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# Effect of size on expression of bistability in mouse spinal motoneurons

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## 22 **Abstract**

23

24 Bistability in spinal motoneurons supports tonic spike activity in the absence of  
25 excitatory drive. Earlier work in adult preparations suggested that smaller motoneurons  
26 innervating slow antigravity muscle fibers are more likely to generate bistability for postural  
27 maintenance. However, whether large motoneurons innervating fast-fatigable muscle fibers  
28 display bistability is still controversial. To address this, we examined the relationship between  
29 soma size and bistability in lumbar (L4-L5) ventrolateral  $\alpha$ -motoneurons of ChAT-GFP and Hb9-  
30 GFP mice during the first four weeks of life. We found that as neuron size increases, the  
31 prevalence of bistability rises. Smaller  $\alpha$ -motoneurons lack bistability, while larger fast  $\alpha$ -  
32 motoneurons (MMP-9<sup>+</sup>/Hb9<sup>+</sup>) with a soma area  $\geq 400\mu\text{m}^2$  exhibit significantly higher bistability.  
33 Ionic currents associated with bistability, including the persistent Nav1.6 current,  
34 thermosensitive Trpm5 Ca<sup>2+</sup>-activated Na<sup>+</sup> current and the slowly inactivating Kv1.2 current, also  
35 scale with cell size. Serotonin evokes full bistability in large motoneurons with partial bistable  
36 properties, but not in small motoneurons. Our study provides important insights into the neural  
37 mechanisms underlying bistability and how motoneuron size correlates with bistability in mice.

38

39 **Keywords:** Motoneuron, Spinal cord, Bistability, Current, Development, Posture, Serotonin,  
40 Neuromodulation

41

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43

44 **New and Noteworthy**

45 Bistability is not a common feature of all mouse spinal motoneurons. It is absent in small, slow  
46 motoneurons but present in most large, fast motoneurons. This difference results from  
47 differential expression of ionic currents that enable bistability, which are highly expressed in  
48 large motoneurons but small or absent in small motoneurons. These results support a possible  
49 role for fast motoneurons in maintenance of tonic posture in addition to their known roles in  
50 fast movements.

51

52

## 53 INTRODUCTION

54

55 Spinal motoneurons not only transmit central commands for movement to muscles but  
56 also shape motor output and muscle contraction through nonlinear firing properties (1). One  
57 such property is bistability, where the motoneuron can switch between stable silent and active  
58 states, depending on transient synaptic input or current injection. Originally detected as plateau  
59 potentials in invertebrate neurons (2-9), bistability was soon found in vertebrates (10),  
60 specifically in spinal motoneurons (11-16). While often induced by neuromodulators such as  
61 serotonin (13, 15-17), bistability can also arise when motoneurons are depolarized to near  
62 threshold and recorded under natural ionic and temperature conditions (18, 19).

63 The active state in bistable motoneurons is mainly mediated by slow ionic currents  
64 including persistent Cav1.3 (13-16) and Nav1.6 currents (20, 21), and the Trpm5-mediated Ca<sup>2+</sup>-  
65 activated Na<sup>+</sup> current (18, 19, 22). Drawing from a series of our previous investigations, we can  
66 summarize the process as follows: The initial depolarization, caused by the slow inactivation of  
67 Kv1.2 potassium channels (23), activates the persistent Nav1.6 current leading to spiking activity  
68 (24). This then prompts Ca<sup>2+</sup> entry through the recruitment of Cav1.3 channels, initiating a Ca<sup>2+</sup>-  
69 induced Ca<sup>2+</sup>-release process (18). This process ultimately activates thermosensitive Trpm5  
70 channels, which are the primary source of the plateau depolarization to sustain repetitive firing  
71 (19). Other currents such as the HCN-type hyperpolarization-activated inward current, I<sub>h</sub> (25,  
72 26) and reduction of calcium-activated outward currents (17, 25) are also involved in different  
73 neurons.

74  $\alpha$ -motoneurons, which drive extrafusal muscle contractions, can be subdivided  
75 functionally into three classes based on size and the muscle type they innervate: large fast-  
76 fatigable (FF), medium fast fatigue-resistant (FR), and small slow (S) motoneurons [reviewed by  
77 (27)]. In decerebrate cats, small motoneurons characterized by slow conduction velocities and  
78 low activation thresholds, appear to have more pronounced bistability than large ones (15, 16).  
79 This observation suggests that S or FR  $\alpha$ -motoneurons show higher bistability than those linked  
80 to FF muscle fibers. Notably, S motoneurons, primarily associated with postural adjustments  
81 and slow movements, have been hypothesized to use their bistability for efficient postural  
82 maintenance, ensuring minimal energy expenditure (15, 16, 28). In mice, the stationary posture  
83 also involves the activation of fast motor units (57). Interestingly, our recent findings indicate  
84 that large motoneurons exhibit bistable firing patterns, which are crucial for the control of  
85 hindlimb movement (19). From as early as the 2<sup>nd</sup> postnatal week, large motoneurons in mice  
86 present an electrophysiological profile that is distinct from that of smaller ones, marked by a  
87 lower input resistance, a higher rheobase, more depolarized spike thresholds, narrower action  
88 potentials, and shorter afterhyperpolarizations (27, 29, 30). They also exhibit delayed onset of  
89 firing and firing acceleration during a step depolarization, and receive a consistently higher level  
90 of recurrent excitation (23, 31-33). Despite this extensive characterization, the comparison of  
91 bistability between large and small motoneurons in mice remains unexplored. While  
92 comparisons with cat studies might lead us to hypothesize increased bistability in smaller  
93 mouse motoneurons, caution is warranted due to the distinct characteristics of mouse spinal  
94 motoneurons that are not seen in cats (34).

95 In the present study, we examined the bistability as a function of size in genetically  
96 labeled L4-L5  $\alpha$ -motoneurons in young mice (postnatal day (P) 1-25). Our data indicate that the  
97 largest  $\alpha$ -motoneurons showed stronger bistable firing properties, while the smallest  
98 motoneurons were rarely bistable. There was a strong correlation between bistability and the  
99 amplitudes of the ionic currents known to support it. Motoneurons of intermediate size often  
100 showed partial bistability, which could be converted to full bistability by serotonin. These  
101 unexpected results suggest new hypotheses regarding the contribution of larger motoneurons  
102 in bistability and postural control.

103

## 104 **MATERIALS AND METHODS**

105

### 106 Animals and ethical standards

107 Hb9-GFP mice were kindly provided by B. Pettmann and Jackson Laboratories (strain 005029).  
108 ChAT-Cre mice were obtained from Jackson Laboratories (strain 007902) and crossed to Rosa-  
109 26-floxed GFP mice. Mice of both sexes were used in all experiments. Animals were housed on  
110 a 12 hr day/night cycle with ad libitum access to water and food. The room temperature was  
111 maintained between 20 and 22°C.

112 Cornell: All animal protocols were approved by the Cornell Institutional Animal Use and Care  
113 Committee and were in accordance with NIH guidelines. Marseille: All animal care and use  
114 conformed to French regulations (Décret 2010-118) and were approved by the local ethics

115 committee (Comité d’Ethique en Neurosciences INT-Marseille, CE71 Nb A1301404,  
116 authorization Nb 2018110819197361).

