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Molecular and electrophysiological properties of mouse motoneuron and motor unit subtypes

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Abstract

The field of motoneuron and motor unit physiology in mammals has deeply evolved the last decade thanks to the parallel development of mouse genetics and transcriptomic analysis and of *in vivo* mouse preparations that allow intracellular electrophysiological recordings of motoneurons. We review the efforts made to investigate the electrophysiological properties of the different functional subtypes of mouse motoneurons, to decipher the mosaic of molecular markers specifically expressed in each subtype, and to elucidate which of those factors drive the identity of motoneurons.

Keywords

Spinal motor system; Functional diversity of spinal motoneurons; In vitro and in vivo spinal cord electrophysiology; Molecular markers

Introduction

Although the study of motor unit physiology dates back from the ‘60s and ‘70s, the field has experienced a considerable renewal in the last decade with numerous efforts relying on progress in genetic tools in mice. We will review recent progress in identifying the various types of motoneurons and motor units in mice, based on their electrophysiological properties and the expression of molecular markers. These advances offer unprecedented tools for physiological studies of the spinal motor system under normal and disease conditions.

Electrophysiological properties of motor units

There are several types of motoneurons (MNs) based on the muscle fibers they contact [reviewed in 1]. α-motoneurons (αMNs) innervate extrafusal muscle fibers and are the central components of motor units (MUs), the elementary unit of movements. Depending on the biomechanical properties of the muscle fibers, one can distinguish three types of MUs: slow-contracting, fatigue-resistant MUs (S), fast-contracting, fatigue resistant MUs (FR), and fast-contracting but fatigable units (FF). On the other hand, γ-motoneurons (γMNs)

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innervate intrafusal muscle fibers of spindles (an intramuscular proprioceptive organ), not extrafusal muscle fibers, and they do not generate any force at the muscle tendon.

Seminal studies in cats, and later in rats, had shown that there exist correlations between the electrical properties of motoneurons and the contractile properties of their MU [2]. However, how much of these correlations held true in mice, and how to apply them to identify mouse MN types was unknown. The task was made even more arduous by the fact that, for the longest time, mouse spinal MNs could only be recorded in vitro. In these conditions, MNs are disconnected from their muscle fiber, which makes identification of their type difficult. Furthermore, for technical reasons, these recordings were restricted to animals younger than two weeks, i.e. during the postnatal development.

Electrophysiological properties of S- and F-type motoneurons in neonatal mice

Neonatal mouse MNs were shown to display different discharge patterns in response to long current pulses at intensities close to the rheobase [3–5]. In these conditions, they either start to discharge immediately at the onset of the current pulse (immediate firing pattern, 33% of the MN population) or discharge with a delay of a few seconds (delayed firing pattern, 67% of the population [4]). We have recently demonstrated that delayed firing MNs have larger input conductances, higher rheobases, more depolarized voltage thresholds for spiking, narrower action potentials and shorter AHPs, longer dendrites and more dendritic branches than immediate firing MNs (Figure 1). Together with the expression of molecular markers (see below, and Figure 1C), these differential features identify delayed- and immediate-firing MNs as F-type and S-type, respectively [4]. The delayed firing pattern was shown to be caused by a combination of two potassium currents: an A-like current that acts at a short time scale (less than 100 ms) and a slowly-inactivating current that acts at a longer time scale (several seconds) [6]. The channel responsible for the slowly inactivating current has recently been identified as Kv1.2 [7]. This current probably plays a key role in dynamically setting the recruitment threshold of the F-type MNs: the memory effect induced by its long lasting action alters the MN recruitment threshold depending upon its firing history [6,7]. Both patterns of discharge are still present in adults as shown using in vivo intracellular MN recordings [7], but whether they continue to be segregated by type remains to be determined.

