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SYNAPSE–GLIA INTERACTIONS ARE GOVERNED BY SYNAPTIC AND INTRINSIC GLIAL PROPERTIES

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Abstract—It is believed that glial cell activation and their interactions with synapses are predominantly dependent upon the characteristics of synaptic activity and the level of transmitter release. Because synaptic properties vary from one type of synapse to another, synapse-glia interactions should differ accordingly. The goal of this work was to examine how glial cell activation is dependent upon the properties of their respective synapses as well as the level of synaptic activity. We contrasted Ca^{2+} responses of perisynaptic Schwann cells (PSCs) at neuromuscular junctions (NMJs) with different synaptic properties; the slow-twitch *soleus* (SOL) and the fast-twitch levator auris longus (LAL) muscles. Amplitude of PSC Ca^{2+} responses elicited by repeated motor nerve stimulation at 40, 50 and 100 Hz were larger and their kinetics faster at LAL NMJs and this, at all frequencies examined. In addition, a greater number of PSCs per NMJ was activated by sustained synaptic transmission at NMJs of LAL in comparison to SOL. Differences in PSC activation could not be explained solely by differences in levels of transmitter release but also by intrinsic PSC properties since increasing transmitter release with tetraethylammonium chloride (TEA) did not increase their responsiveness. As a whole, these results indicate that PSC responsiveness at NMJs of slow- and fast-twitch muscles differ not only according to the level of activity of their synaptic partner but also in accordance with inherent glial properties.

Key words: perisynaptic Schwann cells, synaptic strength, synaptic plasticity, synapse–glia interactions, neuromuscular junction, neuromuscular properties.

In recent years, the traditional view that glial cells of central (CNS) and peripheral nervous systems (PNS) mainly provide support to neurons and synapses has been challenged. Indeed, glial cells are now considered to be active members of their associated synapses as they have been shown to be activated by synaptic transmission and, in turn, to modulate synaptic activity (Auld and Robitaille, 2003; Halassa et al., 2007). Glial cell activation as a function of synaptic activity is therefore a key feature of these bi-directional interactions. Knowing that synapses differ in terms of probability of release, quantal content, level of activity and plasticity, one would predict that glial properties should be adapted with their environment and tuned with their respective synaptic partners. However, it is unclear whether the properties of perisynaptic glial cells differ at synapses with different properties. Therefore, the aim of the present study was to examine the properties of glial cells at differing synapses and to study their activation as a function of the type of synapses they are associated with.

The neuromuscular junction (NMJ) is a valuable model to test this possibility. Indeed, studies of synapse-glia interactions at the NMJ have provided amongst the strongest evidence for the involvement of glial cells in the regulation of synaptic functions as part of a tripartite synapse (Robitaille, 1998; Castonguay and Robitaille, 2001; Todd and Robitaille, 2006; Todd et al., 2007; Feng and Ko, 2007, 2008). Furthermore, the properties of NMJs of various nerve-muscle preparations have been well characterized, in particular, NMJs of fast- and slow-twitch muscles (Gertler and Robbins, 1978; Wood and Slater, 2001; Slater, 2008). These synapses are both cholinergic in nature but differ in terms of their electrophysiological properties and functions. For instance, studies conducted in freely moving rats demonstrated that motor neurons innervating the fast-twitch *extensor digitorum longus* (EDL) muscle preferentially discharge at high frequencies (≈80 Hz) for short periods, therefore behaving in a phasic fashion. On the other hand, motor neurons innervating the slow-twitch soleus (SOL) muscle fire at lower frequencies (20 to 40 Hz) for longer periods of time, exhibiting a tonic behaviour (Henning and Lømo, 1985). Importantly, it has been demonstrated that synapses of fast- and slow-twitch muscles of the rat can also be distinguished by their neurotransmitter release properties where quantal content at NMJs of fast-twitch muscles was shown to be greater than that of NMJs from slow-twitch muscles (Reid et al., 1999). Interestingly, differences in neurotransmitter release properties between EDL and SOL NMJs of the rat have been shown to influence the outcome of post-tetanic plasticity (Lev-Tov, 1987).

