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Neuronal and network activity in networks of cultured spinal motor neurons

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Abstract

This is the first report of multielectrode recordings from networks of cultured motor neurons. Neurons isolated from the ventral horns of spinal cords of E15 rats were cultured on MED64 probes. The majority of the neurons in the cultures are positive for neurofilament, choline acetyltransferase, and Hb9, characteristics of motor neurons. The activity of the motor neuron network is characterized by spiking of individual cells as well as spontaneous, synchronized bursts involving all active electrodes. Both spiking and network bursts are stimulated by GABA antagonists and acetylcholine, and are inhibited by GABA itself and glutamate antagonists. Networks of cultured embryonic motor neurons make a good model system for studying motor neuron development and physiology as well as the pathophysiology of motor neuron disease.

Keywords

cell culture; MEA; MED64; motor neuron; multielectrode arrays; spinal cord

Introduction

Historically, physiological studies of motor neurons have elucidated many fundamental properties of neurons and neuronal signaling [1]. For nearly a generation, however, intense interest in long-term potentiation and other forms of neuronal integration has led to a focus on cortical regions and a relative neglect of motor neurons [2]. Yet as models for studying neuronal function, motor neurons have advantages, including our precise and detailed understanding of how their output drives muscle activity.

Key drivers of function and output across all neural systems are the patterns of activity in the neuronal networks. Multielectrode recording techniques make it possible to study patterns of activity across neuronal networks and to investigate how cellular events modulate network function. Dissociated cultures from embryonic rat spinal cord show spontaneous, network-wide bursting activity [3] that becomes a regular oscillating activity following pharmacological block of fast synaptic inhibition [4,5]. Although dissociated spinal cord cultures are useful for studying the biophysical mechanisms involved in generating patterns of activity in neuronal networks, the cellular composition of this type of network is uncertain. In these cultures, ventral horn cells make up only 15% of total cultured cells [6] suggesting that mixed spinal cord

cultures are largely sensory neurons and interneurons that modulate them. In this study, we have made multielectrode recordings from cultures of dissociated neurons isolated from the ventral horns of day 15 rat embryos. These cultures are limited to motor neurons and associated interneurons, so the study of network activity in these cultures allows identification of regulatory and modulatory factors important in the development, activity, and survival of spinal motor neurons. Such networks can also serve as in-vitro models for the study of motor neuron disease.

Methods

Procedures involving animals were conducted in conformity with National Institute of Health Guidelines for the Care and Use of Laboratory Animals. Primary cultures from the ventral horns of spinal cords from 15-day-old embryos of Sprague–Dawley rats (Charles River Breeding Laboratories Inc., Wilmington, Massachusetts, USA) were prepared using methods as described earlier [7,8]. Briefly, ventral horns were dissected, trypsinized, and the cell suspension centrifuged in Opti-Prep (Sigma, St Louis, Missouri, USA). A band enriched for motor neurons was harvested, centrifuged on a 4% BSA cushion, and resuspended in motor neuron medium [neurobasal medium plus B27, horse serum, glutamate, β -mercaptoethanol, brain-derived neurotrophic factor and pen-strep (Mediatech, Manassas, Virginia, USA)]. 1×10^5 of neurons were plated on MED probes (Automate Scientific, Los Angeles, California, USA) that had been previously coated overnight with poly-DL-ornithine followed by overnight coating with laminin.

For immunostaining, motor neurons grown on coverslips for 1–3 days were washed with phosphate buffered saline and fixed with 10% neutral buffered formalin. Cells were permeabilized with 0.1% nonyl phenoxypolyethoxylethanol-40 in phosphate-buffered saline, blocked with 5% BSA in Tris-buffered saline containing 0.05% Tween 20, and incubated with one of the rabbit anti-homeobox gene (Hb9), goat anti-choline acetyltransferase (ChAT), or rabbit anti-neurofilament M antibodies overnight at 4°C. After washing, cells were incubated with Alexa Fluor 488-conjugated or 594-conjugated secondary antibodies (Invitrogen, Carlsbad, California, USA) against the appropriate species. Coverslips were mounted with Vecta-shield medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, California, USA).