In vivo intracellular recordings allow functional identification of motor unit type

Over the last decade, technical breakthroughs have made it possible to record spinal MNs in vitro in older animals [8–12], but the problem of identifying MN type without MU output remains. In parallel, preparations allowing intracellular recordings of motoneurons in vivo in adult mice were developed. These preparations allow recording fully mature MNs, which has been difficult to achieve in vitro, as well as to investigate the different compartments of motor units (inputs on motoneurons from specific pathways, intrinsic membrane properties of motoneurons and force output). A number of technical issues had to be solved to reach that point, in particular the maintenance, for several hours, of good physiological conditions

of the mouse despite the invasive surgery necessary to get access to spinal cord, nerves and muscles. Actually, the first paper with in vivo motoneuron intracellular recordings was published in 1975 [13], but we had to wait almost 30 years for the publication of a second paper [14], illustrating how difficult such a work is. Since that time, several groups put a lot of efforts in developing in vivo preparations allowing to investigate the intrinsic properties of motoneurons in anaesthetized mice [15,16], the force output of individual motor units [17,18] and the motoneuron response during fictive locomotion in decerebrated mice [19–21].

These technical breakthroughs allowed us to identify the physiological type of the MU and to correlate, for the first time in mice, the electrical properties of MNs to the physiological type of the MU [22]. MUs were classified in S, FR and FF based on twitch amplitude, twitch contraction time, and fatigability (Figure 2). The electrophysiological properties of the different types of MNs followed a similar pattern as previously described: S MNs have a small input conductance and are the most excitable; FR MNs have input conductance generally higher than S, and are therefore generally less excitable; FF MNs are the least excitable, with high input conductances and large recruitment current [22]. However, the distributions of electrophysiological properties overlap widely, and further work is needed to define good criteria, usable in mice, allowing identification of MN type based solely on their electrophysiological properties.

Molecular markers of motoneuron subtypes

Although electrophysiological experiments are able to provide information about the type of the recorded MNs, these techniques remain challenging, time consuming, and fairly low-throughput. This is the reason why investigators have searched for ways to distinguish the different types of MNs on histological slices.

With the advent of mouse genetics and transcriptomic analysis, it has become possible to identify genes expressed in specific types of MNs (see Figure 3). A first step in this direction was to unequivocally differentiate αMNs from γMNs. Early work in cats had demonstrated that γMNs are smaller than αMNs [23], and they do not receive monosynaptic connection from Ia afferents [24,25], nor C-boutons [26]. These features were key in identifying several specific markers of α and γMNs (Figure 3). αMNs specifically express the neuron-specific nuclear protein NeuN [27] and Osteopontin [28]. On the other hand, γMNs are characterized by the lack of NeuN expression, and the selective expression of GDNF receptor Gfrα1 [29], and the serotonin receptor $5HT_{1d}$ [30]. Interestingly, although both populations are dependent upon the expression of the homeobox gene Hb9 [31,32], γMNs do not express GFP in Hb9::GFP mice [29].

Seminal studies in cats have shown that there are morphological differences between αMNs subtypes [2]. Yet, there are no clear-cut parameters that allow differentiating one type of motoneuron from another. This issue is even more true in neonates where morphological differences are even less pronounced. In order to distinguish S, FR and FF αMNs, investigators have focused on genes that are expressed in some, but not all, αMNs. Doing so, several groups have identified Calcitonin gene-related peptide (CGRP)/calca [33–35],

Chondrolectin [35], and Matrix metallopeptidase 9 (MMP-9) [36] as potential markers of F-type MN, and Estrogen-related receptor beta (Esrrb) [35] as a potential maker of S MNs. Since S- and F-type MN innervate different types of muscle fibers, Chakkalakal et al. [37] have studied the distribution of the isoforms of the synaptic vesicle protein SV2 at the neuromuscular junction and have shown that the expression of the SV2A isoform becomes restricted to S-type MNs postnatally. More fortuitously, as a way to study the function of the UCHL1 gene, Yasvoina et al. [38] have generate a mouse expressing eGFP under the UCHL1 promoter. In this mouse, eGFP is expressed by cortico-spinal neurons, as well as a subpopulation of small-size spinal MNs. Observation of their neuromuscular junctions revealed that some of these MNs are γMNs, while others are presumably S-type αMNs [38].

