

1 **Manuscript Title**

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3 **Orphan nuclear receptors Err2 and 3 promote a feature-specific terminal differentiation**
4 **program underlying gamma motor neuron function and proprioceptive movement control**

5 Short Title

6 Err2/3 tune gamma motor neuron function underlying proprioceptive movement control

7 **Authors**

8 Mudassar N. Khan^{1,2*}, Pitchaiah Cherukuri^{1,2,8}, Francesco Negro^{2,3}, Ashish Rajput^{4,9}, Piotr
9 Fabrowski^{1,2}, Vikas Bansal^{4,5}, Camille Lancelin², Tsung-I Lee², Yehan Bian^{2,8}, William P. Mayer⁶,
10 Turgay Akay⁶, Daniel Müller^{1,2}, Stefan Bonn⁴, Dario Farina⁷, Till Marquardt^{1,2**}.

11

12 **Affiliations**

13 ¹Interfaculty Chair for Neurobiological Research, RWTH Aachen University: Medical Faculty
14 (UKA), Clinic for Neurology & Faculty for Mathematics, Computer and Natural Sciences, Institute
15 for Biology 2, Worringer Weg 3, 52074 Aachen, Germany

16 ²Developmental Neurobiology Laboratory, European Neuroscience Institute (ENI-G),
17 Grisebachstraße 5, 37077 Göttingen, Germany

18 ³Department of Clinical and Experimental Sciences, Università degli Studi di Brescia, Viale Europa
19 11, 25123, Brescia, Italy

20 ⁴University Medical Center Hamburg Eppendorf, Center for Molecular Neurobiology Hamburg
21 (ZMNH), Institute of Medical Systems Biology, Falkenried 94, 20251 Hamburg, Germany

22 ⁵Biomedical Data Science and Machine Learning Group, German Center for Neurodegenerative
23 Diseases, Tübingen 72076, Germany

24 ⁶Atlantic Mobility Action Project, Brain Repair Centre, Department of Medical Neuroscience,
25 Dalhousie University, Halifax, Nova Scotia, Canada

26 ⁷Department of Bioengineering, Imperial College London, Royal School of Mines, London, UK

27 ⁸SRM University Andhra Pradesh, Mangalagiri -Mandal, Neeru Konda, Amaravati, Andhra Pradesh
28 522502, India email id: pitchaiah.c@srmmap.edu.in

29 ⁹Maximon AG, Bahnhofplatz, 6300 Zug, Switzerland

30

31 *Correspondence: Khan@fmp-berlin.de

32 **Co-correspondence: tmarquardt@ukaachen.de

33 **Abstract**

34 Motor neurons are commonly thought of as mere relays between the central nervous system and the
35 movement apparatus, yet, in mammals about one-third of them function exclusively as regulators of
36 muscle proprioception. How these gamma motor neurons acquire properties to function differently
37 from the muscle force-producing alpha motor neurons remains unclear. Here, we found that upon
38 selective loss of the orphan nuclear receptors *Err2* and *Err3* (*Err2/3*) in mice, gamma motor neurons
39 acquire characteristic structural (e.g. synaptic wiring), but not functional (e.g. physiological firing
40 rates) properties necessary for regulating muscle proprioception, thus disrupting gait and precision
41 movements *in vivo*. Moreover, *Err2/3* operate via transcriptional activation of neural activity
42 modulators, one of which (*Kcna10*) promoted gamma motor neuron functional properties. Our work
43 identifies a long-sought mechanism specifying gamma motor neuron properties necessary for
44 proprioceptive movement control, which implies a ‘feature-specific’ terminal differentiation
45 program implementing neuron subtype-specific functional but not structural properties.

46

47 **Summary**

48 The transcription factors *Err2* and *3* promote functional properties in a subset of motor neurons
49 necessary for executing precise movements.

50

51 **Introduction**

52 Motor neurons tend to be thought of as passive links between the central nervous system and the
53 movement apparatus by faithfully relaying descending neural commands to the skeletal musculature,
54 where these signals evoke muscle contractions and thereby movement¹. However, in zebrafish,
55 motor neurons have been shown to provide active and direct feedback control for the neural
56 networks driving rhythmic (swimming) locomotion². In mammals, moreover, up to a third of the
57 motor neurons do not connect to the force-generating extrafusal skeletal muscle fibers and do not
58 directly contribute to muscle contractions³⁻⁵. Rather, these neurons, called gamma motor neurons,
59 effectively contribute to the ability of mammals to learn and execute precision movements^{6, 7}.

60 Gamma motor neurons do so by regulating the flow of proprioceptive information the nervous
61 system receives about skeletal muscle (and thus limb and trunk) position and velocity⁸⁻¹¹ via direct
62 synaptic connections onto the intrafusal fibers of stretch-sensitive mechanosensory organs called
63 muscle spindles³⁻⁶. These motor neurons thereby facilitate ‘real-time’ adaptations of movements to
64 changing external conditions by enhancing detection of discrepancies from intended movement
65 trajectories^{6, 7, 12}. In addition, gamma motor neurons⁶ generate muscle tone by recruiting the force-
66 generating (alpha) motor neurons through a monosynaptic spindle afferent feedback ‘servo’ loop,
67 thus assisting movement initiation and postural control⁶.

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69 The ability of gamma motor neurons to control muscle proprioception entails their acquisition of
70 intrinsic functional properties distinguishing them from the muscle force-generating alpha motor
71 neurons¹³. For instance, their low firing thresholds and ability to rapidly gear up high firing rates
72 appear to be exquisitely suited for achieving near-instant intrafusal fiber tension for maintaining or
73 modulating muscle spindle dynamic range^{6, 13}. Another feature allowing gamma motor neurons to
74 effectively control muscle proprioception is their lack of monosynaptic (Ia) afferent feedback, which
75 uncouples them from potential ‘short circuits’ by their own actions on muscle spindle activity^{14, 15}.
76 The acquisition of such specific combinations of functional (activation threshold, firing rate etc.)
77 and structural properties (soma size, synaptic wiring preference etc.) inherent to neuron type or
78 subtype identity¹⁶ is thought to involve overarching terminal differentiation (‘selector’) gene
79 expression programs^{17, 18}. Nevertheless, terminal differentiation programs have also been shown to
80 promote only some but not all neuron subtype-specific structural features, like axonal versus
81 dendritic wiring specificity¹⁹, raising the possibility that at least in some instances neuron type or
82 subtype-specific properties could be specified by such programs in a modular (‘feature-specific’)
83 manner.

84

85 Apart from their overall distinction from the gamma motor neurons, alpha motor neurons
86 themselves exhibit a range of functional properties important for the adjustment of muscle force²⁰.
87 We and others have previously reported mechanisms underlying the diversification of alpha motor
88 neurons proper, which involved both cell-autonomous actions by the non-canonical Notch ligand
89 Dlk1²¹, as well as non-cell autonomous signals by region-specific astrocytes²². Yet, how gamma
90 motor neurons acquire a unique suite of properties that allow them to control muscle proprioception,
91 instead of generating muscle force, remained unaddressed. Here, we studied the specification of
92 gamma versus alpha motor neurons in mice and identify the orphan nuclear receptors Err2 and Err3
93 as drivers of a ‘feature-specific’ terminal differentiation program underlying the acquisition of
94 gamma motor neuron-specific functional but not structural properties required for proprioceptive
95 movement control.

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103 **Results**

104 **Electrophysiological characterization of murine gamma motor neurons**

105 Since the last recordings from mature gamma motor neurons dated from 1978 in the cat¹³, we first
106 sought to establish a baseline for gamma motor neuron functional properties in the mouse. We were
107 aided in this by a fortuitous finding that allowed us to selectively record gamma motor neurons in
108 the mouse spinal cord based on the relative efficacy of retrograde Fluoro-Gold uptake (Figures 1A,
109 1B and S5A-S5G). We observed that indeed gamma motor neurons can be distinguished from alpha
110 motor neurons based on their FG uptake as FG^{high} lacked sensory neuron innervations and expressed
111 low or negligible levels of NeuN when compared to FG^{low} motor neurons (Figure 1B). Upon
112 performing whole cell patch-clamp recordings at 20-22 days postnatally, we observed that the small-
113 soma motor neurons with high-levels of Fluoro-Gold retention (FG^{high}) exhibited an
114 electrophysiological signature matching that previously reported for cat gamma motor neurons¹³
115 (Figures 1C-1E). The FG^{high} motor neurons showed a distinctive combination of lower rheobases
116 with higher firing frequencies and gains, as well as higher instantaneous and steady-state firing rates
117 when compared to the larger (FG^{low}) motor neuron subtypes (Figures 1D, 1E and S6A and S6B and
118 Supplementary Table S1). In contrast, FG^{low} motor neuron subtypes were characterized by a
119 combination of low rheobases with low firing rates (alpha or beta motor neurons)²⁰ (Figures 1D, 1E
120 and S6A and S6B and Supplementary Table S1). Moreover, FG^{high} motor neurons showed
121 significantly lower membrane input resistance, higher membrane capacitance and lower AHP-decay
122 times when compared to FG^{low} motor neurons (Figures 1E and S6C and Supplementary Table S1).
123 In addition to the overall quantitative differences in functional properties, gamma motor neurons in
124 mouse as in the cat^{13,20} further showed a distinctive combination of functional properties (e.g.:
125 relatively very low activation thresholds + very high firing rates), compared to those of the slow
126 (low thresholds + low firing rates) or fast alpha motor neuron subtypes (high thresholds + high
127 firing rates).

128

129 **Correlated expression of orphan nuclear receptors Err2 and Err3 by gamma motor neurons**

130 It had previously been established that the survival of gamma motor neurons beyond the early
131 postnatal period relies on signals released by muscle spindles^{23,24}, which, in addition to the
132 discovery of markers allowing *in situ* detection of gamma motor neurons^{23,24} provided us with entry
133 points for studying mechanisms underlying the diversification of motor neurons into alpha and
134 gamma subtypes. We focused on the estrogen-related receptor (Err) subfamily of orphan nuclear
135 receptors, which primarily function as ligand-independent transcription factors²⁵, because of their
136 contribution to cell type-specific functional (i.e.: electrophysiological) properties in other contexts²⁵
137 and because of the previously reported expression of Err3 by gamma motor neurons²⁴. We further

138 found that the closely related Err3 paralogue Err2, with which it shares virtually the same DNA
139 binding sequences²⁵, was co-expressed with Err3 by gamma motor neurons (Figures 2A-2J and
140 S2A-S2O) upon immunodetection using antibodies specifically recognizing either Err2 or Err3
141 (Figures S1A-S1Q), a co-expression which had been independently observed by others using single-
142 cell RNAseq²⁶. Through deeper analysis by quantitative immunodetection we indeed found high
143 levels of correlated expression (Pearson correlation, $r=0.86$) of both Err2 and Err3 by motor neurons
144 with relatively small somas characteristic for gamma motor neurons²⁷ and low or negligible levels of
145 the alpha motor neuron marker NeuN^{23, 24} (NeuN^{low or negligible}) (Figures 2A-2J and S2A-S2O). The
146 small-soma Err2/3^{high} NeuN^{low or negligible} motor neurons lacked vGlut⁺ varicosities on somatic or
147 dendritic membranes (Figures 1B, 2L), indicating absence of monosynaptic spindle afferent input, a
148 defining characteristic of gamma motor neurons^{14, 15, 23, 24}. Similar to Err3, Err2 was initially broadly
149 expressed by most motor neurons during embryonic development (Figures S3A-S3C), but high Err2
150 levels became increasingly confined to gamma motor neurons during the first two postnatal weeks
151 (Figures S3D-S3R). Consistent with the dependency of gamma motor neuron maintenance on
152 spindle-derived signals^{23, 24}, adult motor neurons failed to retain high levels of both Err2/3 in Egr3-
153 deficient mice with impaired muscle spindle development (Figures 2K, S3S-S3X and S3S'-S3X').
154 We noted that low Err2/3 levels were maintained by the remaining large-soma size NeuN^{high} motor
155 neurons in Egr3-deficient mice (Figures S3S'-S3X'), while high Err2/3 levels persisted in a subset
156 of ventral spinal interneurons in both Egr3-deficient mice (Figures S3S'-S3X') and mice specifically
157 lacking Err2/3 in motor neurons (Figure S1O-S1Q). Furthermore, *Imaris* reconstruction studies in
158 control mice showed that indeed, FG^{high}, Err2^{high}, NeuN^{low or negligible} gamma motor neurons were
159 contacted by few vGlut1⁺ terminals, while FG^{low}, Err2^{low or negligible}, NeuN^{high} alpha motor neuron
160 somas were covered with vGlut1⁺ varicosities (Figures 2L-2N). Mouse gamma motor neurons thus
161 exhibit high levels of correlated Err2 and Err3 expression.