117

### 118 Slice Preparation

119 For electrophysiological experiments, mice were cryoanaesthetized (P2-P7) or anaesthetized  
120 (P8-P25) with intraperitoneal injection of a mixture of ketamine/xylazine (100mg/kg and 10  
121 mg/kg, respectively). They were then decapitated, eviscerated and the spinal cord removed by  
122 laminectomy, and placed in a Sylgard-lined petri dish with ice cold (1-2°) aCSF containing (in  
123 mM): 252 sucrose, 3 KCl, 1.25 KH<sub>2</sub>PO<sub>4</sub>, 4 MgSO<sub>4</sub>, 0.2 CaCl<sub>2</sub>, 26 NaHCO<sub>3</sub>, 25 D-glucose, pH 7.4,  
124 bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The meninges were removed and the posterior cord (L3-S5)  
125 imbedded in agarose. The same solution was used for slicing. 325-350µm sections were  
126 prepared from the L4-L5 region and superfused at 3 ml/min with recording aCSF containing (in  
127 mM): 120 NaCl, 3 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 1.3 MgSO<sub>4</sub>, 1.2 CaCl<sub>2</sub>, 25 NaHCO<sub>3</sub>, 20 D-glucose, pH 7.4,  
128 32-34°C (19, 23). In most experiments (P1-P14), blockers of fast synaptic transmission (CNQX  
129 and D,L-AP5 or kynurenic acid, strychnine, and bicuculline) were added in the aCSF to minimize  
130 synaptic contributions to bistability. To isolate the persistent Na<sup>+</sup> currents (INaP) during  
131 voltage-clamp experiments we used a modified aCSF containing (in mM): 100 NaCl, 3 KCl, 1.25  
132 NaH<sub>2</sub>PO<sub>4</sub>, 1.3 MgSO<sub>4</sub>, 3.6 MgCl<sub>2</sub>, 1.2 CaCl<sub>2</sub>, 25 NaHCO<sub>3</sub>, 40 D-glucose, 10 TEA-Cl and 0.1 CdCl<sub>2</sub>  
133 (24).

134

135 Mature mice (P21-25) were treated as the younger mice, except that the cord was sliced in an  
136 ice cold slicing solution (1-2°) containing (in mM): 130 K-gluconate, 15 KCl, 0.05 EGTA, 20 HEPES,



137 25 D-glucose, 3 kynurenic acid, and pH 7.4 with NaOH (33). After a resting period of 30-60 min,  
138 slices were transferred to the recording chamber and superfused with recording aCSF at 32°C  
139 (35) without addition of fast synaptic transmission blockers.

140

#### 141 Electrophysiological recordings

142

143 Hb9-GFP and ChAT-GFP positive neurons were visualized in the ventrolateral region of lamina IX  
144 in L4-L5 horizontal slices using an Olympus BX51 microscope with appropriate filters. Whole-cell  
145 patch-clamp recordings from visualized fluorescent GFP+ neurons were made with electrodes  
146 (2-6MΩ) pulled from borosilicate glass capillaries (1.5 mm OD, 1.12 mm ID; World Precision  
147 Instruments). They were filled with a pipette solution containing (in mM): 140 K<sup>+</sup>-gluconate, 5  
148 NaCl, 2 MgCl<sub>2</sub>, 10 HEPES, 0.5 EGTA, 2 ATP, 0.4 GTP, pH 7.3. Patch clamp recordings were made  
149 using a Multiclamp 700B amplifier driven by PClamp 10 software (Molecular Devices).  
150 Recordings were digitized on-line and filtered at 10 kHz (Digidata 1322A or 1440A, Molecular  
151 Devices). Pipette and neuronal capacitive currents were canceled, and, after breakthrough,  
152 series access resistance was compensated. The recording was allowed to stabilize for at least 2  
153 min after establishing whole-cell access before recording was started (19, 23). Unless otherwise  
154 indicated, all neurons were held at -70 mV to ensure reproducibility in expression of voltage-  
155 sensitive currents.

156

157 Drug list: All solutions were oxygenated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. All salt compounds, as well as  
158 tetraethylammonium chloride (TEA; #T2265), Triphenylphosphine oxide (TPPO; #T84603),

159 Serotonin creatinine sulfate monohydrate (5-HT; #H7752), 6-Cyano-7-nitroquinoxaline-2,3-  
160 dione (CNQX, #5.04914), D-(-)-2-Amino-5-phosphonopentanoic Acid (DL-AP5; #165304),  
161 strychnine (#S0532), bicuculline (#5.05875), Kynurenic Acid (#K3375) and Amphotericin B  
162 (#A4888) were obtained from Sigma-Aldrich. Tetrodotoxin (TTX; #1078) was obtained from  
163 Tocris Bioscience.

164

### 165 Immunohistochemistry

166 To identify neurons expressing specific proteins, spinal cords from 10-12-day-old Hb9-GFP mice  
167 were removed in ice cold (1-2°) aCSF containing (in mM): 252 sucrose, 3 KCl, 1.25 KH<sub>2</sub>PO<sub>4</sub>, 4  
168 MgSO<sub>4</sub>, 0.2 CaCl<sub>2</sub>, 26 NaHCO<sub>3</sub>, 25 D-glucose, pH 7.4, bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The  
169 spinal cords were fixed for 5-6 h in 4% paraformaldehyde (PFA) prepared in phosphate buffer  
170 saline (PBS), then rinsed in PBS and cryoprotected overnight in 20% sucrose-PBS at 4°C. Spinal  
171 cords were frozen in OCT medium (Tissue Tek), and 30 µm cryosections were collected from the  
172 L4-L5 segments (23). After washing in PBS 3×5 min, the slides were incubated for 1 h in a  
173 blocking solution (BSA 1%, Normal Donkey Serum 3% in PBS) with 0.2% Triton X-100 and for 12  
174 h at 4 °C in a humidified chamber with the knockout-validated primary antibody: mouse-anti-  
175 NeuN (Neuronal Nuclei, Sigma-Aldrich MAB377) (36) or goat-anti-MMP-9 (Matrix  
176 metalloproteinase 9, Sigma-Aldrich M9570)(30, 37). Both antibodies were diluted in the blocking  
177 solution with 0.2% Triton X-100 (1:1000 and 1:500 for anti-NeuN and anti-MMP-9, respectively).  
178 Slides were washed 3×5 min in PBS and incubated for 2 h with an Alexa Fluor® Plus 555-  
179 conjugated secondary antibody (Invitrogen A32816) diluted in the blocking solution. After 3  
180 washes of 5 min in PBS, they were mounted with a gelatinous aqueous medium.

181

182 Data analysis

183 Electrophysiological recording:

184 Electrophysiological data were analyzed with Clampfit 10 software (Molecular Devices). Several  
185 basic criteria were set to ensure optimum quality of intracellular recordings. Only cells with a  
186 stable membrane potential below -60 mV, stable access resistance (no more than 20%  
187 variation), and action potential amplitude larger than 60 mV were analyzed. Reported  
188 membrane potentials were not corrected for liquid junction potentials.

