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Modulation of neurotransmission by reciprocal synapse-glial interactions at the neuromuscular junction

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Abstract

Perisynaptic Schwann cells are glial cells that are closely associated with pre- and postsynaptic elements of the neuromuscular junction. Recent evidence shows that these cells detect and modulate neurotransmission in an activity-dependent fashion. Through G-protein signalling and Ca^{2+} released from internal stores they can decrease or increase neurotransmitter release, respectively. Thus, they help to establish the level of neurotransmission associated with activity dependent short-term synaptic plasticity. We discuss evidence implicating perisynaptic Schwann cells as being active partners in neurotransmission at the neuromuscular junction, with emphasis on the modulation of short-term plasticity and potential implications for long-term changes.

Introduction

The neuromuscular junction (NMJ) consists of three cellular compartments: the nerve terminal, the postsynaptic muscle fibre and the perisynaptic Schwann cells (PSCs). The purpose of the NMJ is to generate a chemical synapse that results in nervous control over muscle contraction. By this functional definition, it is clear that the presynaptic nerve terminal and the postsynaptic muscle fibre are immensely important for the physiological economy of the NMJ. Nevertheless, PSCs are also a component of the NMJ and recent studies show that they play widespread and significant roles in synaptic plasticity, development, and repair following disruption of function. This review will focus on the interactions between PSCs and synaptic plasticity, including: the response of PSCs to neurotransmission, the feedback actions of PSCs on synaptic activity, the potential involvement of novel chemical neuromodulators, and possible links between PSCs and changes in long-term synaptic efficacy.

The neuromuscular junction and perisynaptic Schwann cells

Classically, the NMJ has been described as having two compartments: the presynaptic nerve terminal and the postsynaptic end-plate region of the muscle fibre. The nerve terminal is rich with neurotransmitter containing synaptic vesicles, which are located primarily at active zones near Ca^{2+} entry sites (Robitaille *et al.*, 1990). At frog and mammalian NMJs, acetylcholine (ACh) is the primary neurotransmitter. Indeed, the seminal work of Dr. Bernard Katz and his colleagues established the quantal theory of neurotransmission by studying cholinergic transmission at this synapse (Katz, 1971). In addition to ACh, adenosine triphosphate (ATP) is an important co-transmitter (Smith, 1991). Vesicular co-localization of ACh and ATP was initially identified at the Torpedo electric organ, a specialized NMJ (Dowdall, 1974; Meunier, 1975; Carlson, 1978; Tashiro, 1978). ATP is degraded by ectonucleotidases that give rise to the neurotransmitter-like substances adenosine diphosphate (ADP), adenosine monophosphate

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(AMP) and adenosine (Illes, 2000; Zimmermann, 2000).

Immunostaining has also revealed peptides, such as substance P (SP) and calcitonin gene-related peptide (CGRP), within large dense-core vesicles (LDCVs) of the presynaptic nerve terminal (Matteoli, 1988, 1990). Several neuromodulatory influences have been ascribed to these peptides at the NMJ (Steinacker, 1977; Akasu, 1986; Van der Kloot *et al*., 1998). However, the kinetics of peptide release differ from that of ACh and ATP containing small clear vesicles (SCVs) (Karhunen, 2001). In light of this, it is interesting that Aplysia motor neurons release ACh following a single action potential, but that repetitive firing is required for peptide secretion (Cohen *et al*., 1978; Whim & Lloyd, 1989; Vilim *et al*., 1996). Thus, the most significant actions of peptides likely occur in an activity-dependent manner. In keeping with this, PSC Ca^{2+} responses are activity-dependent and are regulated by endogenous peptide action at the NMJ (see below; Bourque & Robitaille, 1998).

The postsynaptic end-plate is a structural specialisation of the muscle fibre and is closely opposed to the presynaptic nerve terminal. The end-plate region is regularly invaginated by postjunctional folds. Nicotinic acetylcholine receptors (AChRs) are clustered atop these folds and are directly opposed to active zones (Salpeter, 1987). Despite the prominent role of pre- and postsynaptic elements in this specialised region of chemical communication, there is now a growing body of evidence that PSCs make key contributions to this communication (Castonguay *et al*., 2001; Auld & Robitaille, 2003) (Fig. 1).

PSCs do not myelinate the nerve terminal, but they have several proteins that characterise myelinating Schwann cells [*e.g*., P0, myelin associated protein (MAG) and galactocerebroside (Georgiou & Charlton, 1999)]. There are typically 3-5 PSCs found at each NMJ. Using various markers [*i.e*., the 2A12 antibody (Astrow *et al*., 1998)] it has been shown that PSC processes run along nerve terminal branches. In frogs, these processes make finger-like intrusions into the synaptic cleft at irregular intervals (1–3 μm) between active zones (Couteaux & Pecot-Dechavassine, 1974; Peper *et al*., 1974; Jahromi *et al*., 1992; Astrow *et al*., 1998). In mammals, the synaptic cleft is not completely traversed by PSC processes. However, processes still approach the cleft and are in close proximity to neurotransmitter release sites (Salpeter, 1987). PSCs respond to neurotransmission through receptors and ion channel activation. They have functional muscarinic ACh receptors (Jahromi *et al*., 1992; Robitaille *et al*., 1997; Georgiou *et al*., 1999), purinergic receptors (Robitaille, 1995), substance P (SP) receptors (Bourque & Robitaille, 1998) and L-type voltage dependent Ca^{2+} channels (Robitaille *et al*., 1996). Neurotransmitter receptors and the close association of PSCs with other elements of the NMJ facilitate the capacity of these cells to detect changes in the NMJ environment (Auld & Robitaille, 2003).