162

163 **Err2/3 are required for the acquisition of gamma motor neuron electrophysiological** 164 **properties**

165 Due to their correlated expression as well as their molecular similarity, we next asked whether
166 Err2/3 would contribute to gamma versus alpha motor neuron functional diversification by
167 selectively inactivating both *Esrrb* and *Esrrg* genes in motor neurons via Cre-mediated
168 recombination in cholinergic neurons in *Esrrb*^{flox/flox}; *Esrrg*^{flox/flox}; *Chat*^{Cre} (Err2/3^{cko}) mice (Figures
169 S1O-S1Q). Because *Chat*^{Cre}-mediated recombinase activity overlapped with endogenous Err2/3
170 expression in motor neurons but not in other cholinergic neuron types throughout the nervous
171 system (Figures S4A-S4D), we concluded that Err2/3^{cko} mice permitted addressing Err2/3 function
172 in motor neurons. Whole cell patch-clamp recordings performed in 20-22 days-old Err2/3^{cko} mice
173 showed that most FG^{high} motor neurons failed to acquire a gamma motor neuron

174 electrophysiological signature and instead shifted their properties dramatically towards resembling
175 those of FG^{low} alpha motor neurons, with significantly elevated rheobases, as well as lowered gains
176 and firing rates (Figures 3A, 3B and 3C, and S6D-S6H and Supplementary Table S1). At the same
177 time, we did not observe significant changes in the properties of FG^{low} alpha motor neurons in
178 *Err2/3^{cko}* mice when compared to control mice (Figures 3D, 3E and 3F and S6I-S6M and
179 Supplementary Table S1). *Err2/3* thus appear to be prerequisite for the acquisition of gamma motor
180 neuron functional properties, but not for the development of morphologically distinguishable
181 gamma motor neurons proper.

182

183 ***Err2/3*-dependent gamma motor neuron functional properties are required for movement** 184 **accuracy**

185 The biophysical properties of gamma motor neurons are thought to be exquisitely suited to effect
186 instant intrafusal fiber peak tension for maintaining and modulating spindle dynamic range and thus
187 muscle proprioception¹³. We therefore asked how a shift towards an alpha motor neuron-like
188 electrophysiological signature in gamma motor neurons would impact movements relying on
189 proprioceptive feedback from muscles. Because of the exclusive association of high *Err2/3* levels
190 with gamma motor neurons, and the lack of a significant impact of *Err2/3* loss on other motor
191 neuron subtypes, we predicted that beta motor neuron function would be preserved in *Err2/3^{cko}*
192 mice, thus allowing us to study the contribution of gamma motor neuron function to movement
193 control in the otherwise intact animal. Similar to *Egr3*-deficient mice^{9,10}, *Err2/3^{cko}* mice exhibited
194 marked postural and gait alterations, including changes in metrics related to foot placement, weight
195 bearing, stride, stance, braking and propulsion (Figures 4A, 4B, S8D and S8E), consistent with the
196 predicted contributions of gamma motor neuron-assisted spindle function to posture, gait phase-
197 transitions and force generation during locomotion^{6,9-11}. *Err2/3^{cko}* mice were nevertheless able to
198 sustain the same range of speeds as control mice in a treadmill locomotion task with little
199 dependency on muscle proprioception^{9,10} (Figures S8A-S8C). However, *Err2/3* loss from motor
200 neurons triggered a failure to handle precision tasks with extensive reliance on muscle
201 proprioception^{9,10}, such as navigating a narrow horizontal beam (Figures 4C, 4D and Supplementary
202 Movies S1 and S2) or a horizontal ladder (Figures 4E, 4F and Supplementary Movies S3 and S4),
203 indicating that the electrophysiological signature implemented by *Err2/3* in gamma motor neurons is
204 prerequisite for effective modulation of muscle proprioception, and thereby the execution of
205 precision movements. To further test this idea, we recorded spindle afferent responses via suction
206 electrodes in nerve-muscle preparations³¹ derived from *Err2/3^{cko}* or control mice. In these
207 preparations muscle stretch applied by a force transducer elicited similar Ia afferent responses in
208 *Err2/3^{cko}* and control mice (Figures 4G, 4G' and S7A-S7D), consistent with the morphologically
209 normal spindle assembly in these animals (Figures 5K and S7G, S7H). In contrast to control

210 spindles (Figures S7A and S7B), however, $Err2/3^{cko}$ spindle afferents frequently exhibited reduced
211 firing rates at muscle resting length (Figures 4H, 4H' and S7C, S7D), possibly due to a decrease in
212 basal intrafusal fiber contractility caused by chronic disruption of gamma motor neuron input. The
213 $Err2/3$ -dependent implementation of gamma motor neuron functional properties therefore appears to
214 be prerequisite for regulating spindle-mediated muscle proprioception and movement control.

215

216 **Lack of $Err2/3$ in gamma motor neurons does not alter their development and connectivity** 217 **patterns**

218 Since we observed that the loss of $Err2/3$ in gamma motor neurons in $Err2/3^{cko}$ mice led to the loss
219 of a gamma motor neuron electrophysiological identity, including low rheobases, high firing
220 frequencies and gains, we then asked whether $Err2/3$ are necessary for gamma motor neuron
221 morphology and connectivity. In addition to electrophysiological features, gamma motor neurons
222 characteristically possess small soma sizes, lack Ia sensory neuron pre-synaptic innervation and
223 send axons that innervate muscle spindle intrafusal fibers^{24,27}. Remarkably, the lack of $Err2/3$ in
224 $Err2/3^{cko}$ mice did not affect the development of small-soma FG^{high} and $NeuN^{low}$ or negligible motor
225 neurons (Figures S3D-S3R). Similar to control mice (Figures 5E, 5F and 5I), the small-soma FG^{high} ,
226 $Err2^{-}$, $NeuN^{low}$ or negligible motor neurons in $Err2/3^{cko}$ mice (Figures 5G, 5H and 5I) lacked $vGlut1^{+}$
227 varicosities on somatic or dendritic membranes. Moreover, FG^{low} , $Err2^{-}$, $NeuN^{high}$ motor neurons
228 retained their $vGlut1^{+}$ terminals in both $Err2/3^{cko}$ mice (Figures 5C, 5D and 5I) when compared to
229 control mice (Figures 5A, 5B and 5I). We next observed the morphology of the muscle spindles.
230 The muscle spindle appearance, notably the innervation by motor axon presynaptic termini within
231 the peripheral contractile segments of the intrafusal muscle fibers was preserved in $Err2/3^{cko}$ muscle
232 spindles (Figures 5K) when compared to control mice (Figures 5J). Taken together, we surmise that
233 $Err2/3$ are not necessary for gamma motor neuron structural identity, since the lack of $Err2/3$ in
234 gamma motor neurons did not affect the development of morphologically distinct gamma motor
235 neurons and did not perturb gamma motor neuron pre- and post-synaptic innervation patterns in
236 $Err2/3^{cko}$ mice.

237

238 **$Err2/3$ are sufficient in forcing gamma motor neuron functional properties in chick**

239 We next tested whether $Err2/3$ would also be sufficient to promote a gamma motor neuron
240 electrophysiological signature by performing whole cell patch-clamp recordings on chick motor
241 neurons²¹ engineered to stably express elevated $Err2/3$ levels (Figures 6A-6D'). Indeed, forced
242 expression of $Err2/3$ partially shifted motor neuron properties towards an electrophysiological
243 signature recapitulating that of mouse or cat gamma motor neurons^{13,20}, including a combination of
244 high gains and firing rates (Figures 6E-6I, S6N and Supplementary Table S2), suggesting that high

245 Err2/3 levels are not only necessary but also sufficient to promote a gamma motor neuron
246 electrophysiological signature. This effect on motor neuron properties was enhanced by fusing Err2
247 to the heterologous transcriptional activation domain VP16 (Figures 6G-6I, S6N and Supplementary
248 Table S2), but not by its fusion to the *engrailed* transcriptional repressor domain (EnR) (Figures 6G-
249 6I, S6N and Supplementary Table S2), suggesting that in this context Err2 primarily functions as a
250 transcriptional activator. Thus, Err2/3 appear to be not only necessary but also sufficient to promote
251 gamma motor neuron functional properties by operating as transcriptional activators.

252

253 **Err2/3 drive gamma motor neuron functional properties by activating the expression of the** 254 **shaker K⁺ channel subunit gene *kcna10***

255 Reasoning that in these gain-of-function experiments in chick, Err2 would likely operate through the
256 same intermediate factors through which it would normally tune motor neuron electrophysiological
257 properties, we performed comparative transcriptome profiling by RNA sequencing of chick motor
258 neurons forcedly expressing Err2 (Figures 7A and S9G-S9J, Supplementary Table S3). In these
259 experiments, elevated Err2 levels significantly activated a set of genes largely distinct from the gene
260 signature activated by the previously identified (fast) alpha motor neuron determinant *Dlk1*²¹
261 (Figures 7A and S9G-S9J, Supplementary Table S3). This included activation by Err2 of *Kcna10*,
262 which encodes a member of the shaker family of voltage-gated K⁺ channels implicated in neuronal
263 excitability²⁹ (Figures 7A and S9H), the promoter of which contained an evolutionary conserved
264 region (ECR) with three clustered Err2/3 DNA binding motifs (Figures 7B and S9K). In chick
265 motor neurons, moreover Err2 boosted reporter gene activity driven by the *Kcna10* ECR (Figures
266 7C, S9C and S9D), but not upon introducing mutations into the ECR's Err2/3 binding motifs
267 (Figures 7B, 7C, S9E and S9F). Ultimately, similar to *Err2*, forced expression of *Kcna10* shifted
268 motor neuron electrophysiological properties towards lower rheobases and higher firing rates typical
269 for gamma motor neurons (Figure 7D and Supplementary Table S2), together suggesting that
270 Err2/3, like *Dlk1*²¹ or *Islet* in *Drosophila*³⁰, chiefly operate through voltage-gated K⁺ channels to
271 promote neuron subtype-specific electrophysiological properties (Figure 7E). Taken together, these
272 data suggest that Err2/3 operate as transcriptional activators to implement a feature-specific gene
273 expression program encoding neural activity modulators to promote gamma motor neuron
274 functional but not structural properties.

