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Molecular and electrophysiological properties of mouse motoneuron and motor unit subtypes

Short title: Properties of motoneuron subtypes in mice

Authors:

Marin MANUEL* & Daniel ZYTNICKI

Center for Neurophysics, Physiology and Pathology, Paris Descartes University, CNRS UMR 8119, Paris, France

* corresponding author:

CNRS UMR 8119

45 rue des Saints-Pères

75006, Paris

FRANCE

marin.manuel@neurobio.org

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, transcriptomic analysis, and 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. The recent development of *in vivo* mouse preparations, in which one can record simultaneously one motoneuron and of the force generated by its motor unit, now allows, for the first time, correlating the functional type of a motoneuron with the expression of specific genes.

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

1 Introduction

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

8 Electrophysiological properties of motor units

9 There are several types of motoneurons (MNs) based on the muscle fibers they contact
10 [reviewed in 1]. α -motoneurons (α MNs) innervate extrafusal muscle fibers and are the central
11 components of motor units (MUs), the elementary unit of movements. Depending on the
12 biomechanical properties of the muscle fibers, one can distinguish three types of MUs: slow-
13 contracting, fatigue-resistant MUs (S), fast-contracting, fatigue resistant MUs (FR), and fast-
14 contracting but fatigable units (FF). On the other hand, γ -motoneurons (γ MNs) innervate
15 intrafusal muscle fibers of spindles (an intramuscular proprioceptive organ), not extrafusal
16 muscle fibers, and they do not generate any force at the muscle tendon.

17 Seminal studies in cats, and later in rats, had shown that there exist correlations between
18 the electrical properties of motoneurons and the contractile properties of their MU [2].
19 However, how much these correlations held true in mice, and how to apply them to identify
20 mouse MN types was unknown. The task was made even more arduous by the fact that, for
21 the longest time, mouse spinal MNs could only be recorded in vitro. In these conditions, MNs
22 are disconnected from their muscle fiber, which makes identification of their type difficult.

1 Furthermore, for technical reasons, these recordings were restricted to animals younger than
2 two weeks, i.e. during the postnatal development.

3 Electrophysiological properties of S- and F-type motoneurons in 4 neonatal mice

5 Neonatal mouse MNs were shown to display different discharge patterns in response to
6 long current pulses at intensities close to the rheobase [3-5]. In these conditions, they either
7 start to discharge immediately at the onset of the current pulse (immediate firing pattern, 33%
8 of the MN population) or discharge with a delay of a few seconds (delayed firing pattern,
9 67% of the population [4]). We have recently demonstrated that delayed firing MNs have
10 larger input conductances, higher rheobases, more depolarized voltage thresholds for spiking,
11 narrower action potentials and shorter AHPs, longer dendrites and more dendritic branches
12 than immediate firing MNs (Figure 1). Together with the expression of molecular markers
13 (see below, and Figure 1C), these differential features identify delayed- and immediate-firing
14 MNs as F-type and S-type, respectively [4]. The delayed firing pattern was shown to be
15 caused by a combination of two potassium currents: an A-like current that acts at a short time
16 scale (less than 100 ms) and a slowly-inactivating current that acts at a longer time scale
17 (several seconds) [6]. The channel responsible for the slowly inactivating current has recently
18 been identified as Kv1.2 [7]. This current probably plays a key role in dynamically setting the
19 recruitment threshold of the F-type MNs: the memory effect induced by its long lasting
20 action alters the MN recruitment threshold depending upon its firing history [6,7]. Both
21 patterns of discharge are still present in adults as shown using in vivo intracellular MN
22 recordings [7], but whether they continue to be segregated by type remains to be determined.

1 In vivo intracellular recordings allow functional identification of 2 motor unit type

3 Over the last decade, technical breakthroughs have made it possible to record spinal MNs
4 in vitro in older animals [8-12], but the problem of identifying MN type without MU output
5 remains. In parallel, preparations allowing intracellular recordings of motoneurons in vivo in
6 adult mice were developed. These preparations allow recording fully mature MNs, which has
7 been difficult to achieve in vitro, as well as to investigate the different compartments of
8 motor units (inputs on motoneurons from specific pathways, intrinsic membrane properties of
9 motoneurons and force output). A number of technical issues had to be solved to reach that
10 point, in particular the maintenance, for several hours, of good physiological conditions of
11 the mouse despite the invasive surgery necessary to get access to spinal cord, nerves and
12 muscles. Actually, the first paper with in vivo motoneuron intracellular recordings was
13 published in 1975 [13], but we had to wait almost 30 years for the publication of a second
14 paper [14], illustrating how difficult such a work is. Since that time, several groups put a lot
15 of efforts in developing in vivo preparations allowing to investigate the intrinsic properties of
16 motoneurons in anaesthetized mice [15,16], the force output of individual motor units [17,18]
17 and the motoneuron response during fictive locomotion in decerebrated mice [19-21].