Neurotransmission induces responses in perisynaptic Schwann cells

High frequency stimulation of the motor nerve is associated with rapid elevation of PSC Ca^{2+} in *in situ* frog muscle preparations (where NMJ anatomy is preserved as it is *in vivo*) (Fig. 1) (Jahromi *et al*., 1992; Reist & Smith, 1992). These Ca^{2+} responses are related to neurotransmitter release, as opposed to other causes (*e.g*., changes in extracellular ion concentration associated with repetitive neuronal depolarisation) because when transmitter release is blocked, PSC Ca^{2+} responses are greatly reduced (Jahromi *et al*., 1992). Further supporting this is the observation that PSCs responded with Ca^{2+} elevation to exogenous application of ACh and ATP (Jahromi *et al*., 1992). Mouse PSCs respond in a similar fashion to synaptic activity (Rochon *et al*., 2001), implying that PSC sensitivity to neurotransmission is a fundamental, evolutionarily-conserved feature of NMJs.

Frog and mammal PSC Ca^{2+} responses involve intracellular Ca^{2+} stores, as responses to local applications of ATP or ACh were unaffected by removal of extracellular Ca^{2+} (Jahromi *et al*., 1992). Interestingly, unlike the pharmacological profile of the muscle fibre, amphibian and mammalian PSCs do not respond to nicotinic stimulation with Ca^{2+} elevation (Jahromi *et al*., 1992; Reist & Smith, 1992). They do, however, respond to muscarinic stimulation with Ca^{2+} elevation. At the frog NMJ, muscarinic agonists readily induce Ca^{2+} elevation (Robitaille *et al*., 1997), but only gallamine—a subtype non-specific muscarinic antagonist—was able to block this effect. Thus, a receptor with unique pharmacology mediates the PSC response to muscarine at the normally innervated amphibian NMJ (Robitaille *et al*., 1997; Georgiou *et al*., 1999). By contrast, the mammalian PSC muscarine-induced Ca^{2+} response is blocked by atropine, thus conforming to a more typical pharmacology.

PSCs are also responsive to purinergic agonists. At frog NMJs, adenosine induces Ca^{2+} responses through A1 receptors, whereas ATP acts through P2X and P2Y receptors (Robitaille, 1995). After nerve-evoked transmitter release, antagonism of adenosine receptors did not prevent frog PSC Ca^{2+} responses. However, block of ATP receptors reduced the Ca^{2+} responses, indicating these cells are sensitive to endogenous ATP released during synaptic transmission (Robitaille, 1995).

At mammalian PSCs, adenosine also induced Ca^{2+} responses during synaptic transmission. This required A1 receptor activation since Ca^{2+} responses to endogenous nerve-activity were partially blocked by A1 antagonism (Rochon *et al*., 2001). Thus, in contrast to the frog NMJ, endogenous adenosine helps to activate mammalian PSCs (Rochon *et al*., 2001). As it does in the

frog, ATP evokes a significant Ca^{2+} response in PSCs at mouse NMJs. However, its role and mode of action remain unclear (Rochon *et al*., 2001). These experiments show that there have been evolutionary modifications in the details of how PSCs respond to neurotransmission at the NMJ. These differences are expected to be related to—and contribute to—the different physiology of amphibian and mammalian NMJs. Despite the variations, there is a strong conservation of function between these synapses that is reflected by the similarities of their responses (*e.g*., sensitive to ACh through muscarinic, not nicotinic receptors; frequency-dependence of synaptic-activity induced Ca^{2+} responses).

PSCs also respond to other neurotransmitter-like substances that are released during synaptic activity. For instance, SP induced Ca^{2+} responses in frog PSCs. These responses were blocked by an NK-1 receptor antagonist and, like ACh- and ATP-induced responses, they were dependent upon intracellular stores (Bourque & Robitaille, 1998). Although SP induced Ca^{2+} responses by itself, exposure to SP actually reduced Ca^{2+} responses associated with synaptic activity, muscarine or ATP exposure (Bourque & Robitaille, 1998). This effect was similar to the reduction in the Ca^{2+} response normally associated with high-frequency nerve stimulation. Moreover, the normal run-down that accompanies repetitive nerve stimulation was attenuated by an NK-1 antagonist (Bourque & Robitaille, 1998), showing that endogenous SP makes an important contribution to this reaction to activity. These data imply that SP released from the nerve terminal during high frequency stimulation modulates the strength of the PSC response to classic neurotransmitters that are released concurrently with the peptide. Accordingly, it appears that PSC responses are controlled by different signals from the nerve terminal.

Depending on the duration and intensity of synaptic activity—resulting in secretion of different neurotransmitters, peptides, and possibly different retrograde messengers released from the muscle fibre—PSC reaction to neurotransmitter exposure is likely to be altered by variable activation of intracellular signalling pathways. In keeping with this, the Ca^{2+} response of PSCs to synaptic activity varies according to the intensity of that activity (Rousse & Robitaille, 2001). But the PSC Ca^{2+} response is likely only one of many reactions to neurotransmission. Indeed, given that several receptors are activated on PSCs during synaptic activity (summarized in Table 1), complex patterns of signal transduction are likely generated. An important question is whether these different signalling responses result in different regulation of synaptic function by PSCs. Evidence suggests that this is the case. In fact, PSCs differentially regulate neurotransmission according to activation of different signal transduction pathways in response to synaptic activity.