275

276 **Discussion**

277 Neuronal specification is thought to involve gene expression programs that coordinate the
278 acquisition of both functional (activation threshold, firing rate etc.) and structural properties (soma
279 size, synaptic wiring preference etc.) associated with neuron type or subtype identities¹⁶⁻¹⁸. Here, we

280 have shown that during the diversification of motor neurons into gamma and alpha subtypes, the
281 acquisition of neuron subtype-specific functional properties can (at least to a large degree) be
282 achieved independently from the acquisition of structural properties (Figure 7E). The disruption of
283 proprioceptive movement control we observed upon eliminating gamma motor neuron-specific
284 functional (but not structural) properties therefore suggests that homeostatic plasticity, which to
285 some extent can compensate for quantitative fluctuations in neural activity²⁸, is unable to offset a
286 qualitative shift in the biophysical properties of one neuron subtype towards those of another. Since
287 the ‘feature-specific’ action of *Err2/3* in specifying neuron subtype-specific properties is apparently
288 important for the function of the entire neural network involved in muscle spindle-dependent
289 movement control, it will be interesting to determine whether a similar separation of functional and
290 structural terminal differentiation programs exists elsewhere in the mammalian nervous system.
291 This, in turn, would open the intriguing possibility of utilizing ‘feature-specific’ terminal
292 differentiation factors as indicators for a certain set of biophysical properties (i.e.: as ‘markers for
293 neuronal function’), instead of as markers for neuron type or subtype identities proper.

294
295 Two types of gamma motor neuron output, static or dynamic, are thought to normally tune muscle
296 spindle sensitivity during different movement tasks^{6,33}. Three observations led us to conclude that
297 the actions of *Err2/3* do not distinguish between both types of gamma motor neuron output. First,
298 we found *Err2/3* to be broadly expressed by all gamma motor neurons. Second, consistently, gamma
299 motor neuron biophysical properties were disrupted as a whole upon loss of *Err2/3*. Third, the range
300 of movements affected by *Err2/3* removal from motor neurons (from running to ‘skilled’ movement)
301 further suggest that *Err2/3* function would be required for both static and dynamic modulation of
302 spindle function. The mechanistic bases underlying the two different outputs of gamma motor
303 neurons to spindles thus remain to be addressed. How about the beta motor neurons? Beta motor
304 neurons connect to both extrafusal and intrafusal muscle fibers but are otherwise morphologically
305 indistinguishable from alpha motor neurons³⁴⁻³⁶. Since beta motor neurons, like the alpha motor
306 neurons, possess relatively large soma sizes and receive monosynaptic Ia afferent input³⁶, we were
307 able to rule out that *Err2/3* were also operating in beta motor neurons. Since beta motor neurons so
308 far have mostly been studied in the cat, and have been identified solely based on their simultaneous
309 innervation of intrafusal and extrafusal fibers³⁴⁻³⁶, both the prevalence of beta motor neurons and
310 their significance for movement control in rodents awaits further study.

311
312 The remarkable contribution of a subset of motor neurons to proprioceptive movement control in
313 mammals has long been recognized⁴⁻⁶, but in over 75 years since the description of gamma motor
314 neurons, little has been learned about how these neurons acquire properties that allow them to
315 function differently from the force-generating motor neurons in the first place. In the present study,

316 we identified a long-sought mechanism driving the functional divergence of gamma and alpha
317 motor neurons, by showing that the orphan nuclear receptors Err2/3 implement a ‘feature-specific’
318 terminal differentiation program promoting the acquisition of gamma motor neuron functional
319 properties (Figure 7E). These properties, in turn, allow the nervous system to engage muscle
320 spindles for regulating muscle proprioception and thus movement accuracy. The action of Err2/3 in
321 gamma motor neuron functional specification could ultimately serve as a blueprint for how such
322 feature-specific terminal differentiation programs impart either functional or structural properties
323 elsewhere in the nervous system.

324

325 **Experimental Procedures**

326 **Mouse models**

327 Mouse husbandry and experiments involving mice were approved by and conformed University,
328 state, federal and European Union animal welfare regulations. For experiments using wild-type
329 mice, C57BL/6J and CD1 strains (both purchased from Charles River Laboratories, Inc.,
330 Wilmington, USA) were used. “*Chat::tdTomato*” mice were generated by interbreeding mice
331 carrying “*Rosa26^{flx/tdTomato}*”³⁷ and “*Chat^{Cre}*”³⁸ targeted alleles (both purchased from Jackson
332 Laboratories, Bar Harbor, USA). *Egr3^{ko/ko}* mice³⁹ were a gift from Warren G. Tourtellotte
333 (Northwestern University, currently Cedars Sinai Medical Center). *Err2/3^{cko}* (*Esrrb^{flx/flx}*;
334 *Esrrg^{flx/flx}*; *Chat^{Cre}*) mice were obtained by interbreeding mice carrying *Chat^{Cre}* and floxed
335 *Esrrb^{loxP/loxP}* (exon 2 of *Esrrb* gene (chromosome 12) has been flanked by two loxP sites)⁴⁰ alleles
336 with mice carrying floxed *Esrrg^{loxP/loxP}* (in which exon 2 of *Esrrg* gene (chromosome 1) has been
337 flanked by two *loxP* sites alleles, and were derived from the ES cell repository of the Institut
338 Clinique de la Souris (ICS), Alsace, France). Unless otherwise indicated, “controls” for comparison
339 with *Err2/3^{cko}* mice were of the genotype *Esrrb^{flx/flx}*; *Esrrg^{flx/flx}* (unrecombined alleles) and
340 *Egr3^{ko/+}* or C57bl6/J (wild type) mice for comparison with *Egr3^{ko/ko}* mice.

341

342 **Immunodetection**

343 30-60 μm frozen OCT (Sakura Finetek GmbH, Umkirch, Germany) sections were incubated
344 overnight in PBS containing 1% BSA and 0.5% Triton-X 100 as described⁴¹⁻⁴³, using the following
345 antibodies: mouse anti-NeuN (1:1500, Cat. # MAB377, Merck KGaA, Darmstadt, Germany), chick
346 anti-GFP (1:2000, Cat. # ab13970, Abcam plc., Cambridge, UK), rabbit anti-dsRed (1:1000, Cat. #
347 632496, Takara Bio Europe SAS, Saint-Germain-en-Laye, France), mouse anti-Err2 IgG2b (1:4000,
348 Cat. # PP-H6705-00, R&D Systems Inc., Minneapolis, USA), mouse anti-Err3 IgG2a (1:1000, Cat.
349 # PP-H6812-00, R&D Systems Inc., Minneapolis, USA), rabbit anti-VACHT (1:750, Cat. # 139103,
350 Synaptic Systems GmbH, Göttingen, Germany), guinea pig anti-VGLUT1 (1:1000, Cat. # AB5905,

351 Merck KGaA, Darmstadt, Germany), rabbit anti-Isl1/2 (1:2500, Gift from S. L. Pfaff, Salk Institute
352 La Jolla, CA USA), mouse anti-V5 (1:1000, Cat. # 37-7500, Thermo Fisher Scientific Inc.,
353 Waltham, USA). Secondary detection of anti-Err2 and anti-Err3 IgG isoforms was accomplished by
354 Alexa Fluor-conjugated anti-mouse IgG2a (1:2000, Cat. # A-21131, Thermo Fisher Scientific Inc.,
355 Waltham, USA) or IgG2b (1:2000, Cat. # A-21147, Thermo Fisher Scientific Inc., Waltham, USA)
356 antibodies.

357

358 **Microscopy, image analysis and quantification**

359 Fluorescence microscopy was performed using Zeiss LSM 710 and LSM 800 laser scanning
360 microscopes. 6-28 optical sections were obtained at a step-size of 0.8-1.5 μm . Care was applied to
361 avoid oversaturation and distortion of relative expression levels during image acquisition. Raw
362 images were imported into ImageJ and z projected at maximum intensity. For quantification of
363 expression levels raw pixel intensities were quantified in individually outlined motor neuron nuclei
364 “region of interests” (ROIs) using Adobe Photoshop CS5.1. using background levels and the neuron
365 with the highest fluorescent intensity for normalization. For quantification of VGLUT1⁺ varicosities,
366 a step-size of 0.80 μm was used to obtain an average of 20 optical sections. Raw Z-stack Carl Zeiss
367 files (.czi) were imported into *Imaris 8.0* (Bitplane AG, Zurich, Switzerland). Neuronal surfaces
368 were rendered to detect vGlut1 varicosities (“spots”) on motor neuron somas and dendrites using the
369 “find spots close to surface” function⁴⁴ and guided by specific parameters²⁴.

370

371 **Electrophysiology of gamma vs. alpha/beta motor neurons in mice**

372 Mice (P20-22) were intraperitoneally injected with 0.5%-2% (w/v) FluoroGold (FG, Flurochrome
373 LLC., Denver, CO) dissolved in PBS (pH=7.2) at a volume of 0.10 ml/10 g body weight. The
374 animals (1-day post-FG injection) were intraperitoneal injected with 100 mg/kg body weight of
375 Ketamine, 20 mg/kg body weight Xylazine in PBS pH=7.2 at a volume of 0.10 ml/10 g of body
376 weight. After losing their righting reflex, they were placed on a bed of ice until loss of toe pinch
377 response. Immediately after, the animals were decapitated and quickly eviscerated. The torso was
378 placed in chilled Dissecting aCSF (DaCSF) solution (in mM): 191 sucrose, 0.75 K-gluconate, 1.25
379 KH₂PO₄, 26 choline bicarbonate (80% solution), 4 MgSO₄, 1 CaCl₂, 20 dextrose, 2 kynurenic acid,
380 1 (+)-sodium L-ascorbate, 5 ethyl pyruvate, 3 myo-Inositol. The solution was maintained at pH ~7.3
381 using carbogen (95% O₂-5% CO₂), and osmolarity was adjusted to ~305-315 mOsm with sucrose.
382 Vertebrectomy was performed to extract the spinal cord. Ventral roots were cut and the meninges
383 were removed from the spinal cord. The thoracolumbar region (T10-L5) of the spinal cord was
384 isolated and embedded in agar block (4% agar in Recording aCSF (RaCSF)) using 20% gelatin in
385 RaCSF). Slices (370 μm) were obtained using Leica VT1200 S (Leica Biosystems, GmbH,

386 Nussloch Germany). The slices were incubated in 35°C RaCSF for 30 minutes and 30 minutes at
387 room temperature before the recordings. Motor neurons (MNs) were recorded in the RaCSF solution
388 (mM): 121 NaCl, 3 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 1.1 MgCl₂, 2.2 CaCl₂, 15 dextrose, 1 (+)-
389 sodium L-ascorbate, 5 ethyl pyruvate, 3 *myo*-Inositol. The solution was maintained at pH ~7.4 using
390 carbogen (95% O₂-5% CO₂), and osmolarity was adjusted to ~305-315 mOsm with sucrose. Whole
391 cell patch-clamp recordings were performed from FG-labeled motor neurons in the ventral horn
392 from control and *Err2/3^{cko}* animals. FG^{high} and FG^{low} MNs were visually identified using Olympus
393 BX51W1 microscope (Olympus Europa SE & Co. KG, Hamburg, Germany) equipped with an FG
394 longpass filter set (350nm bandpass and 425nm longpass filter) (AHF analysentechnik AG,
395 Tübingen Germany). The patch pipette (resistances of 3-6 MΩ) was filled with intracellular solution
396 (mM): 131 K-methanesulfonate (or MeSO₃H), 6 NaCl, 0.1 CaCl₂, 1.1 EGTA-KOH, 10 HEPES, 0.3
397 MgCl₂, 3 ATP-Mg²⁺ salt, 0.5 GTP-Na⁺ salt, 2.5 L-glutathione reduced, 5 phosphocreatine di(tris)
398 salt. The solution pH was adjusted to 7.25 with KOH and the osmolarity was adjusted to 300 mOsm
399 using sucrose. Data analysis was performed offline using Axograph X Version 1.6. Previously
400 established protocols were applied to obtain membrane properties of rheobase, input resistance,
401 capacitance, *F-I* curve, AHP amplitude, half-width and half-decay times^{21, 45-48}. For obtaining the *F-I*
402 curve for discharge properties, spikes were elicited by applying 20 pA, 1000 ms square current
403 pulses to cells. Currents up to 1 nA were injected for all neurons. For the mouse recordings, currents
404 of up to 1 nA were injected into FG^{high} and 3 nA into FG^{low} MNs from control and *Err2/3^{cko}* spinal
405 cord slices. The firing frequency (Hz) was defined as the inverse of the duration between first two
406 spikes (instantaneous firing frequency), or 0.25-0.75 seconds of the current pulse (steady-state firing
407 frequency), or 1 second current pulse (mean firing frequency). The gain (Hz/nA) was defined as the
408 slope of the regression line of mean firing frequency upon current injection^{49,65}.