Multielectrode recordings were made with the MED64 system (Automate Scientific) with a sampling rate of 20 kHz. The MED probe contains 64 electrodes in an 8×8 grid with interelectrode spacing of 75 μ m. Data were preprocessed in Matlab (The Mathworks Inc., Natick, Massachusetts, USA) by detrending, and non-biological high frequency components (> 5 kHz) were removed using a finite impulse response low-pass filter with linear phase. A high-pass filter was also applied with cutoff frequency of 1 Hz to remove residual decaying offset when spikes occur close to each other. Rasterization of spike trains used a spike extraction protocol that involved calculating the standard deviation, σ , and setting a voltage threshold for spike detection to $\pm 5.5 \sigma$ [9]. Unsupervised spike sorting was performed with wavelets and superparamagnetic clustering [10]. Significance was determined by paired two-tailed Student's *t*-test or one-way analysis of variance with *P* value of less than 0.05 considered to be statistically significant.

Chemicals were obtained from Sigma-Aldrich, except: neurobasal medium, horse serum, and the anti-neuro filament and secondary antibodies from Invitrogen; brain-derived neurotrophic factor from R&D Systems (Minneapolis, Minnesota, USA) Laminin and ChAT antibody from Millipore (Billerica, Massachusetts, USA) and Hb9 antibody from Abcam Inc. (Cambridge, Massachusetts, USA).

Results

Characterization of cultures

In culture, the neurons send out processes and make synapses on other cells. Immunofluorescence shows that the cultures are entirely neuronal (as identified by neurofilament antibodies), and the great majority are motor neurons (Fig. 1). Antibodies to ChAT, an enzyme in the biosynthetic pathway for acetylcholine, and Hb9, a homeobox gene expressed selectively by motor neurons [11], were used as motor neuron markers. The neurofilament antibody labels the long axons characteristic of motor neurons, whereas the Hb9 antibody is expressed in the nucleus and overlaps with the DAPI staining. The larger ChAT and HB9-positive cells tended to cluster together with the smaller, nonreactive cells widely scattered around the culture.

Electrophysiology of motor neuron cultures on MED64 probes

Extracellular action potentials from single neurons can be recorded with the MED64 starting around 7 days of culture. Initially, only sporadic spontaneous activity is visible in a few electrodes. After 9 days in culture, both burst firing and single spikes could be recorded, and the number of active electrodes and the number showing bursting activity increased as the cultures matured. Once burst firing appeared, it occurred simultaneously in almost all active channels (Fig. 2). The frequency of spontaneous bursts ranged from 1.0 to 15/min, and burst duration ranged from 0.25 to 9.41 s as measured from 72 electrodes in five separate probe cultures. Among the five probe cultures analyzed, the number of active electrodes ranged from eight to 23. All spike and burst activities were blocked by the sodium channel blocker tetrodotoxin (Fig. 3f).

Pharmacology of culture activity

A pharmacological characterization of the network properties of the cultured motor neurons was carried out on 13-day old cultures. Basal activity was measured, a drug was added, activity was recorded for 3 min, the drug was washed out, and the activity was recorded again. After the application of γ -aminobutyric acid (GABA, 20 μ M), the average frequency of both bursts and tonic activity were decreased (Table 1, Fig. 3), resulting in a significant decrease in the overall spike frequency (from 3.07 ± 0.37 to 0.53 ± 0.12 Hz). GABA also decreased the burst duration (from 2.72 ± 0.49 to 0.81 ± 0.18 s), the only drug tested that affected that measure.

To determine whether there is a tonic GABA control on the network, the GABA_A receptor antagonist bicuculline was added to the cultures. Bicuculline (20 μ M) increased the average burst frequency, whereas tonic spike activity and burst duration did not significantly change. The large increase in the burst frequency resulted in a significant increase in average overall spike frequency (from 0.84 ± 0.09 to 1.88 ± 0.14 Hz).