189 Confocal imaging:

190 Immunofluorescent staining was quantified from confocal images acquired with the 40X  
191 objective (LSM700 microscope, Zeiss, Germany) on at least two slices per mouse and within the  
192 ventro-lateral area (300 X 300  $\mu\text{m}^2$ ) from each slice. The cell body cross-sectional area was  
193 manually segmented and quantified from stacked confocal images (5 steps ; Z-step, 3  $\mu\text{m}$ ) with  
194 Zen software (Zeiss). An intensity threshold in each slice was applied to determine the presence  
195 of NeuN or MMP9 staining from each segmented GFP+ motoneuron.

196

197

198 Statistics

199 No statistical method was used to predetermine sample size. Statistical analysis was carried out  
200 using GraphPad Prism and Matlab (MathWorks) software. When two groups were compared we  
201 used, the Mann-Whitney test. Fisher's exact test was used for comparing cell proportions  
202 between two groups. In some cases, we also compared the slope of the simple linear  
203 regressions between two groups. The level of significance was set at  $p < 0.05$ . Each statistical test

204 is indicated in the figure legends and provided in more detail in the Source Data File. In the  
205 figures and in the main text, data are presented as mean  $\pm$  SD for the histograms. Median and  
206 quartiles are represented in each violin plot.

207 Source Data File:

208 <https://github.com/remibos/Source-Data-File-HW.git>

209

210

## 211 RESULTS

212

### 213 Immunohistochemical identification of fast $\alpha$ -motoneurons

214

215 Motoneurons in the ventrolateral spinal cord were initially identified in unfixed L4-L5  
216 horizontal slices by their GFP fluorescence in choline acetyltransferase (ChAT)-tagged ChAT-GFP  
217 mice. We found a number of labeled neurons of many sizes (from  $<200 \mu\text{m}^2$  to  $>800 \mu\text{m}^2$ ).  
218 However, these include both  $\alpha$ -motoneurons driving extrafusal muscle fibers and smaller  $\gamma$ -  
219 motoneurons innervating intrafusal muscles that regulate muscle spindle responsiveness to  
220 stretch. These motoneuron subtypes can be distinguished by differential expression of a  
221 number of proteins. While the transcription factor Hb9 is a marker for both  $\alpha$ - and  $\gamma$ -  
222 motoneuron in Hb9-nls-LacZ animals (38), only  $\alpha$ -motoneurons express GFP labeling in the Hb9-  
223 GFP mice (38). We thus used Hb9-GFP mice to identify  $\alpha$ -motoneurons in the L4-L5  
224 ventrolateral spinal cord, preferentially linked to hindlimb muscles (39, 40), from postnatal day 1  
225 (P1) to P25.

226 The transcription factor NeuN is commonly used to distinguish neurons from glial cells.  
227 In the spinal cord,  $\alpha$ -motoneurons express NeuN, whereas 2/3 of  $\gamma$ -motoneurons do not (41).  
228 We examined co-expression of Hb9-GFP and NeuN immunolabeling in fixed tissues and,  
229 regardless of age, we found that virtually all ventrolateral Hb9-GFP-labeled neurons exhibited  
230 NeuN labeling ( $98 \pm 2.4\%$ ,  $n = 144$  cells), indicating they are likely  $\alpha$ -motoneurons (Fig. 1A, B1).  
231 We observed a preponderance of small labeled  $\alpha$ -motoneurons during P4-6 ( $n = 571$  neurons;  
232 Fig. 1B2) and P8-10 ( $n = 425$  neurons; Fig. 1B2), with 80.5% and 79.6% respectively having a

233 maximal cross sectional area of  $400 \mu\text{m}^2$  or less. The percentage of large  $\alpha$ -motoneurons  
234 increased with age (38, 41), and by P12, only 50.4% of them remained under  $400\mu\text{m}^2$  ( $n = 288$   
235 neurons; Fig. 1B2).

236 Only fast motoneurons express the marker matrix metalloproteinase-9 (MMP-9) (30,  
237 37). Our observations confirmed that larger motoneurons co-expressed Hb9-GFP and MMP-9,  
238 while the smaller ones did not (Fig. 1 C1-C4). The mean cross-sectional area of MMP-9-negative  
239 neurons was  $280 \pm 12 \mu\text{m}^2$ , while that of MMP-9-positive neurons was  $907 \pm 26 \mu\text{m}^2$  ( $P < 0.001$ ,  $n$   
240  $= 288$ ; Fig. 1C2). Consistent with previous observations (38), we noted a fairly clear demarcation  
241 in motoneuron characteristics around the  $400 \mu\text{m}^2$  cross-sectional area. Specifically, most of the  
242 motoneurons smaller than  $400\mu\text{m}^2$  were  $\text{HB-9}^+/\text{MMP-9}^-$  (86.75%,  $n=144/166$ ), which can be  
243 presumed to be slow motoneurons. Conversely, those with a cross-sectional area exceeding  
244  $400\mu\text{m}^2$  were virtually all  $\text{HB-9}^+/\text{MMP-9}^+$  (99.2%,  $n=121/122$ ; Fig. 1C3, C4), suggesting their  
245 classification as putative fast motoneurons. This pattern led us to adopt the  $400 \mu\text{m}^2$  threshold  
246 as a practical and empirically supported cut-off, allowing us to grossly distinguish between fast  
247 and slow motoneurons in our study.

248

## 249 **Features of bistable motoneurons**

250 We previously showed that under experimental conditions mimicking natural *in vivo*  
251 conditions, most large motoneurons exhibit bistability in the absence of added  
252 neuromodulators. This was achieved by maintaining *in vivo* calcium concentrations (1.2 mM)  
253 (42) and keeping the preparation temperature above  $30^\circ\text{C}$  (18, 19, 43, 44). Four distinct features  
254 characterized bistable motoneurons, which we have previously characterized (18, 23) (Fig. 2): 1)

255 self-sustained firing triggered by a brief (2 sec) excitation when the motoneuron was pre-  
256 depolarized near the spike threshold (Fig. 2A1); 2) a slow afterdepolarization (sADP) following  
257 the current step if the motoneuron was not pre-depolarized sufficiently to trigger the self-  
258 sustained firing (Fig. 2A1); 3) negative hysteresis during slow triangular current ramp injections,  
259 where spiking stopped at lower currents than where it began (Fig. 2A2); 4) a slowly depolarizing  
260 potential causing delayed spiking acceleration in response to a near-threshold depolarizing step  
261 (Fig. 2A3). We assigned each feature one point, with fully bistable motoneurons scoring 4  
262 points. To be considered bistable, the motoneuron scored at least 3 points, including the  
263 presence of a self-sustained firing and sADP, and either negative hysteresis or delayed firing.