Perisynaptic Schwann cells and regulation of neurotransmission

The NMJ is characterised by several short-term plastic events that are coincident with—or immediately follow—intense synaptic activity (Regher & Stevens, 2001; Zucker & Regehr, 2002). These changes in neurotransmission are primarily the result of effects that are ultimately presynaptic. Prominent among these is

Table 1. Perisynaptic Schwann cell responses to synaptic activity.

	Physiological effect on perisynaptic Schwann cells	*Methods*	*References*
Frog NMJ			
Nerve stimulation	$[Ca^{2+}]_i$ increase elicited by transmitter release	Ca^{2+} imaging	Jahromi *et al.* (1992) and Reist and Smith (1992)
Acetylcholine	Activation of muscarinic AChRs, increase of $[Ca^{2+}]_i$	Ca^{2+} imaging	Jahromi *et al.* (1992) and Robitaille (1997)
	Downregulation of GFAP expression	GFAP immunostaining	Georgiou *et al.* (1994, 1999)
ATP	Activation of P2X, P2Y, increase of $[Ca^{2+}]_i$	Ca^{2+} imaging	Jahromi *et al.* (1992) and Robitaille (1995)
Substance P	Activation of NK1 receptors, increase of $[Ca^{2+}]_i$	Ca^{2+} imaging	Bourque and Robitaille (1998)
Deprivation of synaptic activity	Increase of GFAP expression	GFAP immunostaining	Georgiou *et al.* (1994, 1999)
Mouse NMJ			
Acetylcholine	Activation of muscarinic AChRs, increase of $[Ca^{2+}]_I$	Ca^{2+} imaging	Rochon *et al.* (2001)
Adenosine	Activation of A1 receptors, increase of $[Ca^{2+}]_i$	Ca^{2+} imaging	Rochon *et al.* (2001)

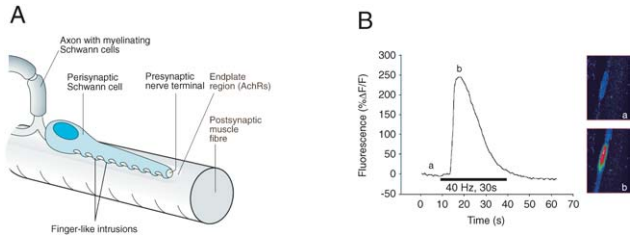

Fig. 1. Perisynaptic Schwann cells at the frog neuromuscular junction and their Ca^{2+} response to synaptic activity. (A) PSCs, non-myelinating glial cells located at the NMJ, are closely apposed to nerve terminals and extend intermittent finger-like intrusions into the synaptic cleft. These intrusions are located near nerve terminal active zones, where synaptic vesicles are concentrated. Thus, PSCs are well placed anatomically to interact with neurotransmission. (B) A typical PSC Ca^{2+} response to high-frequency motor nerve stimulation at the frog NMJ. Intracellular fluorescence changes were monitored using the Ca^{2+} indicator Fluo3-AM. Nerve stimulation at 0.2 Hz did not elicit a PSC Ca^{2+} increase, whereas stimulation at 40 Hz for 30 sec elicited a robust increase. Insert *a*. shows a false color confocal image of a PSC at rest. Insert *b*. shows the same PSC at the peak of the Ca^{2+} response accompanying 40 Hz stimulation. Blue represents low levels of fluorescence. and red high levels. Insert *a*. and *b*. depict the same cell as in the graph.

depression (Betz, 1970), characterised by reduced neurotransmitter release coincident with depletion of the readily releasable pool of synaptic vesicles. In contrast to depression, neurotransmitter release is increased by facilitation when the nerve terminal is invaded by successive action potentials within a very short time frame

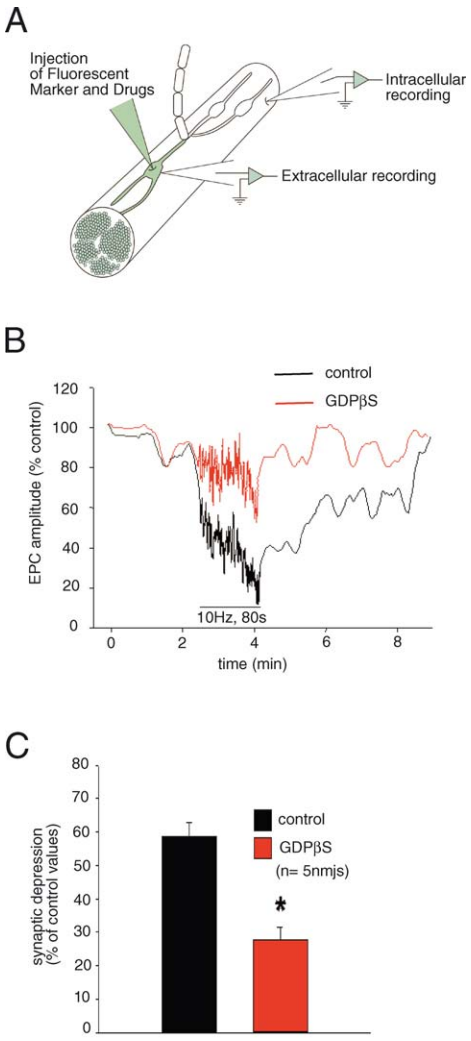

Fig. 2.