As acetylcholine is the main neurotransmitter released by spinal motor neurons, active neurons in the culture would be expected to release acetylcholine onto other motor neurons. Acetylcholine does provide an activating drive in the culture, as bath application increased both the average burst frequency and the average tonic spike frequency, thereby significantly increasing the overall spike frequency (from 0.53 ± 0.14 to 1.79 ± 0.25 Hz). However, basal activity was not affected by the nicotinic receptor antagonist mecamylamine (10 μ M) and muscarinic antagonist atropine (10 μ M). In the presence of both antagonists, burst frequency and tonic spike frequency did not change, resulting in no significant change in the average overall spike frequency (4.65 ± 0.21 vs. 4.85 ± 0.23 Hz).

To determine whether the spontaneous activity recorded from neurons in the culture was due to glutamate-mediated excitation, we tested two glutamate receptor antagonists. After the

application of *N*-methyl-D-aspartic acid (NMDA) receptor antagonist AP5 (2R)-amino-5-phosphonovaleric acid (AP5) 50 μ M), both the burst frequency and the tonic spike activity decreased, whereas the overall spike frequency decreased from 1.90 ± 0.36 to 1.03 ± 0.15 Hz. When the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (20 μ M) was applied with the AP5, all residual burst activity and most tonic activity disappeared, resulting in an average overall spike frequency of 0.17 ± 0.05 Hz.

Discussion

We have cultured spinal motor neurons from E15 rat embryos on the multielectrode probes of the MED64 system, and have characterized the spiking and bursting activity typical of the cultures. Our results with the MED64 system represent the first multielectrode recordings from networks of cultured motor neurons. Our immunofluorescence experiments demonstrate that most of the cultured neurons are motor neurons, as most neurofilament-positive cultured cells also express ChAT, and most DAPI-stained cell nuclei also stain for Hb9.

Although the pattern of spiking and bursting activity in the neurons is variable from probe to probe, a characteristic of all of the cultures is a prominent network bursting activity that emerges after 7–9 days of culture. The bursting activity typically involves all active electrodes on the probe and indicates the presence of recurrent excitatory and inhibitory connections among the cultured neurons [5,12]. Pharmacological characterization of the network activity shows that blocking GABA_A receptors results in a large increase in burst frequency, suggesting that a tonic GABA-mediated inhibition limits basal activity in cultured motor neuron networks. This basal inhibition is most likely because of the activity of inhibitory interneurons present in the culture. The large variation in spontaneous bursting activity between probe cultures suggests that the level of tonic GABA inhibition is highly variable, and likely dependent on the number and activity of inhibitory interneurons present, and the unique structure of each network.

Exogenous acetylcholine stimulates both cell spiking and network bursting behavior, suggesting that motor neurons in the culture can exert a strong excitatory influence on each other. However, the lack of effect of antagonists for either nicotinic or muscarinic acetylcholine receptors on the spontaneous bursting and spike activity indicates that acetylcholine released by the motor neurons does not drive the spontaneous activity in the network. Our finding that blocking glutamate receptors inhibits both bursting and spiking activity suggests that both the spontaneous activity of individual motor neurons and the network bursts arise from the actions of glutamatergic neurons in the culture. These glutamatergic cells are most likely excitatory interneurons that normally participate in motor reflex pathways in the ventral horn. The basal glutamate activation seems to be mediated by a combination of NMDA and non-NMDA receptors, as the combined action of antagonists for both types of receptors is required to significantly inhibit neuronal activity in the culture.

Conclusion

Motor neurons in culture maintain largely the same properties as in vivo, including inhibition by GABA, activation by glutamate through both NMDA and non-NMDA receptors, and little spontaneous activity in the absence of glutamatergic activation. Pharmacological characterization of the activity of cultured ventral horn networks indicates that inhibitory and excitatory inter-neurons, though not large in number, have a large and widespread influence on the level of spontaneous activity.

Acknowledgments

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Fig. 1.