264         Nearly a third of motoneurons (36%,  $n=71/199$ ) did not express any of the bistability  
265 criteria, scoring 0. They typically showed a decelerating firing rate during the 2 sec depolarizing  
266 pulse, leading to a post-step afterhyperpolarization (Fig. 2B1). Their activity during ramp current  
267 injections showed positive hysteresis, where spiking ceased at higher current values than where  
268 it started (Fig. 2B2). They also began spiking immediately during a suprathreshold long step,  
269 with continuous firing deceleration (Fig. 2B3). Some motoneurons, despite having one scoring  
270 feature, lacked self-sustained firing and were also considered as non-bistable. A significant  
271 number of motoneurons also displayed intermediate characteristics, scoring 2. The vast  
272 majority of them (90 %,  $n=22/25$ ) were not bistable, lacking self-sustained spiking, but meeting  
273 two other criteria. This shows that bistability is not an all-or none property, but can manifest  
274 with somewhat different properties.

275

## 276 **Effects of size and age on bistability**

277 As previously described, bistability in motoneurons can emerge early in development  
278 (18). Fig. 2C1 shows the average bistability scores of Hb9-GFP<sup>+</sup>  $\alpha$ -motoneurons during the initial  
279 postnatal month, comparing small neurons (<400 $\mu\text{m}^2$ ) to large neurons (>400 $\mu\text{m}^2$ ). Small  
280 motoneurons consistently showed minimal bistability scores across all developmental ages (Fig.  
281 2C1), rising up to a mean of  $1.14 \pm 0.31$  by P9-P14, then nearly vanishing by early adulthood  
282 (P21-25). 54 out of 81 small cells (<400  $\mu\text{m}^2$ ) displayed a bistability score of 0, and only 9 out of  
283 81 small cells showed bistability (scores 3-4) at any age, with 55% appearing after P9. In  
284 contrast, large motoneurons (>400  $\mu\text{m}^2$ ) exhibited increasing bistability, reaching near maximal  
285 levels around P9 with a mean score of  $3.24 \pm 0.28$ . Remarkably, 83% (n = 24) of these large  
286 motoneurons exhibited high bistability scores (3-4) at P9-P14, with all displaying bistability by  
287 P21-25 (n = 7). Note that the bistability scores of large motoneurons were significantly higher  
288 compared to small ones when we pooled all ages ( $P < 0.001$ , n=199).

289 These data demonstrate a correlation between motoneuron soma size and the  
290 emergence of bistability in young mice. Fig. 2C2 shows the bistability score distribution by size  
291 across all ages. As described above, the large majority of small neurons (<400  $\mu\text{m}^2$ ) were not  
292 bistable, averaging a score of  $0.67 \pm 1.21$  (n = 81); only 11 % achieved scores of 3-4. In contrast,  
293 larger neurons (>400  $\mu\text{m}^2$ ) consistently had high bistability scores (mean  $2.54 \pm 1.43$ , n = 118;  
294  $P < 0.001$ ); the largest (>800 $\mu\text{m}^2$ ) were the most bistable (mean  $3.07 \pm 1.11$ , n = 44) with 75%  
295 scoring 3-4. Intermediate size neurons (400-800  $\mu\text{m}^2$ ) showed largely a mixture of bistable and  
296 intermediate bistability scores (mean  $2.33 \pm 1.51$ ; n = 75). A clear gradient emerged: as neuron  
297 size increased, the proportion of bistable neurons rose, while the proportion of non-bistable  
298 cells decreased, especially above 400  $\mu\text{m}^2$ . Another way to see this correlation was to measure



299 bistability score as a function of input resistance, which was lower in large motoneurons and  
300 higher in small motoneurons. A strong negative correlation between input resistance and  
301 bistability score was seen (Fig. 2C3;  $R^2 = 0.99$ ). As a further control for the possible effects of  
302 cell dialysis during whole cell recordings, we used the amphotericin B perforated patch method  
303 (35) to record bistability from HB9-GFP neurons, whose contents were less disturbed. This  
304 method reaffirmed the trend of increasing bistability with  $\alpha$ -motoneuron size ( $n = 26$ ;  $p < 0.05$ ;  
305 Fig. 2C4).

306  
307 Similar results collected from ChAT-GFP mice reinforce the size-related nature of bistable  
308 properties (Fig 2C5). Before P7, small motoneurons ( $< 400 \mu\text{m}^2$ ) showed no bistability, with an  
309 average score of  $0.56 \pm 1.13$ ,  $n = 9$ . In contrast, larger neurons ( $> 400 \mu\text{m}^2$ ) showed higher  
310 bistability (score  $2.1 \pm 1.69$ ,  $n = 16$ ;  $p = 0.02$ ).

311

### 312 **Currents associated with bistability**

313 The fact that large mouse motoneurons showed greater levels of bistability than small  
314 motoneurons is unlikely to arise from the size difference alone, as larger neurons inherently  
315 show lower overall excitability owing to their lower input resistance (45). Instead, we proposed  
316 that large neurons selectively express higher levels of ionic currents that sustain the bistable  
317 state than small neurons. Several ionic currents have been demonstrated to support the active  
318 state during bistability. Among others, these include a persistent inward current (PIC) that may  
319 comprise both sodium and calcium components (16, 21, 46, 47), a thermosensitive Trpm5

320 calcium-activated inward current (19), and a slow inactivation of the Kv1.2 potassium current  
321 (23).

322 We measured the PIC by delivering a slow ramp depolarization in voltage clamp (Fig.  
323 3A). The PIC amplitude was measured at the inward peak from the extrapolated passive  
324 component at the same voltage (See Fig. 3A). The PIC amplitude was very small or absent in  
325 small Hb9-GFP<sup>+</sup> motoneurons (<400 $\mu\text{m}^2$ :  $33 \pm 58$  pA, n = 17; Fig. 3B), but much larger in large  
326 motoneurons ( $165 \pm 172$  pA, n = 24; p = 0.014), with its amplitude correlating positively with  
327 cell size without any change in the activation threshold (Fig. 3C). Moreover, the amplitude of the  
328 PIC was also found to be proportional to the bistability score (Fig. 3D). Specifically, non-bistable  
329 neurons (scores 0-1) had small to absent PICs (mean  $11.7 \pm 19.4$  pA; n = 15, 9 with no PIC),  
330 whereas bistable neurons (scores 3-4), especially those that are fully bistable (score 4),  
331 exhibited a dramatic rise in the PIC amplitude (mean  $198 \pm 160.6$  pA, n = 22, 0 with no PIC; p <  
332 0.001). . These findings highlighted the strong association between PICs and bistability in large  
333  $\alpha$ -motoneurons and the absence or low amplitude of PICs in smaller non-bistable neurons.