Considering that motoneurons at NMJs of phasic and tonic muscles have different patterns of activity that are suited to their normal functioning, we compared the properties of perisynaptic Schwann cell (PSC) activation by monitoring Ca^{2+} responses at NMJs of the predominantly fast-twitch *Levator auris longus* (LAL) muscle (Erzen et al., 2000) and NMJs of the predominantly slow-twitch SOL

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Abbreviations: ACh, acetylcholine; EDL, *extensor digitorum longus* muscle; Em, membrane potential; EPPs, end plate potentials; HEPES, N′-2-Hydroxyethylpiperazine-N′-2 ethanesulphonic acid; LAL, *levator auris longus* muscle; NMJ, neuromuscular junction; PNS, peripheral nervous system; PSCs, perisynaptic Schwann cells; PTP, post-tetanic potentiation; SOL, *soleus* muscle; TEA, tetraethylammonium chloride; TPEN, tetrakis-(2-pyridylmethyl) ethylenediamine.

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muscle (Bishop and Milton, 1997) at different levels of synaptic activity. We observed that PSC Ca^{2+} responses elicited at all frequencies tested were larger and displayed faster kinetics at NMJs of the fast-twitch LAL in comparison to NMJs of the slow-twitch SOL. Additionally, a greater number of PSCs was activated by synaptic activity at NMJs of the fast-twitch LAL. Increasing synaptic activity at NMJs of the slow-twitch SOL muscle did not enhance the ability of PSCs to respond to synaptic activity.

EXPERIMENTAL PROCEDURES

Nerve–muscle preparations

LAL and SOL muscles, with their respective motor nerve, were dissected from CD-1 mice (males, 22–24 g; Charles River Laboratories, St.-Constant, QC, Canada) under deep anaesthesia (0.1 ml g^{-1} midozolam and hypnorm dissolved in distilled water, administered i.p.). This procedure was performed in accordance with the regulations of the Canadian organization for animal welfare and the Animal care committee of the Université de Montréal such that number of animals as well as their suffering were minimized. Nerve-muscle preparations were pinned in an experimental Sylgard-coated recording chamber filled with normal Ringer's solution containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 1$NaHCO_3$, 2 $CaCl_2$, 25 HEPES and 10 glucose, oxygenated with 100% O_2. In some experiments, a solution with no Ca^{2+} added (low Ca^{2+}/high Mg^{2+}) was used. It contained: 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 5 $MgCl_2$, 1 $NaHCO_3$, 25 HEPES and 10 glucose. All experiments were performed within the same regions of the muscles to minimize variability between experiments.

Calcium imaging of PSCs

For Ca^{2+} imaging of PSCs, both types of nerve-muscle preparations described above were incubated for 90 min in an oxygenated Ringer's solution containing 10 or 20 μM fluo-4 AM (Invitrogen Canada Inc., Burlington, ON, Canada), 0.02% pluronic acid (Invitrogen Canada Inc., Burlington, ON, Canada), and 1% DMSO (Sigma, St.-Louis, MO, USA), at room temperature (21 °C). Following the loading period and prior to the experiment, nerve-muscle preparations were perfused for 20 min with normal Ringer's solution containing 20 μM TPEN (tetrakis-(2-pyridylmethyl) ethylenediamine; Invitrogen) to buffer heavy metals which diminish binding of fluo-4 to Ca^{2+} ions. Experiments were performed with Ringer's solution heated to 30 °C with an automatic temperature controller (Warner Instrument; Hamben, CT, USA). Excitation of fluo-4 was provided by the 488 nm line of the argon ion laser of a Bio-Rad MRC 600 laser-scanning confocal microscope attenuated to 1% of its maximal intensity. Emitted fluorescence was detected using a long-pass filter with a cut-off at 515 nm. Localization of NMJs was achieved using transmitted light microscopy with a 40× water immersion objective (0.75 NA: Olympus, Tokyo, Japan). Changes in fluorescence were measured over PSC somata and expressed as:

$$\% \Delta F/F = (F - F_{rest})/F_{rest} \times 100.$$

The level of resting fluorescence was always within the same range for all experiments and all preparations. These values range between 20 and 30 pixel intensity values and experiments were deemed successful only when this basal value remained constant within each experiment. Importantly, the hardware adjustments were also standardized from experiment to experiment where the offset (black level) was between 4.9 and 5.1 and the gain was between 8 and 9. No other hardware amplifications and filtering were performed. A cell was considered responsive when the amplitude of the response was more than 5 pixel intensity above the resting level.