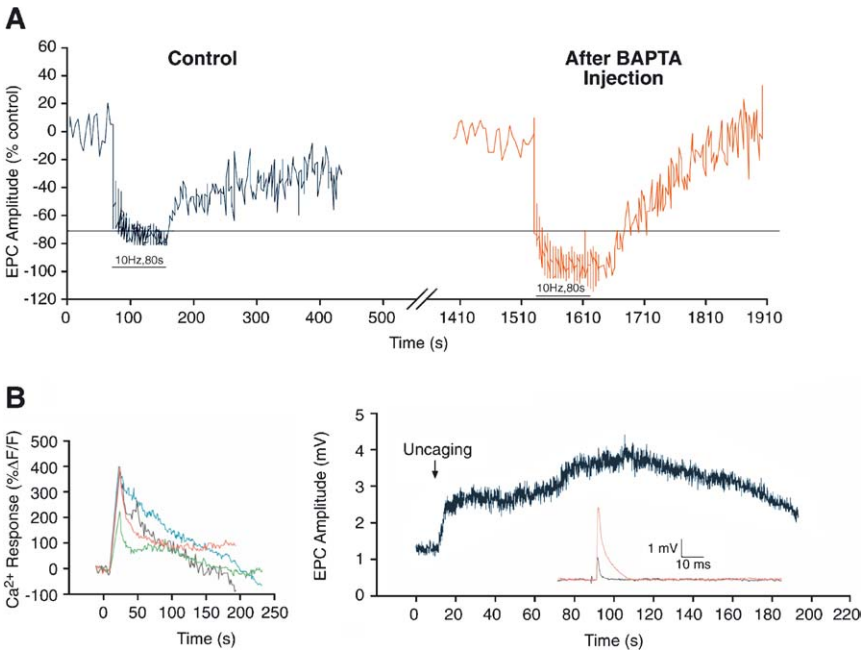

Fig. 3.

(tens of milliseconds) (Mallart & Martin, 1968). Under these conditions, presynaptic changes in the probability of release augment the number of quanta of neurotransmitter released and thus augment the size of the end-plate potential following action potential invasion of the nerve terminal. There is considerable evidence to suggest that residual terminal Ca^{2+} plays a role in this facilitation (Regher & Stevens, 2001; Zucker & Regehr, 2002). Other activity-dependent effects that enhance neurotransmission include augmentation (Magleby & Zengel, 1976) and potentiation (Rosenthal, 1969) and occur following more prolonged stimulation. Potentiation is initiated following a fairly sustained train of inputs. Here, the presynaptic increase in neurotransmitter release is larger and longer (up to several minutes) than that associated with facilitation. Presynaptic Ca^{2+} changes are also likely to play key roles in augmentation and potentiation, but the factors that influence these changes have not been fully elucidated (Regher & Stevens, 2001; Zucker & Regehr, 2002). Recent data suggest that PSCs can influence short-term plasticity at the NMJ, likely through secreted molecules that ultimately cause changes in the presynaptic terminal (Robitaille, 1998, Castonguay & Robitaille, 2001).

G-proteins and reduction of neurotransmission by perisynaptic Schwann cells

The contribution of PSCs to short-term synaptic efficacy was evaluated by selective modulation of PSCs by specific intracellular injections that did not perturb the nerve terminal or muscle fibre. Considering that the most significant neurotransmitters responsible for activating PSCs during high-frequency stimulation act by G-protein coupled receptors, these cells were injected with GTP analogues to mimic their activation by neurotransmitter or with GDP analogues to inhibit this activation (Robitaille, 1998). Immediately following PSC activation by injection of GTPγS (a non-hydrolysable GTP-analogue), neurotransmitter release associated with low frequency-stimulation (0.2 Hz) was reduced. This decrease was related to a reduced probability of release and is thus consistent with a presynaptic effect (Robitaille, 1998). These data suggest that PSCs, upon activation, influence nerve terminal release of neurotransmitter.

By contrast, inhibition of G-proteins by GDPβS (a non-hydrolysable GDP analogue) injection did not affect neurotransmitter release during low-frequency nerve terminal firing (Robitaille, 1998). Interestingly, similar low-frequency activity does not activate PSCs as indicated by the absence of detectable Ca^{2+} elevation (Jahromi *et al*., 1992). Hence, PSCs do not modulate neurotransmitter release in a tonic fashion. Rather, their acute influence on synaptic activity is dependent upon G-protein activation, presumably in a manner directly related to synaptic activity. It should be indicated that even though low levels of synaptic activity are not associated with detectable PSC Ca^{2+} responses or PSC-dependent neuromodulation, PSCs can detect and respond to low-levels of synaptic activity in a different fashion. Indeed, low-frequency nerve firing helps to establish specific patterns of PSC gene expression (Georgiou *et al*., 1994, 1999).

The activity-dependent hypothesis of feedback modulation of neurotransmission by PSCs was further investigated using injections of GDPβS into PSCs during high-frequency nerve terminal firing (10 Hz, 80 s) (Fig. 2), a level of activity normally associated with depression of neurotransmission (Robitaille, 1998).