334 To further understand the contribution of the sodium component of PICs in bistability,  
335 we added 10 mM TEA-Cl and 100  $\mu\text{M}$  CdCl<sub>2</sub> to the recording ACSF (24). Large  $\alpha$ -motoneurons  
336 displayed a more pronounced persistent sodium current (INaP; Fig. 3E). Although the  
337 motoneuron size did not influence the INaP threshold activation (Fig. 3G), the peak amplitude of  
338 INaP was significantly greater in large  $\alpha$ -motoneurons (>400  $\mu\text{m}^2$ ,  $1126 \pm 530$  pA, n = 11)  
339 compared to small motoneurons (<400  $\mu\text{m}^2$ ,  $221 \pm 104$  pA, n = 10; p<0.001, Fig. 3F). This  
340 increase is not merely a reflection of the greater surface area of the bigger neurons that could  
341 express more channels, as the PIC density (corrected by the cellular capacitance) was also larger

342 in large than in small  $\alpha$ -motoneurons ( $4.84 \pm 1.51$  pA/pF,  $n = 11$  vs.  $2.38 \pm 1.09$  pA/pF,  $n =$   
343  $10$ ; Fig. 3H;  $P < 0.001$ ). These findings highlighted the significant contribution of INaP in  
344 bistability from large  $\alpha$ -motoneurons and its diminished presence in smaller, non-bistable  
345 neurons.

346 A calcium-activated inward current was shown to be pivotal in sustaining the plateau  
347 depolarization underlying the tonically firing active state in bistable crab (22) and turtle  
348 motoneurons (48); this current has recently been shown to be mediated by Trpm5 channels in  
349 mice (19). To measure the effect of this current, we induced a 2-sec depolarization in the  
350 presence of tetrodotoxin (TTX,  $1 \mu\text{M}$ ) and tetraethylammonium-chloride (TEA,  $10 \text{ mM}$ ) to  
351 minimize sodium and potassium currents. This depolarization was large enough to elicit a series  
352 of slow calcium-driven spikes to fully activate Trpm5. Subsequently, we measured the resulting  
353 afterdepolarization (sADP; Fig. 4A), which slowly declined as the Trpm5 current decayed (19).  
354 Notably, the sADP was partially blocked by Triphenylphosphine oxide (TPPO,  $50 \mu\text{M}$  Fig. 4A), a  
355 known Trpm5 channel blocker (19, 49). The residual TPPO-insensitive sADP appeared to  
356 predominantly arise from channels that are not yet identified (see discussion in (19)). Thus, to  
357 accurately estimate the Trpm5 component of the sADP, we subtracted the sADP measured in  
358 TPPO from the total sADP. The resulting Trpm5 sADP was only detectable in one of 15 smaller  
359 neurons ( $<400 \mu\text{m}^2$ :  $0.2 \pm 0.92$  mV,  $n = 15$ ), but was evident in most of the larger neurons ( $>400$   
360  $\mu\text{m}^2$ :  $4.9 \pm 5.47$  mV,  $n = 9$ ;  $p = 0.0017$ ; Fig. 4A-B). Neurons with lower bistability scores (scores 0-  
361 1) lacked Trpm5 sADP ( $n=10$ ), but this current was present in most bistable neurons (scores 3-4:  
362  $4.6 \pm 5.32$  mV,  $n = 10$ ;  $p = 0.0108$ ; Fig. 4C). We isolated the Trpm5 calcium-activated inward  
363 current in response to a 2 sec depolarizing voltage step in presence of TTX ( $1\mu\text{M}$ ) and TEA

364 (10mM) (Fig.4D). We confirmed that the Trpm5 inward current amplitude and density  
365 (corrected by cell capacitance) were significantly bigger in large  $\alpha$ -motoneurons ( $>400 \mu\text{m}^2$ ,  $324$   
366  $\pm 146.3 \text{ pA}$  &  $2.7 \pm 1.38 \text{ pA/pF}$ ,  $n = 8$ ) compared to the small ones ( $<400 \mu\text{m}^2$ ,  $26 \pm 7.51 \text{ pA}$  &  
367  $0.7 \pm 0.67 \text{ pA/pF}$ ,  $n = 3$ ) (Fig. 4E-F;  $P < 0.05$ ). Thus, the calcium-activated inward current mediated  
368 by Trpm5 channels (19) appeared associated with bistability in large  $\alpha$ -motoneurons, but was  
369 low or absent in smaller non-bistable neurons.

370 We further assessed the Kv1.2 potassium current, whose slow inactivation delays the  
371 initiation and acceleration of firing of bistable neurons during long current steps (23).  
372 Interestingly, in our earlier work, only bistable neurons showed the delayed initiation and  
373 acceleration of firing during long current steps, induced by closure of the Kv1.2 channels; non-  
374 bistable neurons showed immediate spike onset with spike deceleration (23). We tested  
375 whether the Kv1.2 inactivating current was larger in large, bistable neurons than small neurons.  
376 This was measured in voltage clamp as the amplitude of the inward current over a 7 sec step,  
377 measured after inactivation of the transient potassium current, in the presence of TTX ( $1\mu\text{M}$ )  
378 and TEA (10mM) (Fig. 4G). Again, the amplitude of the Kv1.2 current scaled with cell size.  
379 Smaller  $\alpha$ -motoneurons ( $<400 \mu\text{m}^2$ ) had a lower current amplitude ( $68 \pm 86.9 \text{ pA}$ ;  $n = 19$ ) than  
380 the larger neurons ( $159.2 \pm 155.5 \text{ pA}$ ,  $n = 26$ ;  $P < 0.02$ ; Fig. 4H). Moreover, the smaller  
381 motoneurons had a lower current density ( $2.19 \pm 0.33 \text{ pA/pF}$ ;  $n = 3$ ) compared to larger  
382 motoneurons ( $3.61 \pm 0.94 \text{ pA/pF}$ ,  $n = 7$ ;  $P < 0.05$ ; Fig. 4 I).

383 All together, our findings highlight that INaP, the Trpm5 mediated calcium-activated  
384 inward current, and the slow inactivating Kv1 current are pivotal indicators of bistability in large  
385  $\alpha$ -motoneurons.

386

### 387 **Serotonin effects on bistability in partially bistable neurons**

388 We found that bistability is not strictly binary, as 26% (52 out 199) of  $\alpha$ -motoneurons  
389 displayed some, but not all, of the defining features described above, especially the absence of  
390 persistent firing after the brief depolarization. It has been known for many years that  
391 neuromodulators such as serotonin (5-HT) can evoke bistability in  $\alpha$ -motoneurons which show  
392 only partial bistable properties (14, 17, 25, 50).

393 We analyzed the effects of 10  $\mu$ M 5-HT on  $\alpha$ -motoneurons ( $n = 45$ ) that had bistability scores  
394 below 4 and lacked the self-sustained spiking. Fig. 5A shows an example of an  $\alpha$ -motoneuron  
395 that, under control conditions (i.e. recording aCSF), did not show acceleration of spiking during  
396 a 2-sec depolarization, and only showed a modest sADP at the end of the depolarization. Seven  
397 minutes after application of 5-HT, this motoneuron demonstrated accelerating spiking during  
398 the current step, and self-sustained spiking activity after the end of the step. This activity was  
399 terminated only by a hyperpolarizing step, indicating that the neuron became fully bistable only  
400 in the presence of 5-HT. Fig. 5B shows the ability of 5-HT to evoke bistability in  $\alpha$ -motoneurons  
401 of different sizes. Notably, small  $\alpha$ -motoneurons ( $< 400\mu\text{m}^2$ ;  $n = 19$ ) remained non-bistable even  
402 with 5-HT. In contrast, larger neurons exhibited the potential for a transition to bistability upon  
403 5-HT exposure. Specifically, of non-bistable  $\alpha$ -motoneurons of larger size ( $>400\mu\text{m}^2$ ), 38.5%  
404 switched to full bistability with 5-HT ( $n = 26$ ;  $p < 0.001$ ). It is noteworthy that the majority of  
405 neurons transitioning to bistability with 5-HT already expressed an sADP before 5-HT addition,  
406 as illustrated in Fig. 5A. Thus, 5-HT was able to evoke full bistability in a subset of neurons

407 already leaning towards a bistable state, but could not evoke bistability in smaller or less  
408 bistable neurons.

