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Interneuronal synapses formed by motor neurons appear to be glutamatergic

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Abstract

Acetylcholine release at motor neuron synapses is long established, however recent discoveries indicate that synaptic transmission by motor neurons is more complex than previously thought. Using whole-cell patch clamp we show that spontaneous excitatory post-synaptic currents of rat motor neurons in primary ventral horn cultures are entirely glutamatergic, although the cells respond to exogenous acetylcholine. Motor neurons in culture express the vesicular glutamate transporter VGlut2, and culturing motor neurons for weeks while blocking glutamate receptors up-regulates glutamate signaling, without increasing cholinergic signaling. In spinal cord slices, motor neurons showed no decrease in spontaneous excitatory synaptic potentials after blocking acetylcholine receptors. Our results suggest that motor neuron synapses formed on other neurons are largely glutamatergic in culture and the spinal cord.

Keywords

motor neuron; spinal cord; patch clamp; primary culture; slice recording

Introduction

For generations it has been accepted that motor neurons release acetylcholine (ACh) at the neuromuscular junction [1] and in the spinal cord [2], however recent data are forcing a reevaluation. Numerous studies have shown that motor neurons and neuromuscular junctions express proteins related to glutamatergic transmission [3-10], and multiple laboratories have confirmed that collateral connections they make on other neurons in the spinal cord include glutamatergic and cholinergic synapses [10-12]. Cholinergic and glutamatergic terminals appear to be spatially segregated, and most central synapses appear to be glutamatergic [10-12].

Our earlier work investigating the network properties of embryonic motor neurons in primary culture on multielectrode probes demonstrated that antagonists for ACh receptors had no effect on the activity of the neurons, while blocking glutamate receptors shut down spiking activity in the culture [13]. This result suggests that ACh signaling plays no role in the neuronal activity driven by synaptic connections among cells in the culture.

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In the present work, we have used whole-cell patch clamp to further investigate synaptic connectivity among motor neurons in primary cultures and within the ventral horn of the spinal cord.

Methods

Animal procedures were conducted in conformity with National Institutes of Health Guidelines for Care and Use of Laboratory Animals. Primary cultures were obtained from ventral horns of spinal cords of Sprague-Dawley rat embryos on embryonic day 14 according to published methods [13].

For Immunofluorescence, cultured motor neurons were fixed and permeabilized as described previously [13]. Cells were incubated overnight at 4°C with rabbit anti-Hb9 and mouse anti-VGlut2 (Abcam) and imaged on an Olympus IX70 fluorescence microscope.

For whole-cell patch clamp, cultured cells were recorded after 7 - 21 days in culture. Postsynaptic currents were recorded using voltage-clamp, while membrane potential and action potentials (AP)s were recorded in current-clamp. Modified Tyrode solution containing (in mM): 140 NaCl, 5.4 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose (pH 7.4 osmolality 320 mosM) was perfused (1ml/min) to the cultured motor neuron during recording. Internal solution: (mM) K-gluconate, 135; MgCl₂, 2.0; HEPES, 10.0; EGTA, 0.5; ATP-Mg, 4.0; Na-GTP, 0.5, pH 7.2-7.4, 290-310 mOsm. Borosilicate glass electrodes had an impedance of 4 - 6 MΩ. Motor neurons were identified by their morphology - a large, triangular-shaped soma (> 20 μm).

Spinal cord slices were prepared from 12-18 day old rat pups as described previously [14] and were preincubated for at least 1 h, and then continuously perfused with Krebs solution containing (mM) NaCl, 117.0; KCl, 3.6; MgCl₂, 1.2; CaCl₂, 2.5; NaH₂PO₄, 1.2; glucose, 11.0; and NaHCO₃, 25.0, oxygenated with 95% O₂ and 5% CO₂ at 34°C. Recordings of postsynaptic currents began 3 minutes after whole-cell access was established and current reached steady state, and were abandoned if input resistance changed more than 15%. Spontaneous postsynaptic currents were recorded in voltage-clamp with QX-314 added to the intra-pipette solution to eliminate action potentials. Excitatory post-synaptic currents (sEPSCs) were recorded in the presence of 20 μM bicuculline and 2 μM strychnine. To record miniature EPSCs 1 μM TTX was added. Inhibitory post-synaptic currents (sIPSCs) were recorded in the presence of 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). APs and sPSCs were analyzed with MiniAnalysis (Synaptosoft), and amplitude and frequency were measured over at least 2 min.