Of particular importance for the study of MU physiology are genes that control the differential electrophysiological properties of the MN subtypes. For example, S-type MNs have a longer afterhyperpolarization (AHP) following each spike than F-type MNs [39]. Deardorff et al. [40] have observed that, at least in rodents, the SK3 isoform of the calcium-activated K^+ channels responsible for this hyperpolarization is specifically expressed in the smallest, presumably S-type, αMNs. The different electrophysiological properties of each MN type suggest that, at some point during their development, each type expresses transcription factors responsible for the expression of a set of membrane conductances. One such transcription factor, Dlk1 is expressed by the large, presumably F-type MNs, and it is both necessary and sufficient to promote a “fast” electrophysiological signature in αMNs [41].

There exists another type of motoneuron, the so-called β-motoneurons that are innervating both extrafusal and intrafusal muscle fibers [1]. Studying this type of MN has been hindered by the extreme difficulty in identifying them. Initially, the α3 isoform of the Na/K ATPase was suggested as a selective marker for γMNs [42]. However, others have observed the expression of that protein in αMNs [43–45]. In particular, Ruegsegger et al. have shown, using retrograde labeling from either a muscle subcompartment containing only FF fibers or a muscle containing both S and FR muscle fibers, that FF MNs express α3, while S MNs only express the α1 subunit [43]. This contradiction may be resolved by the observation, in sections of ventral roots, that α3 is present in the plasma membrane of small-diameter myelinated fibers (i.e. γ), as well as some (but not all) substantially larger fibers, suggesting that this isoform is expressed by both γ- and larger MNs, which could be βMNs [46]. Whatever the case, more work is required to identify unequivocally βMNs.

Despite great strides in the identification of potential markers of MN type, we believe it is necessary to remain cautious. These putative markers rely, particularly for the different types of αMNs, on size differences between MNs; but, as mentioned above, size distributions overlap widely. In vivo recordings open the possibility to validate the selectivity of putative molecular markers in MNs that have been functionally identified in adult animals. Chodl was the first marker to be investigated this way (Figure 4). We found that Chodl is specifically expressed in FF as well as the largest FR MUs but not in the smaller FR, nor in the S-type MUs [22]. In other words, contrary to the initial study presenting Chodl as a marker of F-type MNs [35], we show that Chodl is a marker of size, rather than type: even though all Chodl+ MNs are fast, not all fast MNs are Chodl+, and Chodl− MNs can be either

slow or fast. Interestingly, Chodl has been implicated in motor axon growth during development [47,48], which could explain why it is expressed by large MUs, whose axon displays extensive branching within the muscle.

Conclusion and perspectives

A lot of work has been done in the last decade to identify the mosaic of genes/protein that confer to each motoneuron subtype its identity. Even if electrophysiological validation remains to be done, the studies presented here offer new exciting tools for the study of the spinal motor system. Case in point, being able to recognize MN-type using molecular markers has already allowed demonstrating that vestibular and proprioceptive systems project differently on F- and S-type MNs [49]. Furthermore, we believe that these tools will be especially important for the study of Amyotrophic Lateral Sclerosis (ALS), the most prominent neurodegenerative disease of MNs, where the order of MN degeneration depends on their type: FF MUs degenerate first, followed by FR units, whereas S-type MUs are the most resistant [50,51].

Coupled with the cre/lox or crispr/cas9 technologies, highly selective molecular markers would have potentially limitless applications. For instance, optogenetic tools could be expressed in specific subpopulations of MNs to study their role in various behavioral tasks. The future is bright for the young generation of spinal cord physiologists, whose imagination is the sole limit in applying these new technologies to answering questions that no one has yet dared to ask.