409

## 410 **DISCUSSION**

411

412 Our study reveals a size-based gradient in the bistable ability of GFP+  $\alpha$ -motoneurons  
413 from Hb9-GFP and ChAT-GFP mice lines. Specifically, the likelihood of  $\alpha$ -motoneurons being  
414 bistable increases with the cell body cross-sectional area and over time. The currents, Trpm5  
415 and INaP, which contribute to the active state during bistability, and Kv1, whose inactivation  
416 helps to initiate sustained firing, are more pronounced in larger fast  $\alpha$ -motoneurons which can  
417 be reliably identified with the MMP-9 marker. Conversely, these currents are low or absent in  
418 smaller MMP-9<sup>-</sup>  $\alpha$ -motoneurons. We also showed that serotonin evokes full bistability only in  
419 large motoneurons. These findings highlight an important correlation between motoneuron size  
420 and bistability.

421 Research on bistability has primarily focused on larger  $\alpha$ -motoneurons, identified by  
422 their ventrolateral location or by retrograde stimulation from ventral nerves (18, 27). This might  
423 have led to a failure to record the properties of smaller  $\alpha$ -motoneurons. Neurons recorded in  
424 this study are motoneurons, as evidenced by their ventrolateral location and the expression of  
425 ChAT and Hb9 markers (Fig. 1A-B). Furthermore, they are  $\alpha$ -motoneurons since most  $\gamma$ -  
426 motoneurons do not express NeuN (41), and do not express GFP in Hb9-GFP mice (38, 41).  
427 Finally, virtually all of the neurons larger than  $400\mu\text{m}^2$  also expressed MMP-9, indicative of fast  
428  $\alpha$ -motoneurons (30, 37). While there are no markers available to differentiate between the fast

429 fatigue-resistant (FR) and fast fatigable (FF)  $\alpha$ -motoneurons, we conclude that the largest  
430 neurons are fast motoneurons. The smallest neurons are presumably slow (S)  $\alpha$ -motoneurons.  
431 However, size alone is not a reliable indicator to separate these classes (51-53).

432         Electrophysiologically, large fast  $\alpha$ -motoneurons differ from small slow  $\alpha$ -motoneurons  
433 by exhibiting a delayed and accelerated firing during prolonged stimulation (27, 29, 52). Our  
434 experiments confirm this observation, as near-threshold current steps resulted in a slow  
435 depolarization in larger  $\alpha$ -motoneurons leading to delayed firing acceleration (Fig. 2). In  
436 contrast, smaller  $\alpha$ -motoneurons fired immediately upon reaching spike threshold followed by a  
437 spike frequency deceleration (27, 30). The depolarization and spike delay in large fast  $\alpha$ -  
438 motoneurons were attributed to the slow inactivation of a Kv1 current (23, 30).

439         We now add bistability as a predominant property of large fast L4-L5  $\alpha$ -motoneurons in  
440 young mice. Over 75% of neurons over  $800\mu\text{m}^2$  showed bistability, while only  $\sim 10\%$  of neurons  
441 less than  $400\mu\text{m}^2$  were bistable. Consistent with this, a large majority of fast  $\alpha$ -motoneurons  
442 showed negative hysteresis during triangular ramp steps, where the derecruitment current was  
443 lower than the recruitment current, while far fewer of the slow  $\alpha$ -motoneurons displayed  
444 negative hysteresis (30) (Fig. 2). The frequency of bistability in large  $\alpha$ -motoneurons increased  
445 during the first weeks of postnatal development, as previously described (18); (Fig. 2C1). By P21,  
446 all large  $\alpha$ -motoneurons were fully bistable, while smaller neurons continued to lack bistability  
447 features.

448         The distinct firing behaviors observed between large and small  $\alpha$ -motoneurons can be  
449 attributed to a specific set of ionic currents that are more highly expressed in large fast  $\alpha$ -  
450 motoneurons compared to small slow  $\alpha$ -motoneurons. One key contributor is the activation of

451 slow persistent inward currents (PICs), which may be carried by calcium or sodium. This  
452 activation has been linked to negative hysteresis and bistability (15, 16, 19). Expression of the  
453 PIC increases with the size of the  $\alpha$ -motoneurons (Fig. 3B), and with the bistability score (Fig.  
454 3D). Furthermore, our observations demonstrate the relationship between the size of the  $\alpha$ -  
455 motoneurons and the sodium component of PICs (INaP) (Fig. 3E-H).

456

457         Recently, the thermosensitive Trpm5 current has been identified as a calcium-activated  
458 sodium current sustaining the plateau potential in bistable mouse  $\alpha$ -motoneurons (19). We  
459 found that the Trpm5-evoked afterdepolarization was present in virtually all large bistable  $\alpha$ -  
460 motoneurons, but not detectable in small non-bistable  $\alpha$ -motoneurons (Fig. 4A-C). Finally, the  
461 slowly inactivating Kv1.2-mediated current responsible for the delayed firing acceleration in  
462 large motoneurons (23) (Fig. 2), is less expressed in smaller motoneurons (Fig. 4G-I).  
463 Interestingly, this current positively correlates with the degree of bistability (Fig. 4G-I; (23)). It is  
464 likely, that the slow inactivation of Kv1 will shift the balance of currents towards depolarization,  
465 and will help to sustain continued firing in the bistable state. In sum, the lack of sufficient  
466 expression of these three pivotal currents renders small  $\alpha$ -motoneurons non-bistable. In  
467 contrast, large  $\alpha$ -motoneurons, which robustly express these currents, are bistable. Note that  
468 there may be some duplication of effort in these currents. Indeed, among neurons that showed  
469 continued bistable firing, 20% either did not show a marked negative hysteresis during ramps,  
470 or did not show a delayed firing acceleration. This suggest that bistability might not require the  
471 full spectrum of these currents.



472 Bistability in  $\alpha$ -motoneurons was observed in earlier experiments only in the presence  
473 of neuromodulators (14-16, 54). However, we have revealed inherent bistability in most large  $\alpha$ -  
474 motoneurons, provided the recording temperature is sufficiently high (above 30°C) (18), to  
475 unmask the thermosensitive Trpm5 current responsible for the plateau potential (19). In our  
476 preparations, some  $\alpha$ -motoneurons expressed intermediate properties and were not fully  
477 bistable under these conditions. Addition of 5-HT evoked full bistability in ~30% and ~60% of  
478 the intermediate and large neurons, respectively (Fig. 5). Interestingly,  $\alpha$ -motoneurons  
479 completely lacking baseline bistability characteristics (bistability score 0) never became bistable  
480 with 5-HT. We propose that intermediate  $\alpha$ -motoneurons express some of the essential  
481 currents for bistability, but at lower levels (Fig. 5). The known modulatory actions of 5-HT can  
482 then enhance these currents to sustain prolonged firing in the bistable state.