The different parameters of Ca^{2+} responses were defined as follows: latency is the time interval between the start of the motor nerve stimulation and the start of the Ca^{2+} response determined when $\Delta F/F$ is larger than 5% above baseline; time-to-peak is the time between the beginning of the Ca^{2+} response and its maximal amplitude; maximal amplitude is the maximal value of the Ca^{2+} response obtained at its peak; duration corresponds to the time interval measured at 10–90% of responses amplitudes.

Motor nerve stimulation for Ca^{2+} imaging of PSCs

The cut end of the nerve, for both nerve-muscle preparations, was stimulated with a suction electrode at twice the threshold level for muscle contraction (Grass medical instrument; Quincy, MA, USA) at various frequencies (40, 50 and 100 Hz) while maintaining the duration of the stimulus train constant (30 s). A lower frequency (e.g. 20–30 Hz) that would be more in tune with the properties of the SOL muscle was not used because PSCs of LAL muscle were quite unresponsive at this frequency. Muscle contractions generated by neurotransmitter release were prevented by blocking nicotinic receptors with α-bungarotoxin (20 μM for the LAL and 60 μM for the SOL; Invitrogen Canada Inc., Burlington, ON, Canada). Synaptically evoked PSC Ca^{2+} responses show a rundown whereby the amplitude of Ca^{2+} responses are reduced when elicited at 20 min intervals (Jahromi et al., 1992; Rochon et al., 2001). To avoid rundowns of PSC Ca^{2+} responses elicited by repeated nerve stimulations, experiments performed in the presence of tetraethylammonium chloride (TEA) were compared to a different set of experiments performed in control conditions.

Electrophysiological recordings of synaptic transmission

The distal end of the motor nerve was stimulated using a suction electrode at intensity twice above the threshold for muscle contractions. Muscle contractions were prevented by adding D-tubocurarine chloride (1.0 to 1.5 μM; Sigma) to the perfusion solution. Intracellular recordings of end plate potentials (EPPs) were performed with glass microelectrodes (10–15 MΩ) filled with KCl (2 M). Synaptic signals were amplified 100 to 500 times and filtered at 2 kHz with a Warner DC amplifier. Evoked synaptic responses at various frequencies were digitized at 10 kHz and recorded with Strathclyde electrophysiology software (J. Dempster, University of Strathclyde, Scotland). All experiments were performed on muscle fibres having a membrane potential (Em) more negative than −65 mV. Experiments were discarded if Em was found to be depolarized by more then 10 mV. Experiments were performed with continuous perfusion of normal Ringer solution heated to 30 °C. EPPs were recorded at 0.2 Hz for 15 min to establish the basal level of synaptic transmission.

Synaptic plasticity of the SOL and LAL muscles was studied as follows. After the recording of a control period with stimulation at 0.2 Hz, frequency of motor nerve stimulation was increased to one of the following: 10, 20 or 40 Hz for 30 s or 50 Hz for 60 s. Data for EPP amplitude was smoothed by using a moving bin averaging of three consecutive events. Synaptic depression following high frequency stimulation was defined by the reduction in amplitude measured for the last five EPPs of the train of stimuli and expressed as percentage of the averaged amplitude of the EPPs during the control period. Post-tetanic potentiation (PTP) was determined by calculating the ratio of the averaged amplitude of the 10 EPPs that immediately followed the end of the high frequency stimulation over the averaged amplitude of EPPs during the control period.

Drugs

Acetylcholine (ACh), adenosine, ATP and muscarine (20 μM) were dissolved in the same Ringer's solution used for the perfu-