Characterization of rat primary motor neuron cultures. Spinal motor neurons derived from rat E15 embryonic spinal cords cultured for 2–3 days were analyzed with antibodies against a neuronal marker (neurofilament) and motor neuron markers choline acetyltransferase (ChAT) and Hb9. Secondary antibodies were conjugated with either Texas red (red) or Alexa 488 (green). Blue represents 4',6-diamidino-2-phenylindole (DAPI) staining of nuclei.

Fig. 2. Sample recordings of cultured spinal motor neurons on MED64 probes. (a) Screen shots of MED64 recordings from 64 electrodes on culture days 9 and 13; (b) 50 s sample current traces from representative electrodes after 13 days culture. *Synchronized bursts.

Fig. 3. Pharmacological characterization of cultured motor neurons on MED64 probes. (a–d) Summary data (mean $\pm$ SE) showing the effects of γ -aminobutyric acid (GABA), bicuculline, acetylcholine, and (2R)-amino-5-phosphonovaleric acid (AP5) + 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) on the overall spike frequency. * $P < 0.05$. (e–h) Fifty-second sample traces showing the effects of each drug on neuronal activity. TTX, tetrodotoxin.

Table 1

Effects of receptor agonists and antagonists on activity of cultured motor neurons

	Bursts/min	Spikes/min	Burst duration (s)	Frequency within burst (Hz)	Tonic frequency (Hz)
Control	10.6 ± 1.0	182 ± 22.1	2.72 ± 0.49	9.3 ± 0.5	0.20 ± 0.05
GABA	2.3 ± 0.4*	32 ± 6.1*	0.81 ± 0.18*	5.06 ± 0.94*	0.08 ± 0.03*
Wash (26; 3)	8.0 ± 1.1	141 ± 22.6	2.78 ± 0.33	8.91 ± 1.32	0.13 ± 0.04
Control	3.7 ± 0.5	50.3 ± 5.6	2.40 ± 0.40	8.8 ± 1.59	0.21 ± 0.07
Bicuculline	6.5 ± 0.4*	112 ± 8.5*	1.85 ± 0.14*	11.69 ± 0.62*	0.15 ± 0.06
Wash (25; 3)	4.1 ± 0.3	42.3 ± 3.3	1.98 ± 0.46	8.85 ± 0.85	0.07 ± 0.03
Control	1.5 ± 0.4	26.1 ± 6.0	0.74 ± 0.18	5.355 ± 1.28	0.12 ± 0.05
Acetylcholine	4.6 ± 0.4*	111 ± 15*	0.74 ± 0.08	9.63 ± 0.76*	1.47 ± 0.21*
Wash (18; 3)	2.3 ± 0.3	78.1 ± 8.2	1.39 ± 0.21	9.61 ± 0.59	0.28 ± 0.09
Control	12.6 ± 0.85	231.8 ± 11.0	2.72 ± 0.24	9.03 ± 0.43	0.82 ± 0.08
Atropine + mecamylamine	11.1 ± 0.21	242.8 ± 11.7	1.79 ± 0.10*	17.15 ± 0.74*	0.09 ± 0.03
Wash (22; 2)	13.0 ± 0.67	244.1 ± 14.2	2.26 ± 0.17	14.06 ± 0.96	0.04 ± 0.01
Control	10.8 ± 0.5	114.3 ± 18.2	0.88 ± 0.13	17.88 ± 4.21	0.14 ± 0.06
AP5	6.55 ± 0.33*	51.67 ± 7.8*	0.23 ± 0.01*	41.99 ± 4.18*	0.05 ± 0.03*
AP5 + CNQX	0*	8.6 ± 2.6*	0*	0*	0.17 ± 0.05
Wash (24; 2)	10.55 ± 0.63	154 ± 21.0	1.60 ± 0.20*	10.82 ± 0.94	0.32 ± 0.09*

Numbers in parentheses in left-most column indicate the number of electrodes and probes that were averaged to give the results. 'Tonic frequency' refers to the frequency of single spikes recorded between the bursts. Data are means ± SE.

AP5, (2R)-amino-5-phosphonovaleric acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; GABA, γ -aminobutyric acid.

* $P < 0.05$ compared with control.