Fig. 2. Modulation of synaptic depression by perisynaptic Schwann cells. PSC G-protein pathways were manipulated to assess PSC modulation of synaptic depression. (A) G-protein activity in a single PSC was inhibited by specific ionophoretic injection of GDPβS. Focal electrode recordings of end-plate currents (EPCs) represent neurotransmitter release in the local vicinity of the injected PSC. Intracellular electrode recordings of end-plate potentials (EPPs) were used to monitor the possibility of nerve terminal perturbation during placement of the other electrodes (data not shown). (B) EPC amplitude expressed as a percentage of control value before, during and after a train of high-frequency stimuli (10 Hz, 80 s). In black, traces in control before GDPβS injection in the PSC. In red, traces recorded 25 min after GDPβS injection. Inhibition of PSC G-protein activity led to reduced synaptic depression in the local region of PSC coverage. (C) Average depression in control condition (black) and following PSC GDPβS injection (red). Synaptic depression was significantly reduced by blocking PSC G-protein activity ($p < 0.05$). Adapted from Robitaille (1998).

Fig. 3. Ca^{2+} increase in perisynaptic Schwann cells enhances neurotransmission. (A) EPC amplitude expressed as a percentage of control value before, during and after a train of high-frequency stimuli (10 Hz, 80 s). In black, traces in control condition. Buffering intracellular Ca^{2+} with ionophoretic injection of BAPTA injection into a specific PSC resulted in a more pronounced depression (red trace). This demonstrates the existence of potentiating effects of PSCs on neurotransmitter release during high frequency synaptic activity. Adapted from Castonguay and Robitaille (2001). (B) Ca^{2+} uncaging with DMNP-EDTA-AM in PSCs increases intracellular Ca^{2+} in four PSCs from the same NMJ (each coloured line represents the response of a single PSC). This is associated with potentiation of EPP amplitude evoked at low frequency nerve stimulation (0.2 Hz) recorded in the presence of D-tubocurarine chloride to block muscle contractions. Insert shows a typical EPP before (black trace) and at the peak of glial induced potentiation (red trace). These data suggest that Ca^{2+} increase in PSCs is sufficient to induce synaptic potentiation.

Antagonism of G-protein signalling with GDPβS, a manipulation that effectively isolated the PSC from its most significant input (*e.g.*, ACh, ATP), reduced synaptic depression (increased transmitter release) (Robitaille, 1998). Accordingly, these observations suggest that high-frequency nerve stimulation results in activation of G-proteins in PSCs (probably via neurotransmitter receptors, including muscarinic and purinergic receptors), which causes the production/release of neuromodulators that subsequently reduce transmitter release.

Although these data suggest that PSCs contribute to the extent of synaptic depression, it is important to note that reduction in neurotransmitter release associated with high-frequency firing 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 non-neuronal, glial elements.

These observations led to the proposal that glial cells should be considered important elements of chemical synapses (Ullian & Barres, 1998) and helped give rise to the concept of the tripartite synapse (Araque *et al*., 1999), which contends that functionally as well as morphologically, synapses consist of not only of pre- and postsynaptic elements, but also of perisynaptic glial cells. One possible function for PSC control of neurotransmission at the NMJ could be to slow potentially overworked neurons or muscle fibres. This could contribute to a more rapid recovery from high-frequency firing, enabling reduced fatigue.

Glutamate and nitric oxide as neuromodulators at the NMJ

The evidence discussed previously suggests that PSCs influence a portion of synaptic depression at the NMJ. In light of this, our laboratory has investigated molecules that could be involved in PSC modulation of depression. Notably, glutamate and nitric oxide have been studied in light of an activity-dependent model of depression.

GLUTAMATE MODULATION OF NEUROTRANSMISSION AT THE NMJ

The NMJ has traditionally been considered to be purely cholinergic. However, recent evidence has shown that glutamate is a neuromodulator at this synapse. Application of exogenous glutamate decreases miniature EPP frequency and EPP amplitude, both by approximately 20%, and this is dependent upon metabotropic receptor signalling (Lévesque & Robitaille, 2000; Pinard *et al*., 2002). Moreover, these effects are consistent with an ultimately presynaptic mechanism. Since the immunohistochemical data suggest that metabotropic receptors are located primarily on the postsynaptic muscle fibre, to be consistent with a presynaptic mechanism, there must be a retrograde messenger(s) produced (possibly nitric oxide; see below) that affects the nerve terminal.

In addition to the effects of exogenous glutamate, endogenous glutamate makes a contribution to activity-dependent depression. Antagonists of metabotropic glutamate receptors reduced depression. By contrast, antagonists to the glutamate-aspartate transporter (GLAST) increased depression and this is consistent with reduced clearance and accumulation of excess glutamate in the synaptic cleft. GLAST immunoreactivity was found mainly on PSCs, although it may also be present on the nerve terminal (Pinard *et al*., 2002). The finding of this transporter on PSCs is consistent with the observation that they stain intensely for glutamate (Waerhaug & Ottersen, 1993; Lévesque *et al*., 2000). These observations are particularly poignant in light of the evidence that CNS glial cells (astrocytes) are key regulators of synaptic glutamate levels through action of their uptake systems (Bergles & Jahr, 1998; Danbolt, 2001). Moreover, CNS astrocytes can even release glutamate (Parpura *et al*., 1994; Mazzanti *et al*., 2001). Whether PSCs modulate synaptic depression by release of glutamate or by regulation of its uptake are current research focuses of the laboratory.

The presence of glutamate and its receptors at the NMJ, as well as its effects on neurotransmission, have also been described by other groups in different species and at different developmental stages. It was shown that NR-1 subunits of glutamate NMDA receptors are present at the rat and mouse NMJ (Berger *et al*., 1995; Grozdanovic & Gossrau, 1998) and it has been reported that NMDA receptor activation can suppress non-quantal secretion of ACh at the NMJ (Malomuzh *et al*., 2002). Moreover, glutamate has been shown to be excitatory at immature, developing NMJs (Liou *et al*., 1996). The differences in glutamate action and receptor profile reported in these and our studies is likely related to species (*e.g.*, mouse versus frog) and developmental variations. Nevertheless, the common thread between these studies is that neurotransmission at this classic cholinergic synapse is modulated by glutamate, although the nature of this modulation appears to be context specific.