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● of special interest

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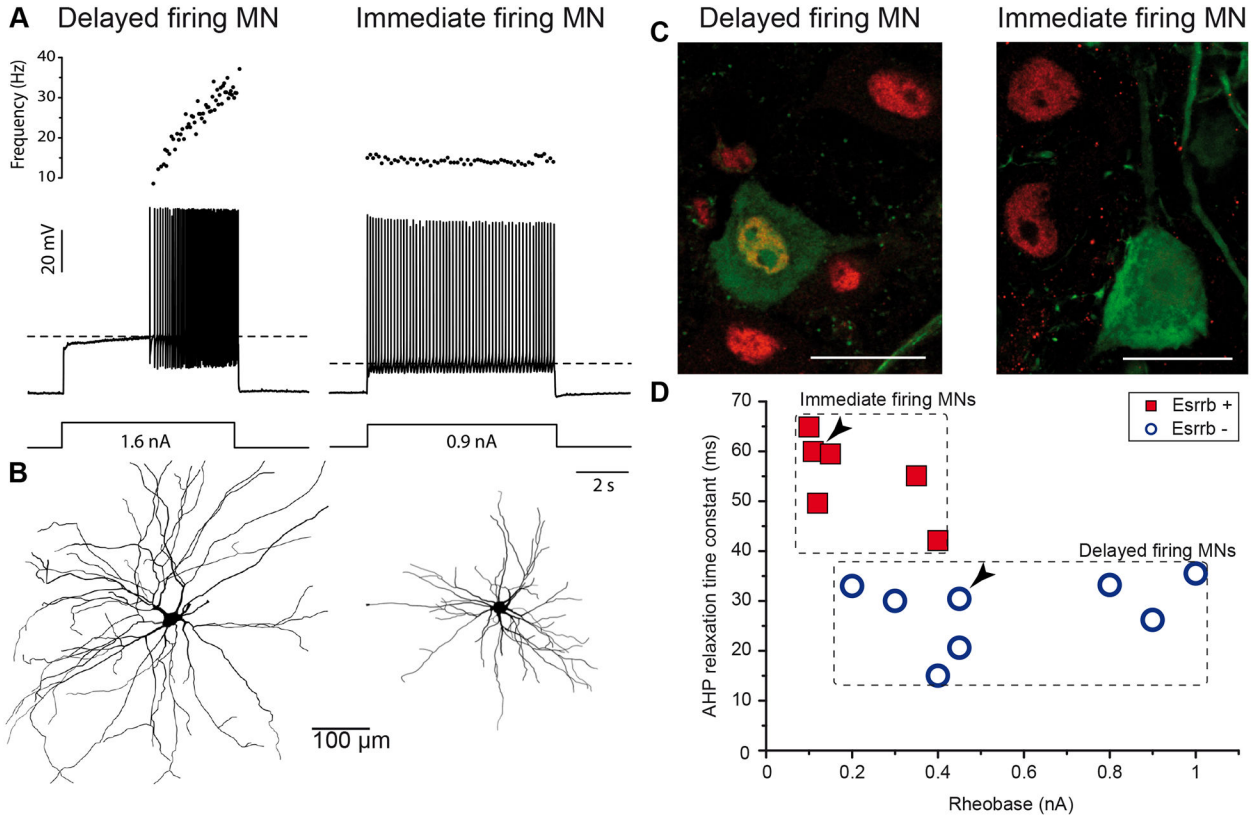

Figure 1. S- and F-type MNs can be distinguished based on their firing pattern in neonatal mice.

A. Response of a delayed-firing (left) and an immediate-firing (right) motoneuron to a 5 s pulse. The current intensity was the minimal intensity necessary to elicit firing in our searching protocol (rheobase). Bottom: injected-current (square pulses), middle: voltage-response and top: instantaneous firing frequency. The horizontal dashed line shows the voltage threshold for spiking. **B.** Reconstructed dendritic trees of delayed- (left) and immediate-firing (right) motoneurons. The axon was not reconstructed in either case. **C.** Examples of Esrrb staining (red) in neurobiotin-filled (green) motoneurons. Scale bar: 30 µm. Both delayed and immediate firing MNs do express NeuN and receive proprioceptive VGluT1-positive synapses (not shown) indicating that they are both α-MNs. However, only immediate firing MNs express Esrrb. In addition, none of the immediate firing MN express Chodl or MMP9 (not shown), whereas half of the delayed firing MN (mainly those with the highest input conductances and rheobases) do. **D.** Plot of the AHP relaxation time constants against the rheobases for labelled motoneurons. Arrowheads point to the motoneurons illustrated in C. Adapted from [4], licensed under CC-BY.