18 These technical breakthroughs allowed us to identify the physiological type of the MU
19 and to correlate, for the first time in mice, the electrical properties of MNs to the
20 physiological type of the MU [22]. MUs were classified in S, FR and FF based on twitch
21 amplitude, twitch contraction time, and fatigability (Figure 2). The electrophysiological
22 properties of the different types of MNs followed a similar pattern as previously described: S
23 MNs have a small input conductance and are the most excitable; FR MNs have input
24 conductance generally higher than S, and are therefore generally less excitable; FF MNs are

1 the least excitable, with high input conductances and large recruitment current [22].
2 However, the distributions of electrophysiological properties overlap widely, and further
3 work is needed to define good criteria, usable in mice, allowing identification of MN type
4 based solely on their electrophysiological properties.

5 Molecular markers of motoneuron subtypes

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

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

21 Seminal studies in cats have shown that there are morphological differences between
22 α MNs subtypes [2]. Yet, there are no clear-cut parameters that allow differentiating one type
23 of motoneuron from another. This issue is even more true in neonates where morphological
24 differences are even less pronounced. In order to distinguish S, FR and FF α MNs,

1 investigators have focused on genes that are expressed in some, but not all, α MNs. Doing so,
2 several groups have identified Calcitonin gene-related peptide (CGRP)/calca [33-35],
3 Chondrolectin [35], and Matrix metalloproteinase 9 (MMP-9) [36] as potential markers of F-
4 type MN, and Estrogen-related receptor beta (Esrrb) [35] as a potential maker of S MNs.
5 Since S- and F-type MN innervate different types of muscle fibers, Chakkalakal et al. [37]
6 have studied the distribution of the isoforms of the synaptic vesicle protein SV2 at the
7 neuromuscular junction and have shown that the expression of the SV2A isoform becomes
8 restricted to S-type MNs postnatally. As a way to study the function of the UCHL1 gene,
9 Yasvoina et al. [38] have generated a mouse expressing eGFP under the UCHL1 promoter. In
10 this mouse, eGFP is expressed by cortico-spinal neurons, as well as a subpopulation of small-
11 size spinal MNs. Observation of their neuromuscular junctions revealed that some of these
12 MNs are γ MNs, while others are presumably S-type α MNs [38].

13 Of particular importance for the study of MU physiology are genes that control the
14 differential electrophysiological properties of the MN subtypes. For example, S-type MNs
15 have a longer afterhyperpolarization (AHP) following each spike than F-type MNs [39].
16 Deardorff et al. [40] have observed that, at least in rodents, the SK3 isoform of the calcium-
17 activated K^+ channels responsible for this hyperpolarization is specifically expressed in the
18 smallest, presumably S-type, α MNs. The different electrophysiological properties of each
19 MN type suggest that, at some point during their development, each type expresses
20 transcription factors responsible for the expression of a set of membrane conductances. One
21 such transcription factor, Dlk1 is expressed by the large, presumably F-type MNs, and it is
22 both necessary and sufficient to promote a “fast” electrophysiological signature in α MNs
23 [41].

24 There exists another type of motoneuron, the so-called β -motoneurons that innervate both
25 extrafusal and intrafusal muscle fibers [1]. Studying this type of MN has been hindered by

1 the extreme difficulty in identifying them. Initially, the $\alpha 3$ isoform of the Na/K ATPase was
2 suggested as a selective marker for γ MNs [42]. However, others have observed the
3 expression of that protein in α MNs [43-45]. In particular, Ruegsegger et al. have shown,
4 using retrograde labeling from either a muscle subcompartment containing only FF fibers or a
5 muscle containing both S and FR muscle fibers, that FF MNs express $\alpha 3$, while S MNs only
6 express the $\alpha 1$ subunit [43]. This contradiction may be resolved by the observation, in
7 sections of ventral roots, that $\alpha 3$ is present in the plasma membrane of small-diameter
8 myelinated fibers (i.e. γ), as well as some (but not all) substantially larger fibers, suggesting
9 that this isoform is expressed by both γ - and larger MNs, which could be β MNs [46].
10 Whatever the case, more work is required to identify unequivocally β MNs.