NITRIC OXIDE MODULATION OF NEUROTRANSMISSION AT THE NMJ

Nitric oxide (NO) is a membrane-permeant gas that has been implicated in various forms of synaptic plasticity (Bredt & Snyder, 1992; Brenman & Bredt, 1997). At the NMJ, NO-synthase (NOS; the enzyme responsible for NO production) is concentrated at the end-plate, in the nerve terminal and in PSCs (Kusner & Kaminski, 1996; Yang *et al*., 1997; Descarries *et al*., 1998). NO is thought to participate in myoblast fusion (Lee *et al*., 1994) and synapse elimination (Wang *et al*., 1995) at immature

NMJs. At mature NMJs, NO is important for muscle metabolism (Young *et al*., 1997) and participates in tissue repair (Anderson, 2000). In addition, NO has been implicated in synaptic depression at crayfish (Aonuma *et al*., 2000) and frog (Lindgren & Laird, 1994; Thomas & Robitaille, 2001) NMJs.

NO-donors reduced EPP amplitude and miniature EPP frequency at the frog NMJ and this is consistent with a presynaptic effect (Lindgren & Laird, 1994; Thomas & Robitaille, 2001). Moreover, NO constitutively modulates neurotransmission, with NO scavengers and NOS inhibitors increasing EPP amplitude. The effect of NO donors on neurotransmission at the NMJ was abolished by prior perfusion with a guanylate cyclase inhibitor, indicating that NO depresses transmitter release by a cGMP dependent mechanism(s) (Thomas & Robitaille, 2001).

In addition to tonic modulation of neurotransmission, NO contributes to the depression in an activity-dependent manner. Application of NO scavengers reduced the level of depression (increased neurotransmission) associated with high-frequency nerve firing (Thomas & Robitaille, 2001). However, NO participated in depression by cGMP-independent mechanisms, since the use of a guanylate cyclase inhibitor did not reduce depression.

Given that PSCs have NOS (Descarries *et al*., 1998) and respond to high-frequency synaptic activity (Jahromi *et al*., 1992), NO production may play a role in activity-dependent modulation of neurotransmission by PSCs. In light of the fact that PSC G-protein activation is coupled to depression of neurotransmission (Robitaille, 1998), it is interesting that NO production has been linked to G-protein receptors (including muscarinic receptors) in a variety of cell types (Balligand *et al*., 1993; Quinson *et al*., 2000).

Although PSCs may produce NO, the presence of NOS in other NMJ compartments implies that there could be multiple NO sources.

COULD NO BE INVOLVED IN GLUTAMATE ACTIONS?

There is considerable evidence that links glutamate to NO production in the CNS (Okada, 1992; Yamada & Nabeshima, 1997) and in the PNS (Malomuzh *et al*., 2002). Moreover, glutamate receptors and NOS are often colocalized (Soares-Mota *et al*., 2001).

There is a remarkable level of similarity between the effects of NO and glutamate on synaptic transmission at the NMJ (Lévesque *et al*., 2000; Thomas & Robitaille, 2001). Application of exogenous glutamate or NO reduces neurotransmitter release at low synaptic-activity levels and both endogenous molecules increase depression induced by high-frequency firing of the nerve. The presence of mGluRs on the muscle fibre and the presynaptic effects of glutamate suggest that there is an intercellular signalling molecule. Owing to the clear similarities in their effects, NO is a good candidate to mediate glutamate neuromodulation.

If the effects of glutamate are dependent on NOS activation, then glutamate effects on neurotransmitter release should be reduced when NO metabolism is impaired. In keeping with this hypothesis, glutamate effects on neurotransmission were markedly reduced in the presence of an NO scavenger or an NOS inhibitor (Pinard *et al*., 2002). In contrast, not all the NO production and its consequent action on neurotransmission are the result of release of glutamate. This is suggested by the more pronounced action of NO versus glutamate on depression. In addition, endogenous NO tonically modulates synaptic transmission (Thomas & Robitaille, 2001), whereas glutamate does not (Lévesque *et al*., 2000). These data suggest that an additional glutamate-independent pathway(s) of NO production contributes to synaptic depression and tonic modulation of neurotransmission.

Calcium and potentiation of neurotransmission by perisynaptic Schwann cells

As discussed previously, either high-frequency nerve activity or application of neurotransmitters induces release of internal Ca^{2+} stores within PSCs (Jahromi *et al*., 1992). Nevertheless, until recently, the functional consequences of these Ca^{2+} transients have been unclear. In contrast to PSC G-protein activation, which decreases neurotransmission (Robitaille, 1998), recent experiments show that PSC Ca^{2+} elevation is associated with *increased* neurotransmission.

Thapsigargin, a potent and irreversible Ca^{2+}-ATPase pump inhibitor, was used as a tool to induce Ca^{2+} release from internal stores (Thastrup *et al*., 1990). When bath applied, thapsigargin induced a delayed and transient Ca^{2+} increase in PSCs. This was likely the result of a gradual leak of Ca^{2+} from internal stores that eventually resulted in their depletion. Following thapsigargin treatment, internal stores likely remained empty, as indicated by the inability of various agonists to induce further Ca^{2+} increases in PSCs. These results suggest that, at rest, PSC Ca^{2+} stores are fully loaded and ready to respond to physiological input (*i.e*., an increase in synaptic activity). Accordingly, internal Ca^{2+} stores likely serve as a trigger mechanism for glial activation by neurotransmission.