409

410 **Gait analysis**

411 Control (n =9) and *Err2/3^{cko}* (n=8) mouse locomotion on a treadmill was recorded through
412 automated high-speed motion-capture (DigiGait, Mouse Specifics Inc., Framingham, USA) as
413 described previously^{21, 50, 66}. This method generates over 50 different gait variables, which exceed
414 the number of observations (8-9 animals per genotype) and are partially redundant. We therefore
415 used the Partial Least Squares (PLS) regression which is optimized for predictive modeling of
416 multivariate data and to deal with multicollinearity among variables. Orthogonal Signal Correction
417 PLS (OSC-PLS) was used as an extension of PLS to separate continuous variable data into
418 predictive and uncorrelated information for improved diagnostics as well as more easily interpreted
419 visualization. The method seeks to maximize the explained variance between-groups in a single
420 dimension and separates the within-group variance (orthogonal to classification goal) into
421 orthogonal dimensions or components. We modified an existing R script^{51,52}, originally designed for

422 chemometrics analysis⁵³, to adapt it to our behavior data. The OSC-PLS method was applied to the
423 complete centered-scaled dataset in order to define a model. This highly complex model was then
424 optimized to establish a robust model, the most parsimonious with the highest prediction
425 performance (not shown). A model with an optimal number of 2 components was used for
426 subsequent analysis in both fore and hindlimbs. Our model coefficient of determination Q², i.e. the
427 model's fit to the training data, and its Root Mean Square Error of Prediction (RMSEP), i.e. the
428 model's predictive ability on the testing data were calculated using the Leave-One-Out method⁵¹.
429 The model was finally validated to ensure that it was performing better than a random model, while
430 not being over-fitted. We conducted an internal cross validation by performing permutations in the
431 original data, from which 2/3 was used to fit a model. This model was then used to predict group
432 memberships on the remaining 1/3 testing set. The process was repeated 100 times and Q² and
433 RMSEP values were averaged over the repeats. We finally compared our model's Q² and RMSEP
434 values to the mean Q² and mean RMSEP values of the permuted models. The results of the two-
435 sample Student's t-tests used for the comparisons indicated a probability much inferior to 0.1% of
436 achieving a performance similar to our model by random chance.

437

438 **Precision Movement Tasks**

439 Precision movements were successively tested using a custom- built setup with a 100 cm horizontal
440 beam of 20, 25 and 30 mm width, respectively⁵⁴. Age-matched control (n=4) and Err2/3^{cko} (n=4) 10-
441 month-old female mice were trained to move across the beam into a home cage at its end. The
442 animals were trained for 3 days (4 trials/animal, bidirectional on the beam) and tested on the fourth
443 day (4-5 trials/animal, bidirectional on the beam). Furthermore, a custom-built setup to record
444 skilled locomotion on a 100 cm horizontal ladder, with 3 mm rungs spaced at 14 mm (similar to the
445 setup previously described)⁹ was used to test age-matched 8 weeks-old female control (n=5) and
446 Err2/3^{cko} (n=5) mice. Mice were trained to move across the ladder into a home cage placed at its end
447 and were trained for 2 days (3 trials/animal, single direction on the ladder) for two-weeks and tested
448 on the third day of the second week (4-5 trials/animal, single direction on the ladder). The animals
449 were rested in their home cage for 1 minute between trials for both tasks. Animal locomotion was
450 recorded using GoPro HD Hero2 (GoPro Inc., San Mateo, U.S.A) fitted to a custom-built slider
451 track. The videos were acquired at 120 fps at an image size of 848x480 and stored as MP4 files. The
452 videos were processed using GoPro Studio Version (2.5.4) and proDAD Defishr Version 1.0
453 (proDAD GmbH, Immendingen, Germany). The figure videos were slowed to 25-40% of original
454 speed and reduced to 60 fps using GoPro Studio Version (2.5.4). The fish-eye view was removed
455 from videos using proDAD Defishr Version 1.0 (proDAD GmbH, Immendingen, Germany) with
456 Mobius A Wide presets and Zoom adjusted to 110.0 or 180.0. A “miss” was scored when the mouse

457 paw failed to locate the rung or the beam leading to the animal to slip or to halt until the paw
458 regained its footing. An average of ~40 steps per trial were analyzed for each mouse.

459

460 **Muscle spindle afferent recordings**

461 *Ex vivo* extensor digitorum longus (EDL) muscle-nerve preparations were used to study the
462 response of muscle sensory neurons to stretch and was essentially performed as described³¹. Briefly,
463 the dissection of muscle (with the nerve attached) was performed in low calcium and high
464 magnesium solution^{31,55}. Then, the muscle was placed in recording solution 22-24 °C and
465 equilibrated with carbogen and was then hooked to a dual force and length controller -transducer
466 (300C-LR, Aurora Scientific Inc., Aurora, Canada) with the help of 5-0 sutures tied to its tendons.
467 Following the determination of resting length (Lo) as described in previous studies, a suction
468 electrode (tip diameter 50-80 µm) connected to an extracellular amplifier (EXT-02F, npi Electronics
469 GmbH, Tamm Germany) was used to sample muscle spindle afferent activity. Data acquisition was
470 performed with LabChart 8 connected to PowerLab 8/35 (ADInstruments Ltd., Oxford, UK).
471 Afferent activity, when obtained was checked for the presence of a characteristic pause following a
472 series of 30 twitch contractions at 1Hz and if the pause was present, the unit was identified as a
473 muscle spindle afferent. Then a series of 9 ramp and hold stretches were delivered to the muscle
474 (2.5% Lo, 5% Lo and 7.5% Lo at 40% Lo/sec, protocols were kindly provided by K.A. Wilkinson
475 lab). The data was recorded and analyzed offline with a custom written MATLAB code. Spikes
476 were detected using KMEANS (2). For each afferent, resting discharge (RD), dynamic peak
477 discharge (DP), dynamic index (DI) and static response (SR) were calculated. A total of 10 control
478 and 10 *Err2/3*^{cko} animals were used for recordings, from which 8 control and 9 *Err2/3*^{cko} spindle
479 afferents were analyzed.

480

481 **Molecular cloning**

482 Mouse *Esrrb* (NM_011934.4) (*Err2*) and Mouse *Esrrg* (NM_011935.3) (*Err3*) open reading frames
483 were isolated using PrimeScript 1st cDNA synthesis Kit Takara Bio Europe SAS, Saint-Germain-
484 en-Laye, France) following manufacturer's directions from E18.5 mouse spinal cord total RNA and
485 cloned after PCR amplification with the following oligonucleotide primers:

486 *Esrrb* Forward 5'-CATGCCATGGATGTCGTCCGAAGACAGGCACC-3',

487 *Esrrb* Reverse 5'-CATGCCATGGCACCTTGGCCTCCAGCATCTCCAGG-3',

488 *Esrrg* Forward 5'-CATGCCATGGATGGATTTCGGTAGAACTTTGC-3' ,

489 *Esrrg* Reverse 5'-CATGCCATGGGACCTTGGCCTCCAGCATTTCC-3'.

490 The chick *Kcna10* (NP_989793) open reading frame was isolated from E5.5 chick embryo total
491 RNA as above using the following primers:

492 *Kcna10* Forward 5'-ATGATGGACGTGTCCAGTTGG-3'

493 *Kcna10* Reverse 5'-TTTTTTGGCCTTGTCTCGAGG-3'.

494 Thus, synthesized cDNAs were subcloned into an expression vector between *CMV* promoter in
495 frame with an Aphthovirus 2A peptide-GFP fusion sequence for co-translational cleavage⁵⁶. This
496 entire cassette was flanked by *Tol2* sites facilitating genomic integration upon co-transfection with
497 *Tol2* transposase as described²¹. Err2VP16 was generated by fusing the open reading frame for the
498 herpes simplex virus-1 (HSV-1) VP16 (amplified from *pActPL-VP16AD* plasmid, Addgene plasmid
499 #15305, Watertown, USA) activation domain to Err2. Err2EnR was generated by fusing the open
500 reading frame of the transcriptional repressor domain of *Drosophila* Engrailed (amplified from
501 *CAG-EnR* plasmid, Addgene plasmid #19715, Addgene, Watertown, USA) to Err2.

502

503 **Chick motor neuron electrophysiology**

504 Chick embryos electroporated at E2.7-E3.0 (HH stages 14-18) with appropriate DNA constructs
505 were harvested at E12-15 (HH stages 38-41) and processed as described previously²¹. Briefly, chick
506 embryos were placed on ice for 5 minutes, extracted from the egg, decapitated and dissected in a
507 petri dish containing cold chick aCSF (CaCSF) solution (mM): 139 NaCl, 3 KCl, 1 MgCl₂, 17
508 NaHCO₃, 12.2 dextrose, 3 CaCl₂. The solution pH was adjusted to 7.25 with KOH and the
509 osmolarity was adjusted to ~315 mOsm using sucrose. The thoracolumbar region of the spinal cord
510 (with the vertebral column intact) was isolated and embedded in an agarose block (4% agarose in
511 CaCSF) using 20% gelatin in CaCSF. Slices (370 μm) were obtained using a Leica VT1200 S
512 vibrating blade microtome (Leica Biosystems GmbH, Nussloch, Germany) and incubated in CaCSF
513 for 30 minutes at room temperature (22°C). Motor neurons were visualized by GFP expression
514 using 4x air objective (Olympus UPlan FL N) and 40x water-immersion objective (Olympus UPlan
515 FI N) equipped on an Olympus BX51W1 microscope. The patch pipette (resistances of 3-6 MΩ)
516 was filled with intracellular solution (mM): 130 MeSO₃H, 10 KCl, 2 MgCl₂, 0.4 EGTA, 10 HEPES,
517 2 ATP-Mg²⁺ salt, 0.4 GTP-Na⁺ salt, 0.1 CaCl₂. The solution pH was adjusted to 7.3 with KOH and
518 the osmolarity was adjusted to ~315 mOsm using sucrose. The intracellular solution contained 25
519 μM Alexa fluor 568 dye (Thermo Fisher Scientific, Inc., Waltham, USA) to label recorded motor
520 neurons. Current-clamp recording signals were amplified and filtered using MultiClamp 700B
521 patch-clamp amplifier (Molecular Devices LLC., San Jose, USA). The signal acquisition was
522 performed at 20 kHz using Digidata 1322A digitizer (Molecular Devices LLC., San Jose, USA) and
523 pCLAMP 10.4 software (Molecular Devices LLC., San Jose, USA).

