PSC TrkC signaling. With normal NT-3/TrkC signaling, the PSC would be maintained in a state where process extension would be unlikely. Reduced NT-3 may be a key signal for PSCs that NMJ physiology has been disrupted and that compensatory measures, such as process extension, are appropriate. In this fashion, activity-dependent secretion of NT-3 acting on PSCs may be an important determinant of NMJ stability.

Conclusions

Neurotransmission and long-term synaptic stability/change have traditionally been studied separately. However, it is now clear that synaptic activity has a powerful influence on changes in synapse state, and PSCs are emerging as central participants in these processes. Not only are these cells important for reciprocal control of neurotransmission in the short term, but their ability to respond to activity- and innervation-dependent signals during reinnervation is critically important for normal recovery after nerve injury. In a complementary manner, the ability of PSCs to monitor and detect appropriate or inappropriate levels of activity may have important influences on the stability or lability of the NMJ. It is likely that activity-dependent expression/secretion of trophic factors, notably the neurotrophins, is important for coordinating these changes.

We predict that PSCs are important for an activity-dependent continuum of synaptic efficacy, stability, and plasticity at the NMJ. We expect that this relationship helps maintain synaptic efficacy under normal conditions and contributes to reestablishing synaptic connections after denervation. It is possible that glial cells in the CNS may play similar roles.

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