Following a rapid initial increase in transmitter release (likely associated with increased terminal Ca^{2+}), a second phase of transmitter release increase was observed (Castonquay & Robitaille, 2001). This was slower to build and was transient. The slow phase of increased transmitter release correlated with the slow increase of PSC Ca^{2+} that was also induced by thapsigargin. To test the hypothesis that thapsigargin-induced PSC Ca^{2+} increases might be responsible for this slow increase in transmitter release, BAPTA was

Table 2. Some mechanisms of synaptic plasticity at the neuromuscular junction: potential Perisynaptic Schwann cell involvement.

	Molecules involved	*Methods*	*References*
Synaptic depression	Glutamate → NO → depression	Antagonist of mGluR and NO scavenger decrease high frequency depression	Thomas and Robitaille (2001) and Pinard *et al.* (2002)
Evidence for potential PSC involvement	– Glutamate	Positive for glutamate staining	Waerhaug and Ottersen (1993) and Lévesque *et al.* (2000)
	– GLAST	Positive for GLAST immunostaining	Pinard *et al.* (2002)
	– nNOS	NADPH diaphorase assay, positive for nNOS immunostaining	Descarries *et al.* (1998)
Enhancement of neurotransmission	Prostaglandins	Indomethacin decreases EPP amplitude, PGE2 increases EPP amplitude	Madden and Van der Kloot (1982, 1985)
Evidence for potential PSC involvement	– PLA2	Positive for PLA2 immunostaining	Pappas *et al.* (1999)
	– COX1	Positive COX1 immunostaining	Pappas *et al.* (1999)

specifically injected into PSCs to chelate Ca^{2+}. This manipulation abolished the delayed and slow increase in PSC Ca^{2+} and neurotransmitter release (Castonguay & Robitaille, 2001). These results strongly suggest that increases in PSC Ca^{2+} are responsible for the concurrent slow increase in nerve terminal transmitter release. The hypothesis that PSC Ca^{2+} responses could positively regulate neurotransmission was supported further by injections of IP_3 into PSCs, which induced Ca^{2+} release in PSCs and caused a slow and transient enhancement of low-frequency neurotransmitter release (Castonguay & Robitaille, 2001). Similarly, photolysis of Ca^{2+} in PSCs, using multiphoton excitation, increased transmitter release (Fig. 3).

Since Ca^{2+} elevation in PSCs resulted in a potentiation of transmitter release, it was hypothesised that the PSCs Ca^{2+} responses associated with high-frequency nerve stimulation would have similar effects. Thus, it was predicted that buffering intracellular Ca^{2+} in PSCs would result in increased in synaptic depression owing to occlusion of the Ca^{2+}-dependent potentiating mechanisms. Indeed, specific BAPTA injection into PSCs resulted in more pronounced synaptic depression (Castonguay & Robitaille, 2001) (Fig. 3). These data suggest that release of Ca^{2+} from internal PSC stores contributes to establishing synaptic efficacy at intact NMJs in an activity-dependent manner. One messenger possibly released by PSCs and involved in this positive feedback regulation is prostaglandin E2. Cyclooxygenase (COX; the prostaglandin E2 synthesis enzyme) is localized to PSCs. Moreover, prostaglandin E2 enhances neurotransmission at the NMJ (Pappas *et al.*, 1999), whereas pharmacological blockade of COX results in more pronounced synaptic depression.

Our data imply that the same G-protein coupled receptors likely activate pathways in PSCs that act to enhance (effected by Ca^{2+}) (Castonguay & Robitaille, 2001) or reduce (unidentified effector mechanism) (Robitaille, 1998) neurotransmission. The different feedback modulatory actions of PSCs on synaptic transmission are summarized in Table 2.

Perspective: Perisynaptic Schwann cells and synaptic plasticity

In response to high-frequency synaptic activity, positive and negative modulation of synaptic efficacy by PSCs likely contributes to the various plastic events associated with a particular level of activity. It may seem contradictory for PSCs to positively and negatively modulate neurotransmission in response to the same synaptic input within a brief time-period. This is, however, in keeping with what is understood regarding short-term synaptic plasticity. Indeed, during high-frequency stimulation, the associated decrease in neurotransmitter release is actually the net product of several plastic processes. These include depression, facilitation, augmentation and potentiation, and each makes a variable contribution according to the frequency and duration of nerve terminal activity (Regher & Stevens, 2001; Zucker & Regehr, 2002). Thus, the apparently contradictory neuromodulation by PSCs is in keeping with a role in providing activity-dependent feedback to control neurotransmission. Indeed, PSCs may make distinct contributions to the differential short-term plastic events associated with high-frequency activity. Most likely, these different effects are mediated by release of different effector molecules.

Since 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.