Results

Characterization of motor neurons in primary cultures

Immunofluorescence using antibodies directed against a motor neuron marker (Hb9) show that in our ventral horn cultures, most of the neurons are motor neurons (~76% of DAPI positive neurons were HB9 positive in 9 preparations). Hb9 positive cells in our culture were usually also positive for the vesicular glutamate transporter, VGlut2 (55% of Hb9 positive cells were also VGlut2 positive in 3 preparations) (Fig. 1).

Spontaneous EPSCs recorded from motor neurons in the cultures provide striking evidence that virtually all synaptic transmission in ventral horn cultures is glutamatergic. The sEPSCs represent excitatory responses to synaptic inputs from other cells, most of which are motor neurons. The frequency and amplitude of sEPSC's is unchanged by blockade of both

muscarinic and nicotinic acetylcholine receptors, while antagonist to glutamate receptors completely silence spontaneous synaptic transmission (Fig. 2).

The lack of spontaneous synaptic currents driven by cholinergic input is not due to a lack of excitatory responses to ACh. In current clamp, ACh increased action potential frequency in most motor neurons (Fig. 3). Cells in which ACh did not increase spike frequency (5 out of 11) showed a significant increase in the frequency of excitatory post-synaptic potentials (EPSPs) from 1.7 ± 0.8 Hz in control to 4.7 ± 1.4 Hz ($p < 0.05$; Student's *t* test). These cells had lower basal firing rates perhaps reflecting a tonic inhibition that ACh could not overcome. The stimulatory effect of ACh appears to be mediated by nicotinic receptors as the stimulation was blocked by the nicotinic antagonist mecamylamine, but not the muscarinic antagonist atropine (Fig. 3C). Bath-applied ACh also increased the frequency of sIPSCs from 0.8 ± 0.2 Hz to 2.1 ± 0.4 Hz ($n = 3$) an effect that was also blocked by mecamylamine, but not atropine.

Chronic blockade of glutamate transmission in culture

Motor neurons dissociated at E14 show little measurable spiking or post-synaptic currents before 5 – 6 days in culture. Given this slow development of synaptic activity, chronic blockade of glutamate receptors during early stages of culture might down-regulate glutamate transmission allowing the development of more cholinergic signaling. To test this, motor neurons were grown in glutamine/glutamate-free media with $20 \mu\text{M}$ CNQX + $100 \mu\text{M}$ AP5 for 14 days beginning 18 hours after establishment of the culture. Growth media was replaced and fresh drug preparations were added every 48 hours.

Chronic blockade of glutamate receptors caused an up-regulation of glutamate signaling as shown by the significant increase in the frequency of sEPSCs (from 3.5 ± 0.9 Hz to 6.0 ± 0.9 Hz; Fig. 4), however, there was no significant difference in the amplitude of sEPSCs between the two groups (56.8 ± 13.5 pA in control Vs 65.9 ± 10.4 pA after blockade). Glutamate blockade also significantly increased the frequency of mEPSCs, which are independent of action potentials in presynaptic neurons, from 0.9 ± 0.3 to 2.9 ± 0.6 Hz. However, growing the neurons for weeks with no glutamate neurotransmission did not increase excitatory cholinergic signaling, as adding glutamate antagonists during recording eliminated all sEPSCs just as in control cultures.

Post-synaptic responses of motor neurons in spinal cord slices

In addition to the excitatory connections to muscle fibers that drive muscle contraction, motor neurons also make collateral connections onto other neurons in the spinal cord. Some collateral connections activate inhibitory interneurons known as Renshaw cells that feed back to inhibit motor neuron pools, but others are recurrent excitatory connections onto other motor neurons [15, 16]. Using whole-cell patch clamp we measured excitatory synaptic inputs to motor neurons in spinal cord slices and found that the average frequency of sEPSCs (10.8 ± 1.4 Hz in baseline conditions) was not changed by blocking ACh signaling with atropine and mecamylamine (10.3 ± 1.1 Hz; $n = 8$; Fig.5). These results suggest little role for ACh in excitatory inputs originating within a slice.