524

525 RNA sequencing and transcriptome analysis

526 E12.5 (HH St. 38-39) chick lumbar spinal motor columns transfected with either *CMV::eGFP*, or
527 *CMV::Err2VP16.2A.eGFP* or *Dlk1.IRES.eGFP* plasmids were identified by GFP fluorescence,
528 dissected and collected. RNA isolation and RNA sequencing were carried out as described
529 previously⁵⁷. Briefly, RNA was isolated using Tri-Reagent (Sigma-Aldrich Chemie GmbH,
530 Taufkirchen, Germany) and Phenol-Chloroform extraction according to the manufacturer's protocol.
531 RNA quality was assessed using Nanodrop 2000 (Thermo Fisher Scientific, GmbH) and RNA
532 integrity number (RIN) was evaluated by using the Agilent 2100 Bioanalyzer (Agilent
533 Technologies, Inc., USA). RNA was reverse transcribed to cDNA using Transcriptor High Fidelity
534 cDNA synthesis kit (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) and RNA-Seq
535 libraries were obtained using TruSeq RNA Sample Preparation v2 kit (Illumina, Inc., San Diego,
536 USA). To analyze the library quality, Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa
537 Clara, USA) was used and the concentration was measured by a Qubit^R dsDNA HS Assay kit
538 (Thermo Fisher Scientific Inc., Waltham, USA). The concentration was adjusted to 2 nM prior to
539 sequencing (50 bp) on a HiSeq 2000 sequencer (Illumina, Inc., San Diego, USA) using TruSeq SR
540 Cluster kit v3-cBot-HS (Illumina, Inc., San Diego, USA) and TruSeq SBS kit v3-HS (Illumina, Inc.,
541 San Diego, USA) based on manufacturer's instructions. RNA-sequencing quality was evaluated by
542 utilizing raw reads using the FastQC quality control tool version 0.10.1⁵⁸. Bowtie2 v2.0.2 using
543 RSEM version 1.2.29 with default parameters was utilized to align sequence reads (single-end 50
544 bp) to chicken reference genome (Galgal5)^{59,60}. Prior to indexing, GFP, Err2, Dlk-1, VP16 and
545 IRES sequences and annotations were added to the reference genome (FASTA file) and annotations
546 (GTF file). Ensembl annotations (version 86.5) with rsem-prepare-reference from RSEM software
547 was used to index chicken reference genome⁶¹. Furthermore, sequence alignment of sequence reads
548 and gene quantity was obtained through the use of rsem-calculate-expression. Rsem-calculate-
549 expression resulted in sequence read count and TPM value (transcripts per million) for individual
550 genes. DESeq2 package was used to carry out differential expression analysis⁶². Finally, genes with
551 less than 5 reads (baseMean) were filtered, while genes with an adjusted p-value < 0.05 were
552 classified as differentially expressed. Gene ontologies and categorization was performed using the
553 DAVID Gene Functional Classification Tool⁶⁷.

554

555 Enhancer identification and promoter assays

556 Evolutionary conserved non-coding genomic regions (ECRs) around the *Kcna10* genomic locus
557 were identified using the ECR Browser⁶³ and screened for potential Err2/Err3 transcription factor
558 binding sites using the JASPAR CORE database⁶⁴. A 240 bp ECR 3.5 kb upstream of the *Kcna10*
559 transcription start site with three putative Err2/Err3 binding sites was amplified from mouse
560 genomic DNA using the following primers: Forward 5'-TCTCACAGCCCTGCTCATC-3' and

561 Reverse 5'-CTTGCCTGAGAACCTGATCTCC-3' and subcloned into a reporter vector containing
562 a minimal promoter followed by tdTomato coding sequence, which together were flanked by *Tol2*
563 sites to facilitate stable genomic integration. To test promoter activity and potential regulation by
564 *Err2/3*, Lohmann LSL fertilized chick eggs were incubated until E2.7-E3.0 (HH stages 14-18) and
565 chick embryo neural tubes were electroporated *in ovo* using the ECM 830 electroporation system
566 (BTX/Harvard Apparatus Inc., Holliston, USA) as described²¹. *Kcna10::tdTomato* reporter plasmids
567 together with either *CMV::2A.eGFP* or *CMV::VP16:Err2.2A.eGFP* at a molar ratio of 1:1 were
568 transfected.

569

570 **Data Availability**

571 All data are available in the manuscript or the supplementary materials. Raw data are available upon
572 reasonable request to the corresponding author.

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779 **Acknowledgments**

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781 We thank Louisa Dorr, Simeon Helgers, Beate Veith, Alexandra Klusowski, Brenda Ross for
782 technical assistance and Rob Brownstone (UC London) for help setting up P20-22 motor neuron
783 recordings, Warren G. Tourtellotte (Northwestern University, currently Cedars Sinai Medical
784 Center) for the *Egr3^{ko}* mice and Stephan Kröger (LMU Munich) for help setting up muscle spindle
785 recordings. This research received funding from the European Research Council under the European
786 Union's Seventh Framework Programme (FP/2007-2013)/ERC Grant Agreement 311710-MU
787 TUNING, the Göttingen Excellence Cluster for Nanoscale Microscopy and Molecular Physiology
788 of the Brain, Deutsche Forschungsgemeinschaft (DFG) Research Center 103, Section B1 and the
789 BMBF. Ashish Rajput, Vikas Bansal, and Stefan Bonn were supported by BMBF IDSN, ERA-Net
790 E-Rare MAXOMOD, and CRC 1286/Z2. Dario Farina received funding from the European
791 Research Council through the Synergy Grant NaturalBionicS (contract #810346). Francesco Negro
792 has received funding from the European Union's Horizon 2020 research and innovation programme
793 under the Marie Skłodowska-Curie grant agreement No 702491 (NeuralCon).

794

795 **Author Contributions**

796 M.N.K., P.C., F.N., A.R., P.F., T-I.L. Y.B. and W.P.M. conducted the experiments, M.N.K., P.C.,
797 F.N., V.B., C.L., D.M., T.A., S.B. and D.F. analyzed the data, M.N.K. and T.M. designed the
798 experiments and wrote the paper.

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800 **Declaration of Interests**

801 The authors declare no competing interests.

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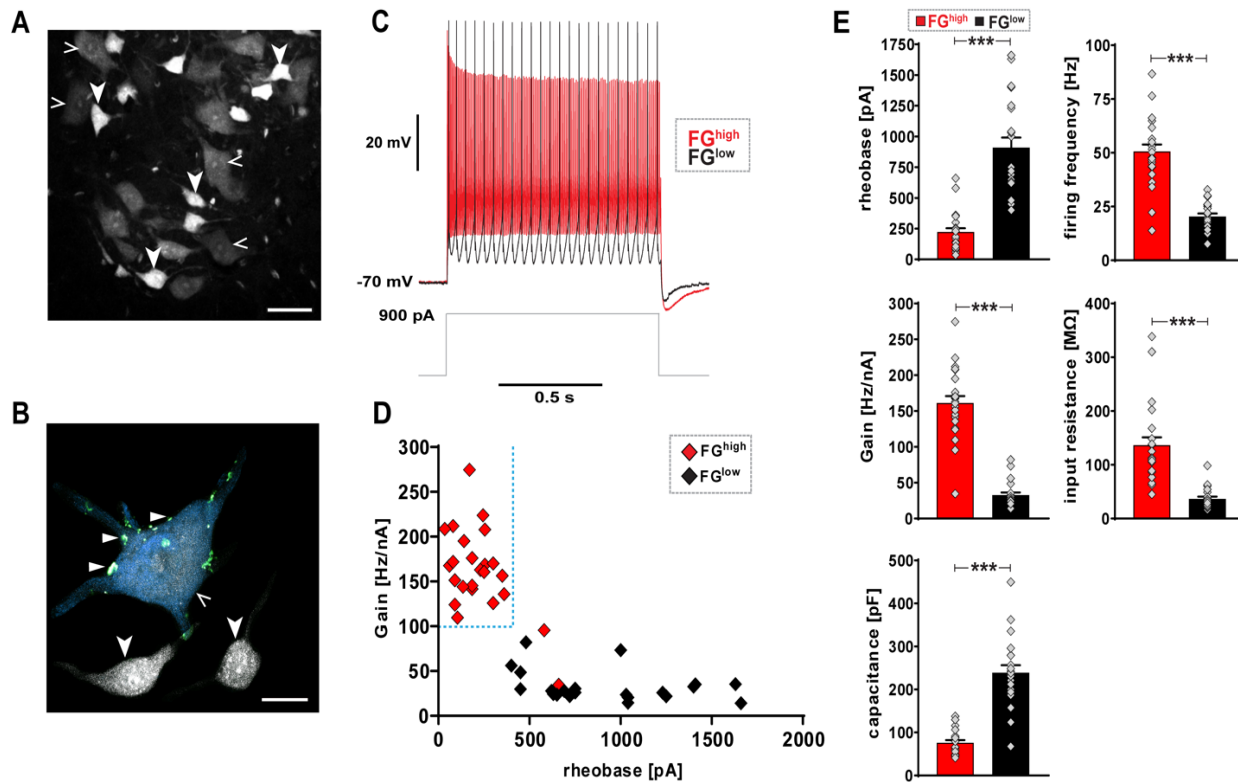
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811 Main Figures

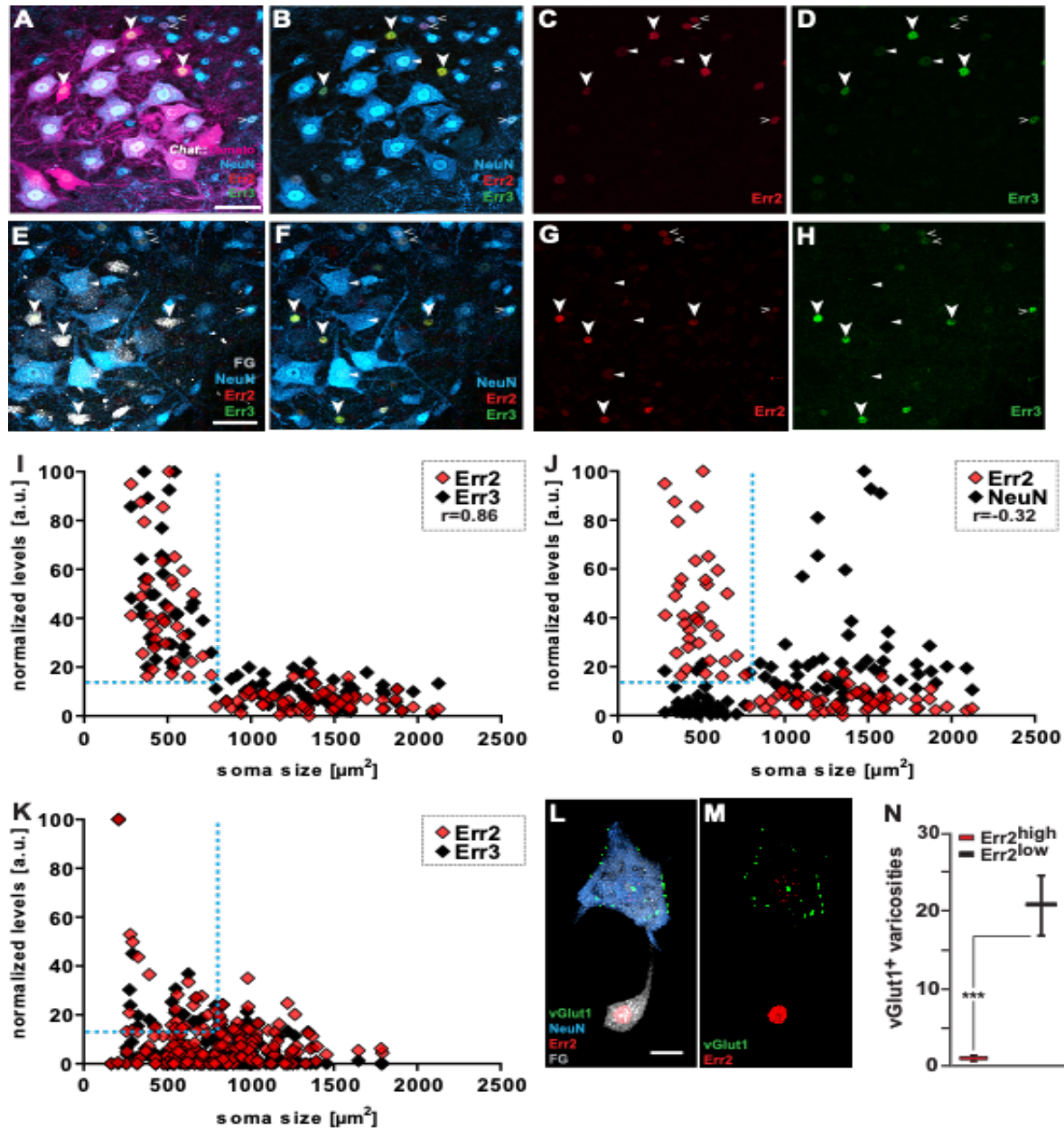


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813 **Figure 1.** Direct electrophysiological interrogation of alpha and gamma motor neurons shows
814 unique electrophysiological properties (A-D).