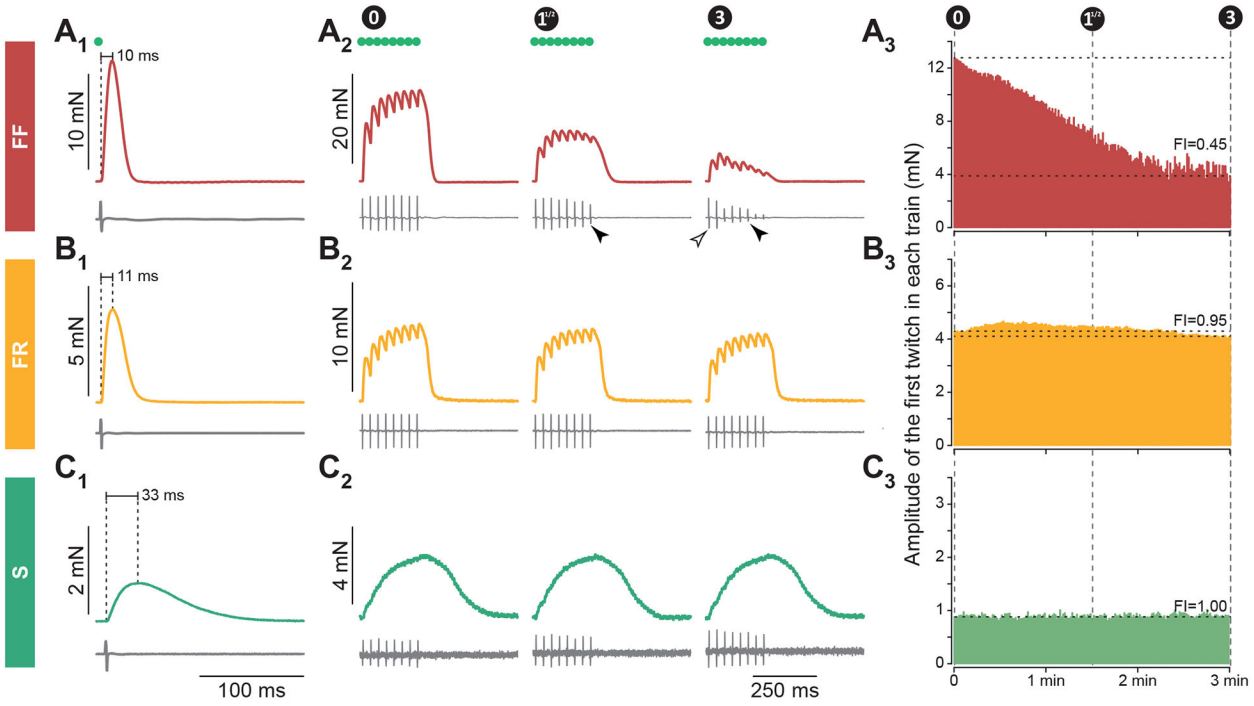

Figure 2. Functional characterization of mouse motor units in vivo.

A–C. Procedure for type-identification of mouse MUs in vivo and examples of three MUs: FF (**A**), FR (**B**) and S (**C**). Individual twitch response allow measurement of twitch amplitudes and contraction time (A_1, B_1, C_1). Series of unfused tetani allow to test the fatigability of the muscle fibers ($A_{2–3}$, $B_{2–3}$, $C_{2–3}$). Motor units were classified as S-type if contraction time ≥20 ms; FR if contraction time <20 ms and twitch amplitude <8 mN; FF if contraction time <20 ms and twitch force ≥8 mN. See [22] for details. Figure from [22], licensed under CC-BY.