Discussion

Many types of neurons release more than one neurotransmitter including multiple small molecule neurotransmitters [17, 18]. Glutamate in particular, has been observed in neurotransmitter pools in dopaminergic, serotonergic and noradrenergic neurons, [19] and recent work suggests that motor neurons release glutamate as well [7, 9-11]. Motor neurons in primary culture with glial cells have been shown to make autaptic synapses (recurrent

synapses on their own somas) that are almost entirely glutamatergic. Only a subset of neurons showed evidence of cholinergic responses, which appeared to be due to extra-synaptic receptors [20]. Here we present results with patch clamp electrophysiology showing that motor neurons grown 2 - 3 weeks in culture in the absence of contact with muscle cells do not form cholinergic synapses with other neurons, as excitatory neurotransmission in the culture is exclusively glutamatergic. Even when motor neurons are grown in culture in the complete absence of glutamate signaling, excitatory cholinergic transmission is not increased, although glutamate transmission is up-regulated. ACh activates motor neurons through nicotinic receptors, as well as inhibitory (Renshaw) cells in the culture, which then increase their inhibition of motor neurons.

Conclusion

In many cells the expression of neurotransmitter phenotype is plastic and influenced by interactions with the cellular environment [21-24]. Motor neurons may be similar so that in culture, in the absence of muscle and neuromuscular junctions, the capability for cholinergic signaling may not fully develop or may decline over time. Alternatively, since motor neurons in our cultures continue to express choline acetyl transferase, a synthetic enzyme for ACh [13], it may be that motor neurons co-release ACh and glutamate, but ACh receptors are not part of the post-synaptic structure of inter-motor neuron synapses.

The lack of spontaneous excitatory cholinergic inputs to motor neurons we observed in spinal cord slices is consistent with activation at inter-motor neuron synapses being driven largely by glutamate, even in motor neurons with neuromuscular junctions.

Acknowledgments

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Figure 1. Immunolabeling of primary motor neuron cultures

Cultured spinal motor neurons are stained with antibodies against the motor neuron marker Hb9, the vesicular glutamate transporter VGlut2. Secondary antibodies (Jackson ImmunoResearch Laboratories) were labeled with DyLight 549 (red) or FITC (green) from. DAPI staining (blue) indicates cell nuclei. “Merge” combines the left two panels in each row. Images taken after 6 days in culture.

Figure 2. Synaptic transmission in ventral horn cultures is entirely glutamatergic

Top: Sample traces showing sEPSCs recorded from motor neurons in 14-day-old cultures with ACh antagonists (10 μ M each atropine and mecamylamine; left) and a glutamatergic antagonist (10 μ M CNQX; right). Bottom: Pooled data showing average sEPSC frequency and amplitude from 5 motor neurons in each condition. ($p < 0.01$, One-way ANOVA).

Figure 3. Cultured motor neurons show excitatory responses to ACh

A. Sample traces of action potentials (APs) recorded in current clamp mode from cultured motor neurons. Notice the depolarization and increase in EPSP frequency stimulated by ACh in the trace 2nd from bottom. B. Top: Average AP frequency for motor neurons that increased their firing in response to ACh ($n = 6$; * $p < 0.05$; one way ANOVA). Bottom. Average AP frequency for motor neurons that did not increase their firing rate ($n = 5$). Notice the overall low action potential frequency. C. ACh (100 μM) was added alone or in the presence of 10 μM atropine to block muscarinic receptors or 10 μM mecamylamine to block nicotinic receptors.

Figure 4. Chronic block of glutamate receptors upregulates glutamate signaling, but does not increase cholinergic signaling

Motor neuron cultures were grown in glutamate/glutamine-free media and treated 20 μ M CNQX plus 100 μ M AP5 for 14 days after establishing culture. “Control media” - cells grown in standard neurobasal media without glutamate antagonists. “Glutamatergic blocking media” – cells grown with glutamate antagonists. No antagonists were present during the recording. “CNQX + AP5 during recording” – cells grown with glutamate antagonists in which the same antagonists were added back during the recording. A. Sample traces of sEPSCs. B. Sample traces of miniature EPSCs. C. Average frequency of EPSCs for control medium (n = 8) and blocking media (n = 9; * = $p < 0.05$ compared to control media; One way ANOVA).

Figure 5. Synaptic activity of motor neurons in spinal cord slices not affected by ACh receptor block

Top: Sample traces of sEPSCs recorded from motor neurons in spinal cord slices from 6 -7 day-old rat pups with and without ACh receptor antagonists atropine + mecamylamine (10 μ M each). Bottom: Pooled data averaged for 8 cells. A + M added for 5 minutes before recording ($p > 0.05$, One way ANOVA).