815 (A) Transversal section of P21 (wild-type) mouse lumbar spinal cord ventral horn: motor neurons
816 labeled by retrograde tracer FluoroGold (FG). Putative alpha motor neurons retain low-levels of FG
817 in soma (FG^{low}) (open arrowheads), while putative gamma motor neurons retain high-levels of FG in
818 soma (FG^{high}) (arrowheads) (scale bar: 50 μm). (B) Imaris 3D reconstruction: vGlut1⁺ synaptic
819 varicosities (triangles) associated with FG^{low} and NeuN^{high} alpha motor neuron (open arrowheads),
820 but not with adjacent FG^{high} and NeuN^{low or negligible} gamma motor neurons (arrowheads) (scale bar: 20
821 μm). (C) Whole cell patch-clamp recordings: example traces of FG^{high} (black) and FG^{low} (gray)
822 motor neurons upon 900 pA, 1 s square current pulse. (D) Scatter plot: FG^{high} (n=24, N=16) and
823 FG^{low} (n=22, N=12) motor neurons exhibit divergent gamma and alpha subtype-defining
824 electrophysiological signatures, respectively, including gamma subtype-specific combination with
825 low rheobase and high gain by FG^{high} motor neurons (see Supplementary Table S1 for details). (E)
826 FG^{high} motor neurons have significantly lower rheobase (pA) (221.87 ± 31.34), higher firing
827 frequency (Hz) (50.65 ± 3.23), higher gain (Hz/nA) (161.01 ± 9.77), higher input resistance (136.62
828 ± 14.63) and lower capacitance (76.07 ± 6.01) when compared to FG^{low} motor neuron rheobase
829 (909.09 ± 82.11), firing frequency (20.41 ± 1.70), gain (32.64 ± 4.02), input resistance ($36.55 \pm$
830 4.16) and capacitance (239.1 ± 17.17), respectively (see Supplementary Table S1 for details). Data

831 is presented as mean \pm SEM. n= # of neurons, N= # of mice. Statistically significant differences
832 between FG^{high} and FG^{low} neurons are indicated as: ***p<0.001, Student's t-test).



833

834 **Figure 2.** High levels of correlated Err2/3 expression by gamma motor neurons.

835 (A-D). Transversal section of P21 *Chat^{Cre}; Rosa26^{floxedTomato}* mouse lumbar spinal cord ventral horn:
 836 motor neurons genetically labeled by tdTomato (scale bar: 50 μm). Arrowheads: high Err2 and Err3
 837 levels in small NeuN^{low} or negligible, tdTomato⁺ motor neuron nuclei. Open arrowheads: relatively
 838 moderate-to-high-levels Err2/3 levels in NeuN^{high} tdTomato⁻ interneurons. Triangles: consistently
 839 lower but detectable levels in some large motor neurons with moderate-to-high NeuN levels. (E-H).
 840 Transversal section of P21 (wild-type) mouse lumbar spinal cord ventral horn: motor neurons
 841 labeled by retrograde tracer FluoroGold (FG) (scale bar: 50 μm). Arrowheads: high Err2 and Err3
 842 levels in small NeuN^{low} or negligible that retain high levels of FG^{high}. Open arrowheads: relatively
 843 moderate-to-high levels Err2/3 levels in NeuN^{high} FG⁻ interneurons. Triangles: consistently lower
 844 but detectable levels in some large motor neurons with moderate-to-high NeuN levels. (I)

845 Quantitative analysis: high Err2 (red) and Err3 (green) levels in motor neurons with small somas
846 (n=93, N=3, Pearson's correlation ($r=0.86$)). **(J)** High Err2 (red) and low NeuN (blue) levels in
847 motor neurons with small somas (n=93, N=3, Pearson's correlation ($r=-0.32$)). **(K)** Loss of high
848 Err2 (red) and Err3 (green) levels by Egr3-deficient small motor neurons (n=184, N=3). **L-N)** Imaris
849 3D reconstruction: vGlut1⁺ synaptic varicosities associated with Err2^{low or negligible} NeuN^{high} motor
850 neuron, but not with adjacent Err2^{high} NeuN^{low or negligible} motor neuron Err2^{low} motor neuron (motor
851 neurons retrogradely labeled by FluoroGold, FG) (scale bar: 20 μ m). **N)** Quantification of vGlut1⁺
852 synaptic varicosities associated with Err2^{high} or Err2^{low} motor neurons. N= # of mice and n= # of
853 neurons. Statistically significant differences between motor neurons are indicated as: * $p<0.05$,
854 ** $p<0.01$, *** $p<0.001$, n.s.= not significant, Student's t-test).

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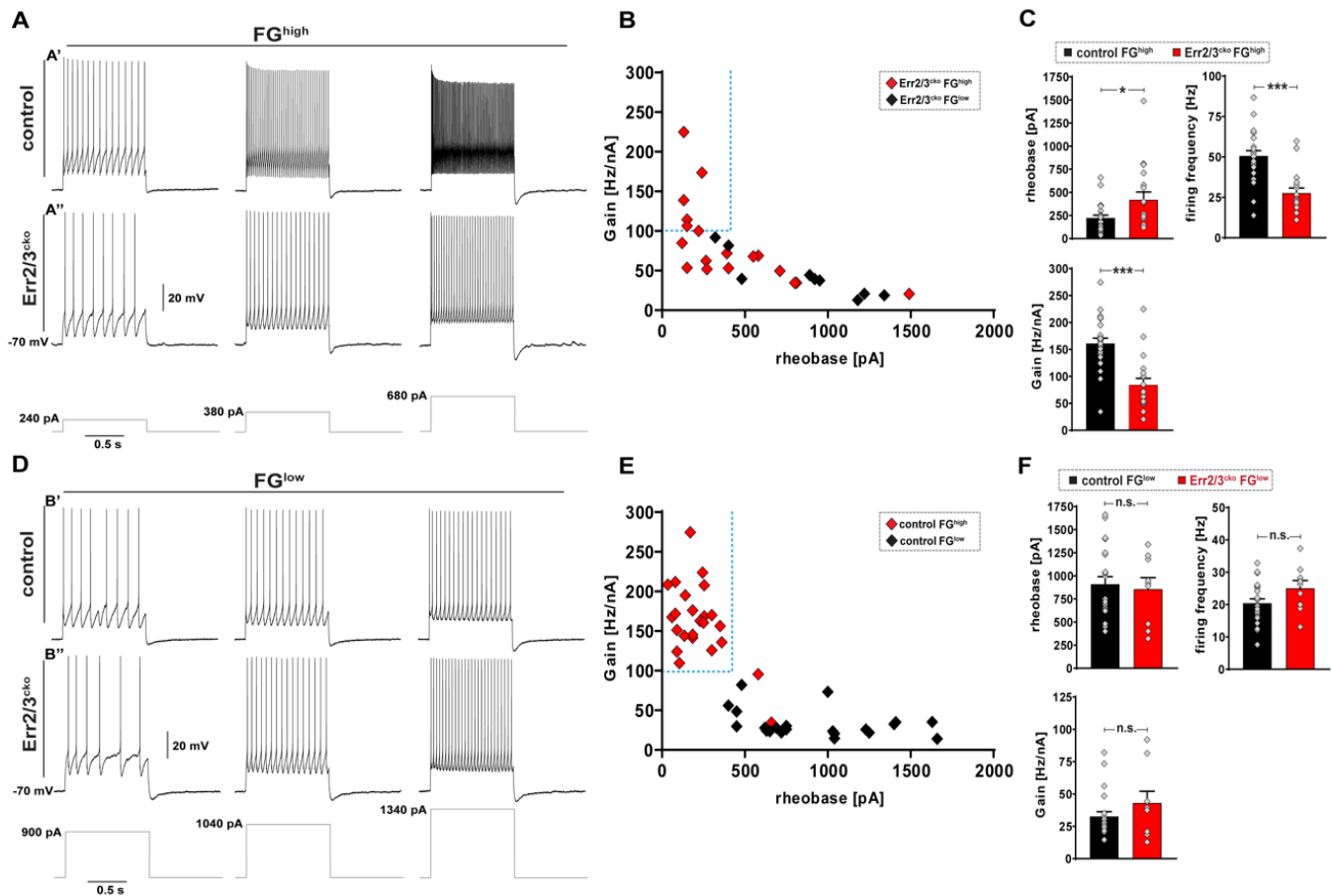
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863 **Figure 3.** Err2/3 are required for the acquisition of a gamma motor neuron functional properties.

864 **(A)** Example traces of whole cell patch-clamp recordings upon 240, 380 or 680 pA, 1 s square
 865 current pulses from small soma motor neurons exhibiting high-levels of FG incorporation (FG^{high})
 866 from control mice or Err2/3^{cko} mice. Control FG^{high} motor neurons (**A'**) exhibit higher firing rates
 867 compared to FG^{high} Err2/3^{cko} motor neurons (**A''**) and gear up their firing rates more rapidly in
 868 response to current pulses. **(B)** Scatter plot: lack of segregation of Err2/3^{cko} FG^{high} gamma motor
 869 neuron and Err2/3^{cko} FG^{low} alpha motor neuron electrophysiological signatures. **(C)** Err2/3^{cko} FG^{high}
 870 motor neurons show higher rheobase (419.72 ± 84.11), lower firing frequency (27.61 ± 3.15) and
 871 lower gain (84.01 ± 12.34) compared control FG^{high} motor neuron rheobase (221.87 ± 31.34), firing
 872 frequency (50.65 ± 3.23), gain (161.01 ± 9.77), respectively. **(D)** Example traces of whole cell
 873 patch-clamp recordings upon 900, 1040 or 1340 pA, 1 s square current pulses from large soma
 874 motor neurons with lower levels of FG incorporation (FG^{low}) from control mice or Err2/3^{cko} mice.
 875 Err2/3^{cko} FG^{high} motor neurons exhibit comparable firing rates and properties to control FG^{low} motor
 876 neurons. **(E)** Scatter plot: segregation of electrophysiological signatures between control FG^{high}
 877 gamma motor neurons versus control FG^{low} alpha motor neurons. (Note: Data from is from Fig. 1D).
 878 **(F)** No significant differences seen in Err2/3^{cko} FG^{low} alpha motor neuron subtype rheobase (855.55
 879 ± 124.85), firing frequency (25.03 ± 3.76) and gain (43.04 ± 9.81) when compared to control FG^{low}
 880 alpha motor neuron subtype rheobase (909.09 ± 82.11), firing frequency (20.41 ± 1.70), gain (32.64

881 ± 4.02), respectively (see Supplementary Table S1 for details). Data is presented as mean \pm SEM.
882 n= # of neurons and N= # of mice. Statistically significant differences between FG^{high} and FG^{low}
883 neurons are indicated as: *p<0.05, **p<0.01, ***p<0.001, n.s.= not significant, Student's t-test).