11

12 Despite great strides in the identification of potential markers of MN type, we believe it is
13 necessary to remain cautious. These putative markers rely, particularly for the different types
14 of α MNs, on size differences between MNs; but, as mentioned above, size distributions
15 overlap widely. In vivo recordings open the possibility to validate the selectivity of putative
16 molecular markers in MNs that have been functionally identified in adult animals. Chodl was
17 the first marker to be investigated this way (Figure 4). We found that Chodl is specifically
18 expressed in FF as well as the largest FR MUs but not in the smaller FR, nor in the S-type
19 MUs [22]. In other words, contrary to the initial study presenting Chodl as a marker of F-type
20 MNs [35], we show that Chodl is a marker of size, rather than type: even though all Chodl+
21 MNs are fast, not all fast MNs are Chodl+, and Chodl- MNs can be either slow or fast.
22 Interestingly, Chodl has been implicated in motor axon growth during development [47,48],
23 which could explain why it is expressed by large MUs, whose axon displays extensive
24 branching within the muscle.

1 Conclusion and perspectives

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

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

18 References

19 Papers of particular interest have been highlighted as:

20 ● of special interest

21 ●● of outstanding interest

22

1 • Martínez-Silva MdL, et al. (2018) Hypoexcitability precedes denervation in the large fast-
2 contracting motor units in two unrelated mouse models of ALS. eLife 7.

3 This study is the first to identify functionally MN subtypes, in mice, based on the force
4 developed by their MUs.

5

6 •• Bos R, et al. (2018) Kv1.2 Channels Promote Nonlinear Spiking Motoneurons for
7 Powering Up Locomotion. Cell Reports 22:3315-3327.

8 This work identified the channel responsible for the slow depolarization observed in delayed-
9 firing motoneurons. It further shows that this channel can act as a gain control mechanism
10 and helps generate a smooth transition from quiescence to steady-state locomotion.

11

12 •• Ruegsegger C, et al. (2016) Aberrant association of misfolded SOD1 with
13 Na(+)/K(+)ATPase- $\alpha 3$ impairs its activity and contributes to motor neuron vulnerability in
14 ALS. Acta neuropathologica 131:427-451.

15 This study reveals that misfolded mutSOD1 proteins interacts with the $\alpha 3$ isoform of the
16 Na/K ATPase, reducing its activity. Inhibition of Na/K ATPase $\alpha 3$, which is highly express
17 in FF MNs (the most vulnerable in ALS), accelerates the disease.

18

19 •• Basaldella E, Takeoka A, Sigrist M, & Arber S (2015) Multisensory Signaling Shapes
20 Vestibulo-Motor Circuit Specificity. Cell 163:301-312.

21 This work used cutting-edge viral technologies and molecular markers to demonstrate that
22 vestibular inputs target preferentially extensor vs. flexor motor pools, and that, within the
23 extensor motor pool, they target preferentially S-type vs. F-type MNs. Interestingly, the
24 density of proprioceptive inputs on F- and S-type MNs is anti-correlated to the distribution of
25 vestibular inputs. They go on to show that the two systems interact, probably through

1 retrograde and homeostatic mechanisms, so that genetically imposed changes to one system
2 are counterbalanced by the other.

3

4 •• Müller D, et al. (2014) Dlk1 promotes a fast motor neuron biophysical signature required
5 for peak force execution. Science (New York, N.Y.) 343:1264-1266.

6 This work demonstrates that the transcription factor Dlk1 is both necessary and sufficient to
7 promote a “fast” signature to MNs. Dlk1 controls an network of genes setting the excitability
8 and firing properties of F-type MNs.

9

10 •• Kaplan A, et al. (2014) Neuronal matrix metalloproteinase-9 is a determinant of selective
11 neurodegeneration. Neuron 81:333-348.