483

484 Our demonstration of a size principle for bistability contrasts with the groundbreaking  
485 work by Lee and Heckman (15, 16), who studied  $\alpha$ -motoneurons in adult cats. In their study,  
486 they achieved a full bistability in about one-third of the  $\alpha$ -motoneurons, which interestingly  
487 exhibited characteristics of smaller motoneurons. There are several potential explanations for  
488 the differences between our findings. First, it is uncertain whether the smallest  $\alpha$ -motoneurons  
489 in the cat were recorded because of the use of sharp electrode recordings. Consequently, the  
490 bistability in the smallest slow  $\alpha$ -motoneurons in cats remains unknown. Second, mouse  $\alpha$ -  
491 motoneurons are inherently more excitable than cat  $\alpha$ -motoneurons, primarily due to their  
492 smaller size (34, 55). Despite this, the PIC amplitude is relatively similar across both species,  
493 suggesting that PIC has a more significant impact on the firing rate in mice. Third, our

494 observations were made on mouse  $\alpha$ -motoneurons during the first 4 weeks of life, while Lee  
495 and Heckman's experiments were made on adult cats. However, bistability in the larger  
496 neurons became more pronounced with age in the mouse neurons. In young adult mice (P21-  
497 P25) all large motoneurons exhibited bistability, whereas none of the smaller ones did. Finally,  
498 our results were made *ex vivo* from slices in the presence of blockers of fast synaptic  
499 transmission (though not at P21/P25) and mostly without neuromodulators. On the other hand,  
500 the cat neurons were recorded *in vivo* in the presence of methoxamine to maximize the  
501 incidence of bistability.

502         The role of bistability in spinal  $\alpha$ -motoneurons remains unclear. It has been recorded  
503 during quiet standing in both rats and cats (56), suggesting a potential significance for postural  
504 control (15, 16, 28, 50). The Henneman Size Principle (57) posits that smaller  $\alpha$ -motoneurons,  
505 due to their lower input conductance, are the first to be recruited, potentially playing a role in  
506 maintaining posture. This can be consistent with earlier work in cats, where smaller neurons  
507 showed full bistability and were possibly active during quiet standing (15, 16, 58). However, we  
508 here demonstrate, in younger mice, that small  $\alpha$ -motoneurons are not bistable. Instead,  
509 bistability increases with the size of motoneurons (Fig. 2). Ritter et al. (58) provided evidence  
510 that large fast  $\alpha$ -motoneurons may also be tonically recruited during quiet standing in mice. An  
511 intriguing study by Bos et al. (19) showed that mice lacking Trpm5 channels in lumbar  
512 motoneurons exhibited compromised postural control. Given the central role of Trpm5 in  
513 bistable properties of larger  $\alpha$ -motoneurons (19) (Fig 4), it is possible that fast  $\alpha$ -motoneurons  
514 may play a role in postural maintenance. Alternatively, the currents responsible for bistability  
515 may play a more important role in recruiting the less excitable large motoneurons. The PIC

516 plays an important role in the initiation of bistable firing, but is also critical for repetitive firing in  
517  $\alpha$ -motoneurons (59), and in synaptic amplification due to their dendritic location (1). Thus, the  
518 currents which together lead to bistability may individually play multiple roles in motor control  
519 in the mouse. These assumptions should be further investigated in the future using more  
520 integrated *in vivo* preparations.

521 **FIGURE LEGENDS**

522

523 **Figure 1: Identification and size distribution of  $\alpha$ -motoneurons in the ventrolateral spinal cord**

524 **at L4-L5. A:** Ventrolateral cord at P12 showing Hb9-GFP (green), NeuN (red) and double labeling

525 (yellow). **B1:** The vast majority (98%, n = 141/144) of Hb9-GFP- labeled neurons co-express

526 NeuN. **B2:** Cross-sectional area distribution of Hb9-GFP neurons at P4-P6 (light green, n=571

527 neurons), P8-P10 (medium green, n=475 neurons) and P12 (dark green, n=288 neurons). **C1:**

528 Ventrolateral cord at P12 showing Hb9-GFP (green), MMP-9 (red) and dual labeling (yellow). **C2:**

529 Size distribution of Hb9-GFP labeled neurons expressing (n=122) or not expressing (n=166)

530 MMP-9. **C3:** Histogram of size distribution of GFP<sup>+</sup>-MMP-9<sup>-</sup> (green) or HB9<sup>+</sup>-MMP-9<sup>+</sup> (yellow)

531 neurons. **C4:** Double-labeled Hb9-GFP<sup>+</sup>/MMP-9<sup>+</sup> neurons are predominantly larger than 400

532  $\mu\text{m}^2$  (yellow, top) while most Hb9-GFP<sup>+</sup>/MMP-9<sup>-</sup> neurons are smaller than 400  $\mu\text{m}^2$  (green,

533 bottom). C2-C4: n=288 neurons. \*\*\* p<0.001 (two-tailed Mann-Whitney test for **C2**; Fisher's

534 exact test for **C4**). Mean  $\pm$  S.D. for B1. Median (red solid line) and quartiles (red dashed lines)

535 are represented for C2.

536

537 **Figure 2: Bistability score in spinal motoneurons varies by age and size. A:** Fully bistable

538 neuron (bistability score 4). **A1:** Baseline depolarization towards spike threshold: a 2-sec

539 suprathreshold stimulation evokes a prolonged afterdepolarization (ADP: 1 point). Slightly

540 higher baseline depolarization followed by a 2 sec stimulation leads to prolonged firing which is

541 only terminated by hyperpolarizing current step (1 point); trace has been offset to be more

542 easily seen. **A2:** Negative hysteresis during ramp stimulation. The current threshold for onset of