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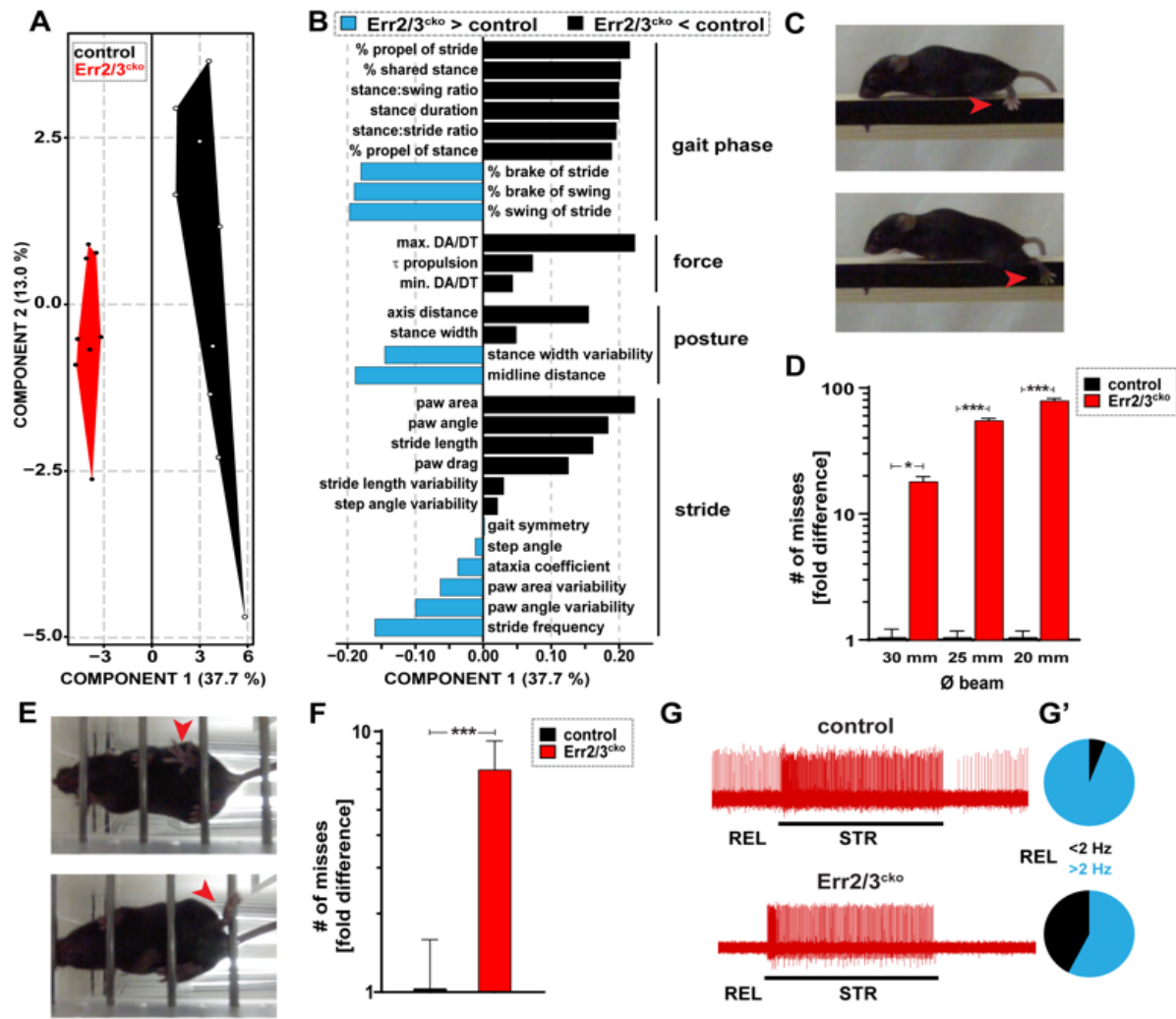
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911 **Figure 4.** Err2/3 is required for the acquisition of gamma motor neuron-specific functional
 912 properties and the execution of precision movements.

913 **(A, B)** **(A)** Polygon graphs based on partial least squares (PLS) analysis of 58 gait variables
 914 measured during treadmill locomotion at 25 m·s⁻¹ reveals significant gait alterations in Err2/3^{cko}
 915 mice (n=8, 3 trials/animal) when compared to control mice (n=9, 3 trials/animal). Each dot
 916 represents a single animal, polygons group animals of the same genotype, the segregation of which
 917 along the x-axis indicate that Err2/3^{cko} mice exhibit significant gait alterations at all speeds tested
 918 (Err2/3^{cko} mice, 3 trials each). **(B)** Negative (black) or positive (light blue) changes (arbitrary units)
 919 in gait variables in Err2/3^{cko} compared to control mice ranked by predictive value independent of
 920 sign, including reduced stance-swing phase ratio (gait phase related), reduced propulsion velocities
 921 (force related), decreased stance width (posture related), increased paw angle variability (stride
 922 related). **(C)** Still images of Err2/3^{cko} mouse navigating a horizontal beam. Red arrow: (example of a
 923 “miss”): foot missing the beam during swing-stance transition, causing the hindlimb and animal to
 924 slip during swing phase (red arrow in C). **(D)** Err2/3^{cko} (18.0 ± 1.75, 55.0 ± 2.20, 79.0 ± 3.49) but
 925 not control (1.0 ± 0.175, 1.0 ± 0.125, 1.0 ± 0.125) mice exhibit dramatically increasing erratic

926 locomotion (18, 55 and 79-fold increase in the # of misses) upon navigating horizontal beams with
927 decreasing width 30 mm, 25 mm and 20 mm, respectively (control N=4, Err2/3^{cko} N=4, 4-5
928 trials/animal). **(E)** Still images of Err2/3^{cko} mouse navigating a horizontal ladder. Examples of a
929 “miss” (red arrow in upper panel): foot missing a rung during swing-stance transition, causing the
930 hindlimb to slip during swing phase. Note: animals frequently attempted to compensate such misses
931 by using the “slipped” hindlimb to push against the rung and propel itself forward (red arrow in
932 lower panel). **(F)** Err2/3^{cko} (7.12 ± 2.06) exhibit significantly more erratic locomotion (7-fold
933 increase in the # of misses) when compared to control (1.0 ± 0.55) upon navigating horizontal
934 ladder (control N=5, Err2/3^{cko} N=5, 4-5 trials/animal). **(G)** Example traces showing Ia spindle
935 afferent responses during resting length (REL) or stretch (STR) applied by force transducer: normal
936 STR responses, but reduced REL firing of Ia afferents in Err2/3^{cko} mice (N=10) when compared to
937 control mice (N=10). **(G')** Pie charts: ratio of Ia afferents firing above or below 2 Hz at REL (total
938 trials: control n=84, Err2/3^{cko} n=118; control >2 Hz n=79, Err2/3^{cko} >2 Hz n=68). Data is presented
939 as mean \pm SEM. N= # of mice, n= # of Ia afferents. Statistically significant differences between
940 control and Err2/3^{cko} mice are indicated as: *p<0.05, **p<0.01, ***p<0.001, n.s.= not significant,
941 Student’s t-test).

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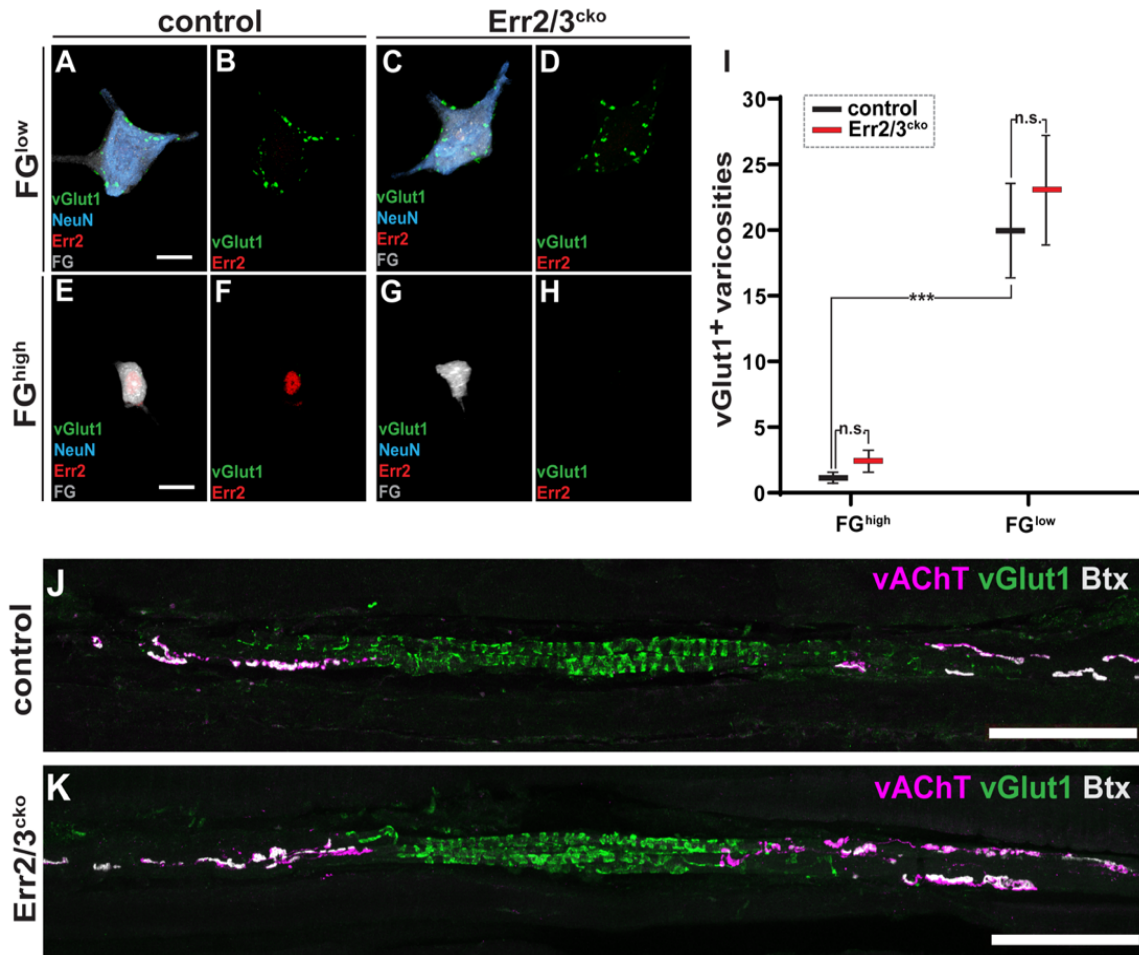
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960 **Figure 5.** Acquisition of gamma motor neuron-specific morphology and synaptic connectivity
 961 without Err2/3.

962 (A-I) Imaris 3D reconstruction: vGlut1⁺ synaptic varicosities (green) associated with large FG^{low},
 963 NeuN^{high} motor neurons in control (A, B) and Err2/3^{cko} mice (C, D) (scale bar: 20 μm). (E-H)
 964 Absence of vGlut1⁺ puncta on FG^{high}, NeuN^{low or negligible} motor neurons in control (E, F) and Err2/3^{cko}
 965 mice (G, H) (scale bar: 20 μm). (I) Significant difference in vGlut1⁺ varicosities associated with
 966 FG^{high}, NeuN^{low or negligible} and FG^{low}, NeuN^{high} motor neurons from control mice. Lack of significant
 967 differences in vGlut1⁺ synaptic varicosities associated with FG^{low}, NeuN^{high} motor neurons in control (20
 968 ± 3.48, n=9) versus Err2/3^{cko} mice (23.1 ± 4.06, n=10). Lack of significant differences in vGlut1⁺
 969 varicosities between control FG^{high}, NeuN^{low or negligible} (1.1 ± 0.36, n=10) and Err2/3^{cko} FG^{high},
 970 NeuN^{low or negligible} motor neurons (2.4 ± 0.78, n=10). (J, K) P70 mouse extensor digitorum longus
 971 (EDL) muscle spindles of control (J) and Err2/3^{cko} (K) mice. (J) Distribution of Ia sensory
 972 annulospiral endings in the central spindle segment (visualized by vGlut1, green), motor innervation
 973 (vAChT, magenta) and their postsynaptic sites (Btx, alpha bungarotoxin, grey) (scale bar: 100 μm).
 974 (K) Normal appearance of Err2/3^{cko} muscle spindle based on the distribution of sensory and motor
 975 innervation (scale bar: 100 μm). Data is presented as mean ± SEM. n= # of neurons. Statistically

976 significant differences control and Err2/3^{cko} motor neurons are indicated as: *p<0.05, **p<0.01,
977 ***p<0.001, n.s.= not significant, Student's t-test).