12 This transcriptomic study attempts to identify ALS resistance and susceptibility genes by
13 comparing resistant and vulnerable motor pools. They identified MMP9 as a susceptibility
14 gene and showed that MMP9 is expressed in the largest, most vulnerable MNs. Furthermore,
15 knocking out MMP9 protects against neuromuscular junction denervation, improve motor
16 performance and lifespan.

17

18 • Leroy F, Lamotte d'Incamps B, Imhoff-Manuel RD, & Zytnicki D (2014) Early intrinsic
19 hyperexcitability does not contribute to motoneuron degeneration in amyotrophic lateral
20 sclerosis. eLife 3.

21 This study shows that S- and F- type motoneurons have a different firing patterns in neonatal
22 spinal slices. In this work, MN identity was established on the basis of their electrical
23 properties, their anatomy and the expression of molecular markers.

24

1 ●● Deardorff AS, et al. (2013) Expression of postsynaptic Ca²⁺-activated K⁺ (SK) channels
2 at C-bouton synapses in mammalian lumbar-motoneurons. *The Journal of physiology*
3 591:875-897.

4 This work shows very high quality immunolabeling pictures showing the restricted
5 expression of the SK3 isoform of the Ca²⁺-activated K⁺ (SK) channels in the smallest
6 αMNs. Using correlations with electrophysiological features (AHP), the authors make the
7 convincing argument that this protein is only expressed in S-type αMNs. Remarkably, these
8 channels are exclusively located on the post-synaptic side of the C-boutons.

9

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32 Declarations of interest: none

1 Figure Legends

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

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

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

19 **A–C.** Procedure for type-identification of mouse MUs in vivo and examples of three
20 MUs: FF (**A**), FR (**B**) and S (**C**). Individual twitch response allow measurement of twitch
21 amplitudes and contraction time (A_1 , B_1 , C_1). Series of unfused tetani allow to test the
22 fatigability of the muscle fibers (A_{2-3} , B_{2-3} , C_{2-3}). Motor units were classified as S-type if

1 contraction time ≥ 20 ms; FR if contraction time < 20 ms and twitch amplitude < 8 mN; FF if
2 contraction time < 20 ms and twitch force ≥ 8 mN. See [22] for details. Figure from [22],
3 licensed under [CC-BY](#).

4 Figure 3. Summary of putative molecular markers of motoneuron 5 subtypes

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

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

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

- 1 proportion of cells expressing Chodl in the population of tested cells, split in two categories,
- 2 large and small. Adapted from [22], licensed under [CC-BY](#).