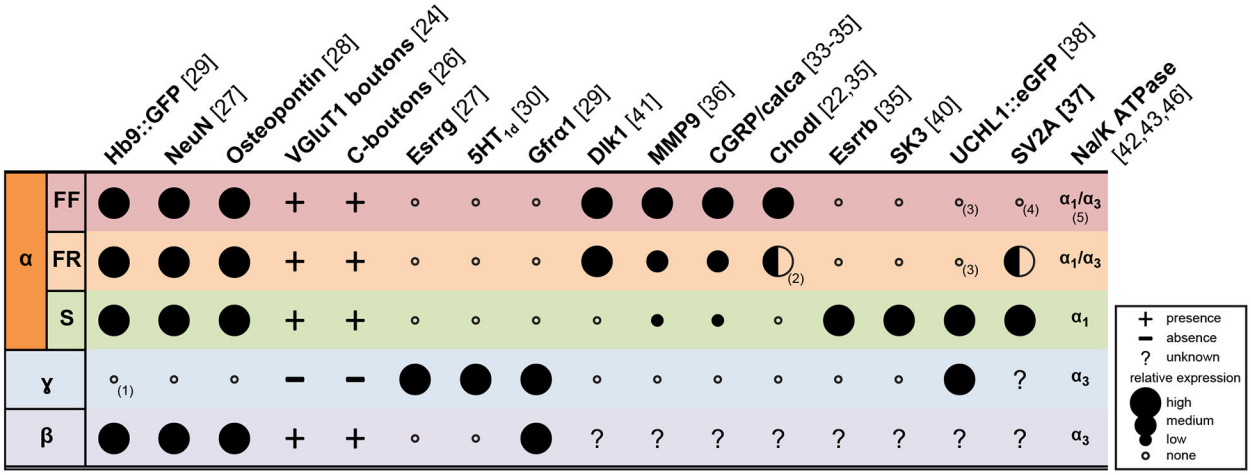

Figure 3. Summary of putative molecular markers of motoneuron subtypes

Notes: (1) No expression of GFP in Hb9::GFP mice despite the expression of Hb9 in γMNs during development [31]. (2) Expression of Chodl is restricted to the largest FR MUs, see [22] and text for details. (3) True only in rodents. SK3 is expressed in all αMN subtypes in cats. (4) expression is restricted postnatally only: signal is present at P0 but disappears at P14. (5) Several authors seem to disagree on this point, see text for details.

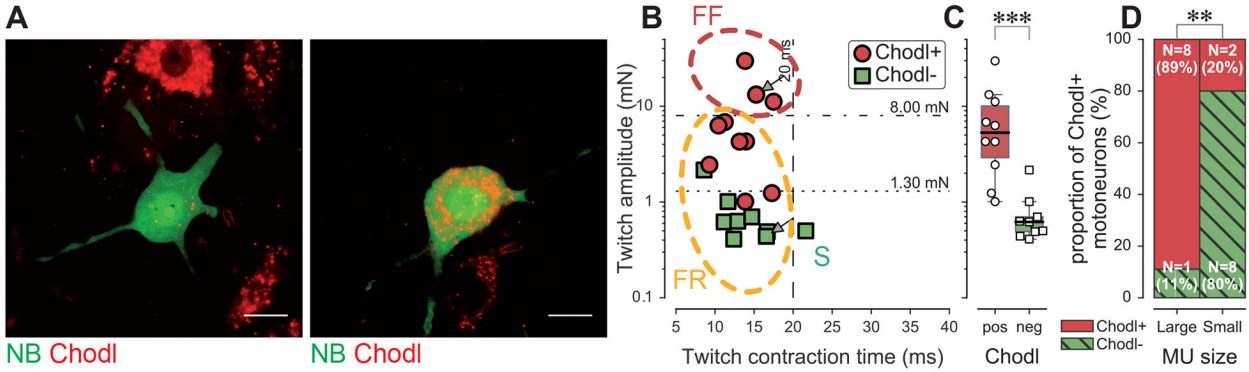

Figure 4. Chondrolectin is a marker of size rather than type.

A. Two examples of intracellular-labeled (neurobiotin, NB, green) motoneurons, coupled with ISH revelation of Chodl RNA (Chodl, red). In each experiment, a single MN was type-identified, labelled with an intracellular dye (NB), in order to recognize it among all the other MNs; and in situ hybridization against Chodl mARN was performed after fixation and slicing of the spinal cord. Left panel: Chodl− small FR motoneuron; Right panel: Chodl+ FF motoneuron. Scale bars: 15 μm. **B.** Contractile properties of the motor units tested for Chodl expression. The motoneurons indicated with arrows correspond to the two cells in D. Red circles are the motoneurons that expressed Chodl, while green squares are those that did not. The dashed lines at 8 mN and 20 ms separate the different types of MUs, and the dash-dotted line at 1.3 mN separates large from small MUs. **C.** Comparison of the average twitch amplitude of motor units split according to their expression of Chodl. **D.** Comparison of the proportion of cells expressing Chodl in the population of tested cells, split in two categories, large and small. Adapted from [22], licensed under CC-BY.