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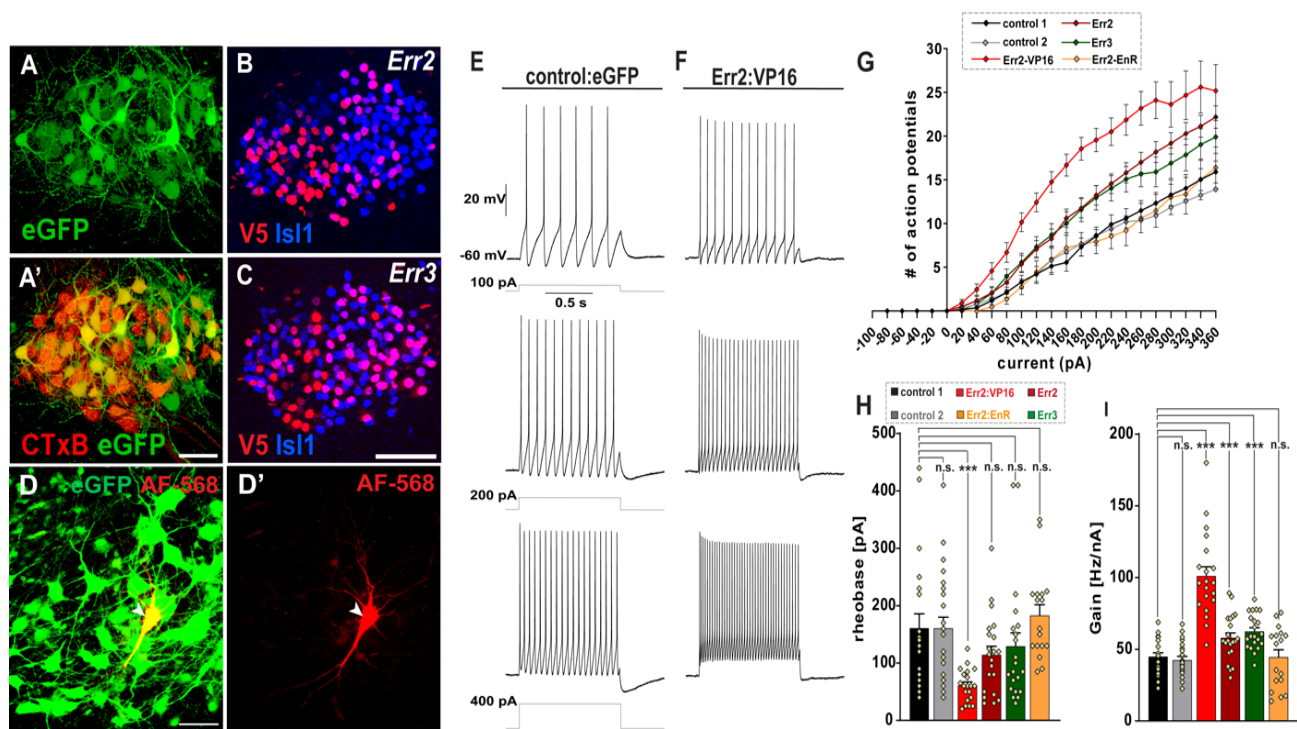
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995 **Figure 6.** Err2/3 promote gamma motor neuron-specific functional properties through
 996 transcriptional activation.

997 (A-D') Overview of E12 chick spinal cord: stable transfection with expression vector driving eGFP
 998 expression (A) in ventral horn motor neurons. Higher magnification of eGFP expressing neurons
 999 further identified as motor neurons by retrograde cholera toxin B (CTxB) tracing upon *in ovo*
 1000 injection into the hindlimb (A') (scale bar: 100 μ m). Examples of expression and nuclear
 1001 localization of mouse Err2 (B) and Err3 (C) in chick motor neurons (detected via N-terminal V5
 1002 epitope tags) (scale bar: 30 μ m). eGFP⁺ motor neuron recorded with patch pipette containing Alexa
 1003 fluor-568 dye (red) (D and D') (scale bar: 50 μ m). (E, F) Example traces of current clamp
 1004 recordings of chick motor neurons (in acute spinal cord slice preparations) forcedly expressing
 1005 eGFP only (control) (E) or Err2:VP16 and eGFP (F), upon 100, 200 and 400 pA, 1s square current
 1006 pulses: motor neurons expressing elevated Err2 levels exhibit higher firing rates and gear up their
 1007 firing rates more rapidly in response to current pulses (F). (G) Current-action potential response
 1008 curves for control transfected motor neurons (black, n=21). Similar leftward shifts in the slope of the
 1009 response curve upon expressing Err2 (dark red, n=21) or Err3 (green, n=21). Exaggerated leftward
 1010 shift in the slope of the response curve upon expressing Err2 fused to a transcriptional activation
 1011 domain (Err2:VP16) (red, n=20), while lack of shift when expressing Err2 fused to a transcriptional
 1012 repression domain (Err2:EnR) (orange, n=17). (H) Compared to control 1 (160.95 ± 25.85),
 1013 significant decrease in rheobase was observed upon over-expression of Err2:VP16 (61 ± 6.36),
 1014 while no significant change in rheobase was observed upon over-expression of Err2 ($114.28 \pm$
 1015 15.10), Err3 (129.28 ± 23.41) or Err2:EnR (182.94 ± 18.81). (J) Compared to control 1 ($44.84 \pm$

1016 2.75), significant increase in gain was observed upon over-expression of Err2:VP16 (101.01 ± 6.58),
1017 Err2 (58.00 ± 3.49), Err3 (62.47 ± 2.66) but not upon the forced expression of Err2:EnR ($44.8 \pm$
1018 5.14). No differences detected between control 1 and control 2 (n=with parameters recorded during
1019 two different experiments at different time points upon expressing “eGFP only” control vectors,
1020 thus demonstrating robustness of the assay. Data is presented as mean \pm SEM. n= # of neurons.
1021 Statistically significant differences are indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.= not
1022 significant, Student’s t-test).

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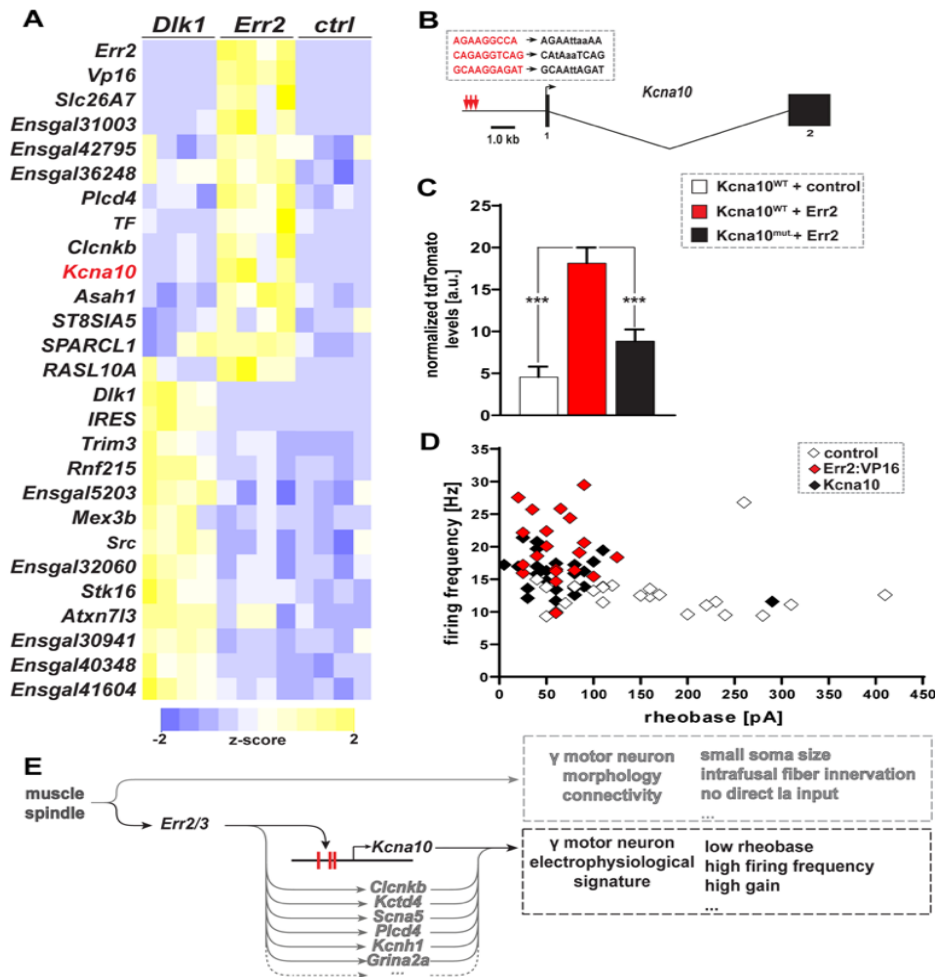
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1034 **Figure 7.** Err2 operates through *Kcna10* to promote a gamma motor neuron electrophysiological
 1035 signature.

1036 (A) Heatmap based on transcript reads (transcript per million (TPM)) detected by RNA sequencing:
 1037 Err2 (N=4) and Dlk1 (N=4) promote different gene expression signatures in chick motor neurons
 1038 when compared to control (N=4), including *Kcna10* (red) by Err2 (only upregulated genes are
 1039 shown). (B) *Kcna10* genomic locus: 3 clustered Err2/3 binding sites within promoter region. (C)
 1040 Reporter tdTomato fluorescence driven by wild-type (WT) *Kcna10* promoter is boosted by Err2 co-
 1041 transfected (18.14 ± 1.85) (red, n=102) in chick motor neurons when compared to control (4.57 ±
 1042 1.23) (gray, n=100) and decrease in tdTomato reporter fluorescence upon mutating the Err2/3
 1043 binding sites (8.85 ± 1.38) (black, n=101). (D) Whole cell patch-clamp recordings: forced
 1044 Err2:VP16 expression (red, n=20) shifts chick motor neuron properties towards a gamma motor
 1045 neuron-like electrophysiological signature (high firing rates, low rheobases) compared to control
 1046 (white, n=23). Forced *Kcna10* (black, n=24) expression recapitulates the promotion of a gamma
 1047 motor neuron electrophysiological signature by Err2 in chick motor neurons when compared to
 1048 control (white, n=23) motor neurons. (E) Summary: Err2/3 bind 3 clustered binding sites within the
 1049 *Kcna10* promoter region to drive *Kcna10* expression that promotes gamma motor neuron
 1050 electrophysiological signature of low rheobases and high firing rates while not affecting the

1051 morphology and connectivity identities of gamma motor neurons. Data is presented as mean \pm SEM.
1052 n= # of neurons, N= # of embryos. Statistically significant differences are indicated as: * $p < 0.05$,
1053 ** $p < 0.01$, *** $p < 0.001$, n.s.= not significant, Student's t-test).

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