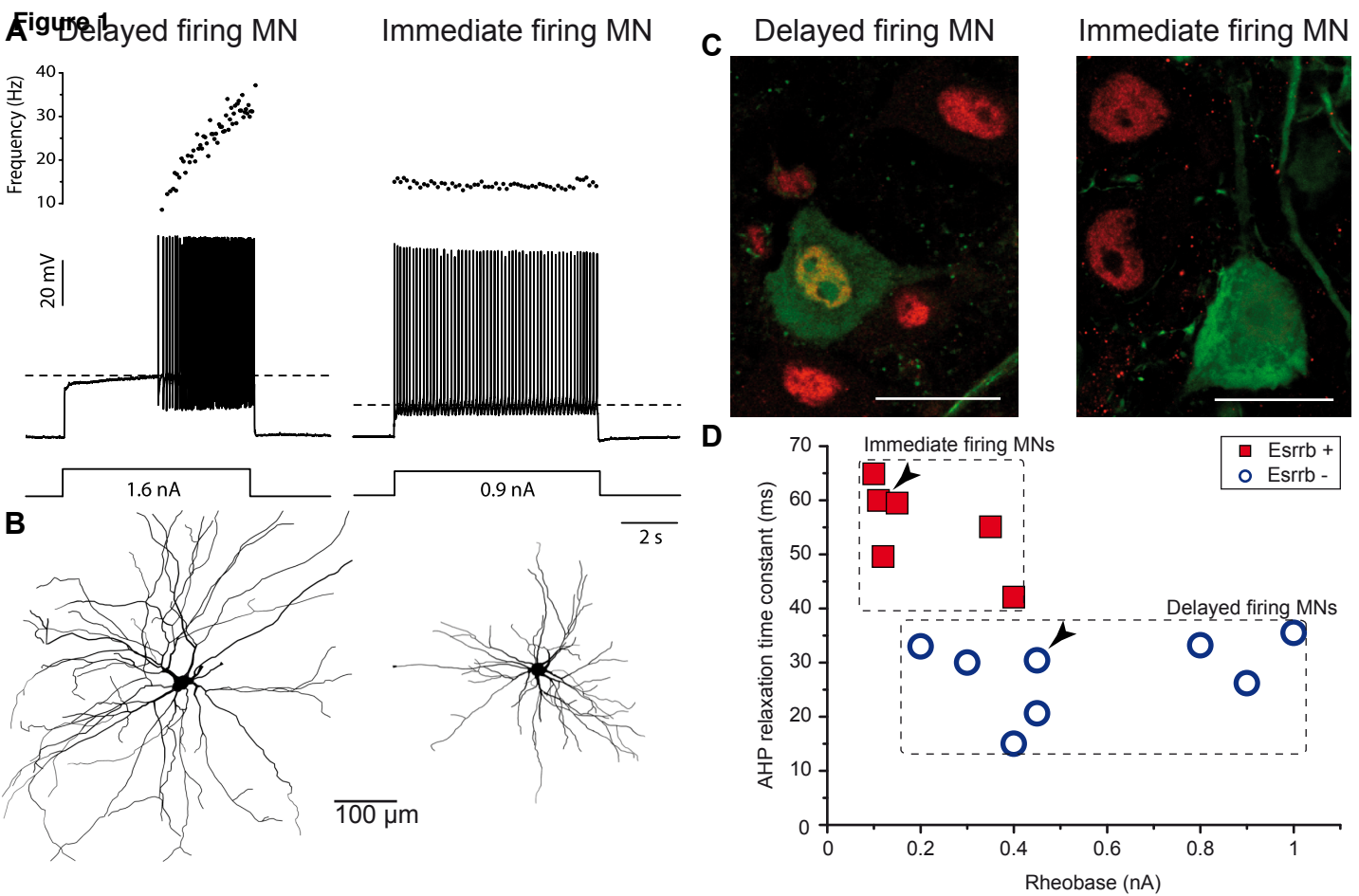


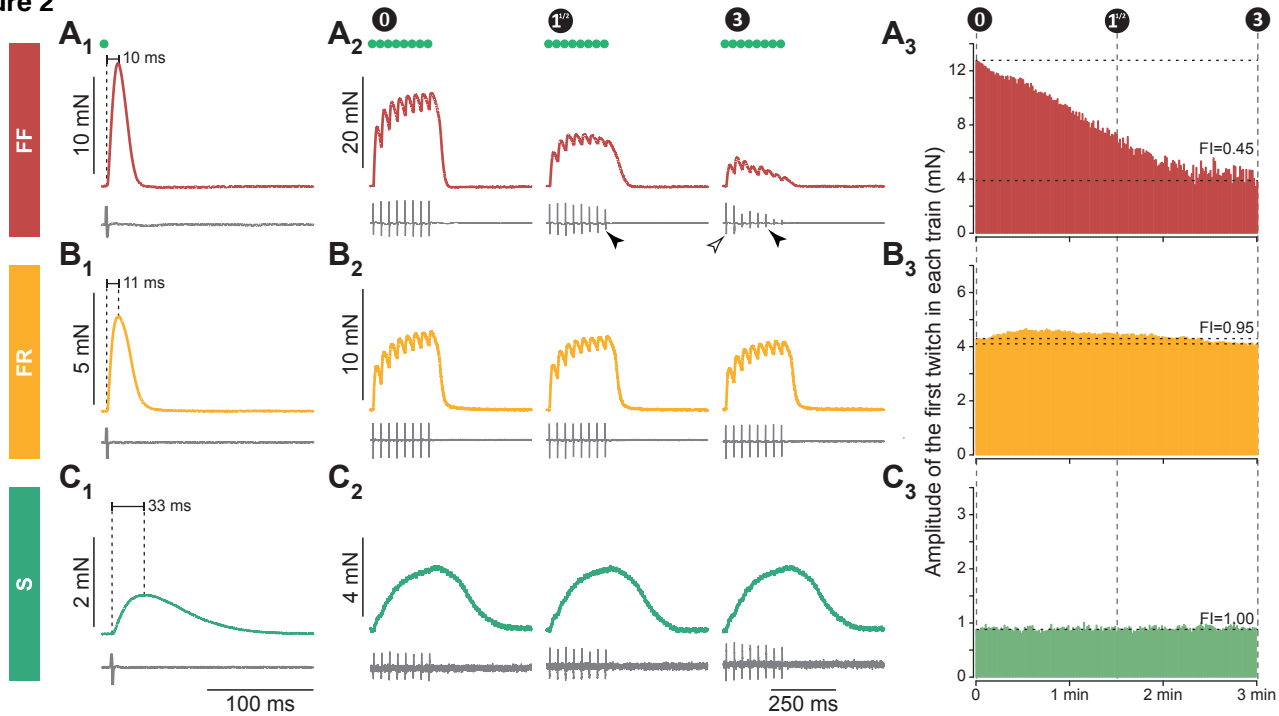
Figure 2

Figure 3

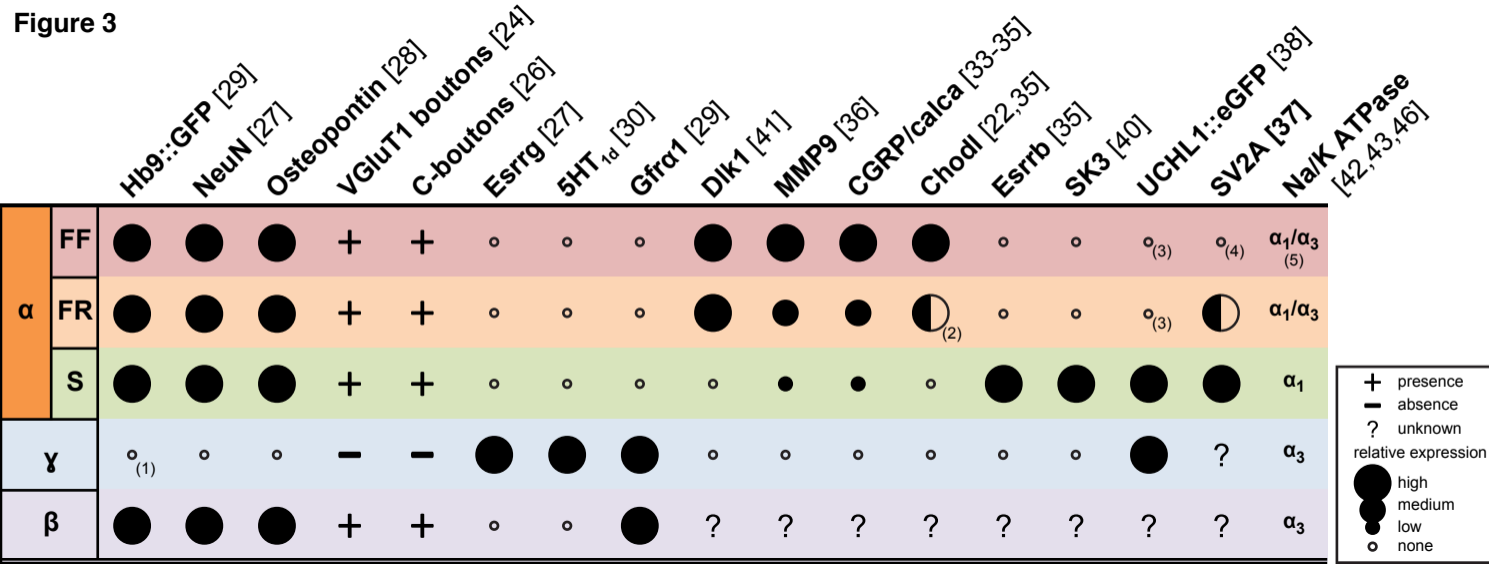
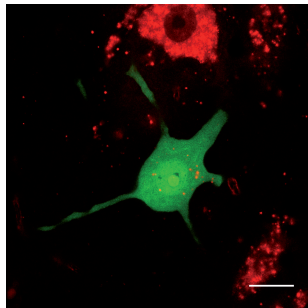
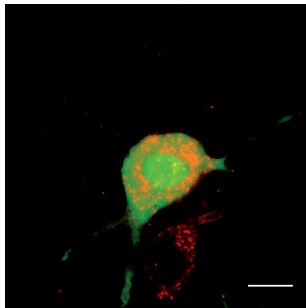


Figure 4

NB Chodl



NB Chodl