Perisynaptic Schwann cells and long-term changes at the neuromuscular junction

Traditionally, neurotransmission and long-term synaptic stability/change have been studied separately. Nevertheless, studies have clearly demonstrated that synaptic-activity is an important determinant of synaptic state. For instance, presynaptic blockade of neurotransmission with botulinum toxin induces both nerve terminal sprouting and PSC process extension (Son & Thompson, 1995a). More dramatically than neurotransmission blockage, denervation has been associated with robust PSC process extension (Reynolds & Woolf, 1992; Woolf *et al*., 1992; Mehta *et al*., 1993; Chen & Ko, 1994; Son & Thompson, 1995a, b; Son *et al*., 1996; Astrow *et al*., 1998; Love & Thompson, 1999; O'Malley *et al*., 1999; Koirala *et al*., 2000). One identified function of PSC processes following denervation-associated loss of nerve contact and neurotransmission appears to be to guide re-innervating nerve terminals to end-plates (Son & Thompson, 1995a, b; Son *et al*., 1996; Astrow *et al*., 1998; Love & Thompson, 1999; O'Malley *et al*., 1999; Koirala *et al*., 2000). There is also evidence that PSCs could guide innervating axons during normal NMJ development (Herrera *et al*., 2000). Given that large changes in PSCs accompany the severe environmental changes associated with denervation, our laboratory is interested in whether PSCs may exhibit subtler differences between NMJs that are naturally dissimilar.

Evidence shows that the NMJ is a structure capable of physiological and morphological changes in response to a modified environment. These include various forms of short-term plasticity that accompany high levels of synaptic activity and last for a few minutes at most (see above). There are, however, longer-lasting forms of plasticity at the NMJ that can persist for several days and require alterations in gene expression and new protein synthesis (Nguyen & Atwood, 1990; Davis *et al*., 1996). Long-lasting modifications of both pre- and postsynaptic elements have been described for many species in response to various factors, including aging, hormonal status and motor activity. When subjected to prolonged nerve stimulation, the rabbit NMJ undergoes morphological changes that include (1) reduced NMJ surface area, length and width, (2) increased number of postsynaptic folds per unit area and (3) increased nerve terminal vesicle and mitochondria density (Somasekhar *et al*., 1996). Chronic nerve stimulation also imposes a decrease in transmitter release and a higher resistance to synapse fatigue at the frog NMJ (Hinz & Wernig, 1988). Moreover, seasonal behavioural changes in the crayfish that are associated with increased neuronal activity result in a switch of phasic NMJs (characterized by large transmitter release and fast fatigue) to tonic NMJs (characterized by smaller transmitter release and slow fatigue) (Lnenicka *et al*., 1986; Lnenicka & Zhao, 1991). By contrast, reduced nerve activity in winter frogs is associated with increased EPP amplitude, lower facilitation and higher depression (Wernig *et al*., 1996). It is not known whether changes in PSCs could directly contribute to changes in neurotransmission observed with activity-dependent transformation. However, given that PSCs can modulate synaptic plasticity at the NMJ—resulting in either an increase or decrease in neurotransmission—it is possible that the balance of this modulation is changed during transformation.

In fact, it seems very likely that some PSC properties will be altered by long-term changes in patterns of neurotransmission. It is already known that reduced nerve activity results in GFAP upregulation (Georgiou *et al*., 1994). Additionally, there are changes in PSC morphology, physiology and gene expression that follow environmental changes such as denervation. Following denervation, PSCs invade the synaptic cleft (Bevan *et al*., 1973; Dennis & Miledi, 1974; Ko, 1981), can release ACh (Bevan *et al*., 1973; Dennis & Miledi, 1974) and extend elaborate networks of processes (Reynolds & Woolf, 1992; Woolf *et al*., 1992; Mehta *et al*., 1993; Chen & Ko, 1994; Son & Thompson, 1995a, b; Son *et al*., 1996; Astrow *et al*., 1998; Love & Thompson, 1999; O'Malley *et al*., 1999; Koirala *et al*., 2000). Moreover, PSCs respond to denervation with functional expression of new neurotransmitter receptors (Robitaille *et al*., 1997).

As discussed above, patterns of nerve activity can result in NMJ size changes (Somasekhar *et al*., 1996). In support of the likelihood that there are PSC changes following altered neurotransmission, it is known that PSC numbers positively correlate to end-plate size. For instance, PSC number increases with end-plate size at testosterone-sensitive NMJs (Lubischer & Bebinger, 1999; Jordan & Williams, 2001) and decreases when end-plates shrink (Lubischer & Bebinger, 1999). Since chronic nerve stimulation is associated with reduced NMJ size (Somasekhar *et al*., 1996), it is quite likely that there is a downregulation of PSC number associated with chronic synapse activity. Whether any changes in PSCs occurring coincident with long-term adjustment of the NMJ are passive (reacting to pre- and postsynaptic changes without reciprocally influencing these elements) or executive (playing an active role in coordinating and producing change) is a research focus of our laboratory. Given that PSCs guide nerve terminals during reinnervation and possibly development, it is likely that they may also play a similar role under less extreme circumstances associated with activity-dependent changes in NMJ size.

Conclusion

At the NMJ, PSCs have emerged as important players in processes relating to neurotransmission. They respond to neurotransmission in an activity-dependent fashion. Moreover, they help control neurotransmission in the short-term by modulating the level of depression associated with high-frequency firing. Given these functions in the short-term, as well as their involvement in the re-establishment of synaptic connections following denervation, we hypothesise that they may also participate in long-term modifications of NMJ morphology and physiology that occur in response to changes in synaptic activity. We predict that PSCs are likely important components of an activity-dependent continuum of synaptic efficacy, stability and plasticity at the NMJ. We expect that this relationship helps maintain synaptic efficacy in the short-term, contributes to re-establishing synaptic connections after denervation and may be involved in less dramatic reorganization of the synapse in response to different patterns of neurotransmission. The central feature of this hypothesis is the capacity of PSCs to detect and respond to neurotransmission.

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