543 spiking is higher than the threshold for offset of spiking ( $-\Delta I$ ; 1 point). **A3**: A small subthreshold  
544 current step leads to a slow depolarization (trace has been offset to be more easily seen). A  
545 slightly higher current step leads to a larger depolarization leading to delayed spike onset and  
546 acceleration of spike frequency during the step (1 point). **B**: Completely non-bistable neuron  
547 (bistability score 0). **B1**: Current steps to near threshold lead to decelerating spike frequency  
548 during a 2 sec suprathreshold pulse, and an afterhyperpolarization at the end of the step (0  
549 point). **B2**: Positive hysteresis during ramp stimulation. The current threshold for onset of  
550 spiking is lower than the threshold for offset of spiking ( $+\Delta I$ ; 0 point). **B3**: A small subthreshold  
551 current step does not evoke a slow depolarization. A slightly higher current step evokes  
552 immediate onset firing with decelerating spike frequency during the step (0 point). Size  
553 markers: 1 sec, 20 mV. **C1**: Bistability score as a function of postnatal age for smaller ( $<400\mu\text{m}^2$ ,  
554 blue,  $n=81$ ) and larger ( $>400\mu\text{m}^2$ , red,  $n=118$ ) Hb9-GFP<sup>+</sup> neurons. Statistical comparison was  
555 done for each age and when all ages were pooled. **C2**: Distribution of bistability as a function of  
556 neuronal cross-sectional area combining all ages. Smaller neurons predominantly have  
557 bistability scores of 0-1 (blue,  $n=95$ ) while larger neurons predominantly have bistability scores  
558 of 3-4 (red,  $n=80$ ). C1-C2:  $n=199$  neurons. **C3**: Bistability score as a function of neuronal input  
559 resistance. A strong negative correlation is seen ( $R^2=0.99$ ,  $n=176$ ). **C4**: Effect of size on  
560 bistability in neurons (P1/9) recorded by perforated patch-clamp. Large neurons show  
561 significantly higher bistability scores than small neurons ( $p<0.05$ ;  $n=27$ ) **C5**: Post-natal (P2/6)  
562 motoneurons identified by expression of ChAT-GFP show similar bistability scores that rise with  
563 cross-sectional area (blue for  $<400\mu\text{m}^2$ ,  $n=16$  and red for  $>400\mu\text{m}^2$ ,  $n=9$ ;  $p=0.02$ ). \*  $p<0.05$ ; \*\*\*

564  $p < 0.001$  (two-tailed Mann-Whitney test for **C1**, **C4** and **C5**; Fisher test and slope comparison of  
565 simple linear regressions for **C2**). Mean  $\pm$  S.D.

566

567 **Figure 3: Persistent inward current (PIC), and its sodium component, is larger in bistable**

568 **neurons. A:** Voltage clamp measure of PIC activation during slow voltage ramp in a bistable but

569 not in a non-bistable neuron. **B-C:** PIC amplitude (B), and threshold (C) as a function of cell

570 cross-sectional area of Hb9-GFP<sup>+</sup> neurons (blue circles,  $< 400 \mu\text{m}^2$ ,  $n=17$  (B),  $n=9$  (C) and red

571 circles,  $> 400 \mu\text{m}^2$ ,  $n=24$  (B),  $n=17$  (C);  $P=0.014$  (B);  $P=0.72$  (C)). **D:** PIC amplitude increases with

572 increasing bistability (Green: bistability scores 0-1,  $n=15$ ; yellow: bistability scores 3-4,  $N=22$ ;

573  $P=0.001$ ). **E:** Superimposed leak-subtracted sodium persistent current (INaP) recorded from

574 Hb9-GFP<sup>+</sup> motoneurons in the presence of 10 mM TEA and 100  $\mu\text{M}$  CdCl<sub>2</sub> (area  $< 400 \mu\text{m}^2$  or

575  $> 400 \mu\text{m}^2$  for the blue and red trace, respectively) in L4-L5 regions at P9 in response to a slow

576 ramp depolarization. **F-H:** INaP peak amplitude (F), threshold (G) and density (H) as a function

577 of cell cross-sectional area of Hb9-GFP<sup>+</sup> neurons (blue circles,  $< 400 \mu\text{m}^2$ ,  $n=10$  and red circles,

578  $> 400 \mu\text{m}^2$ ,  $n=11$ ;  $P=0.001$ ). ns, no significance; \* $p < 0,05$ ; \*\*\* $p < 0,001$  (two-tailed Mann-Whitney

579 test for **B-D** and **F-H**). Median (solid line) and quartiles (dashed lines) are represented in each

580 violin plot.

581

582 **Figure 4: Trpm5-mediated current and slowly inactivating Kv1 current are signatures of**

583 **bistability. A:** Neurons are depolarized for 2 sec in presence of 1  $\mu\text{M}$  TTX and 10 mM TEA.

584 Above a threshold, large calcium-dependent oscillations are evoked. In bistable motoneurons

585 (red trace), these elicit a large, slow afterdepolarization which is partially blocked by 50  $\mu\text{M}$

586 TPPO (black trace). Non-bistable neurons (blue trace) do not express this slow  
587 afterdepolarization. **B:** Amplitude of the Trpm5-induced, TPPO-resistant afterdepolarization is  
588 larger in large motoneurons ( $>400 \mu\text{m}^2$ ,  $n=9$ ) than small motoneurons ( $<400 \mu\text{m}^2$ ,  $n=15$ ;  
589  $p=0.0017$ ). **C:** Trpm5-induced, TPPO-resistant afterdepolarization is larger in bistable  
590 motoneurons (score 3-4,  $n=10$ ) than nonbistable motoneurons (score 0-1,  $n=10$ ;  $p=0.0108$ ). **D:**  
591 TPPO-sensitive Trpm5 current is isolated in response to a voltage step of 2 sec in presence of  
592  $1 \mu\text{M}$  TTX and 10 mM TEA. Superimposed traces of Trpm5 isolated current from large ( $>400 \mu\text{m}^2$ ,  
593 red) vs small ( $<400 \mu\text{m}^2$ , blue) Hb9-GFP<sup>+</sup> motoneurons. Note that the current trace during the  
594 depolarizing step has been truncated for improved visualization of the post-step current. **E-F:**  
595 Amplitude (E) and density (F) of the Trpm5 current increases with cross-sectional area of Hb9-  
596 GFP<sup>+</sup> neurons (blue circles,  $<400 \mu\text{m}^2$ ,  $n=3$ , and red circles,  $>400 \mu\text{m}^2$ ,  $n=8$ ;  $p=0.0121$  and  $p=0.0242$ ,  
597 respectively) **G:** Superimposed traces of the slowly inactivating Kv1 current in bistable (red) and  
598 non-bistable (blue) neuron in response to a long depolarizing voltage step in presence of  $1 \mu\text{M}$   
599 TTX and 10 mM TEA. **H-I:** Amplitude (H) and density (I) of Kv1 current increases with cross-  
600 sectional area of Hb9-GFP<sup>+</sup> neurons (blue circles,  $<400 \mu\text{m}^2$ ,  $n=19$  (H),  $n=3$  (I) and red circles,  
601  $>400 \mu\text{m}^2$ ,  $n=26$  (H) and  $n=7$  (I);  $p=0.0216$  and  $p=0.0167$ ). \* $p<0,05$ ; \*\* $p<0,01$  (two-tailed Mann-  
602 Whitney test for **B-C**, **E-F** and **H-I**). Median (solid line) and quartiles (dashed lines) are  
603 represented in each violin plot.

604

605 **Figure 5: Induction of full bistability by serotonin.** **A:** Non-bistable neuron's response (top,  
606 black trace) to 2 sec depolarizing pulse (bottom). Only a small afterdepolarization is recorded  
607 after the step. During  $10 \mu\text{M}$  serotonin (5-HT), this neuron became fully bistable, with

608 continuous firing (top, red trace) following the 2 sec depolarizing step (bottom). **B**: Not all non-  
609 bistable neurons respond to serotonin (n=45). Smaller neurons (<400  $\mu\text{m}^2$ , n=19 of 19) fail to  
610 show full bistability (score 4) with 5-HT. 38% of larger (>400  $\mu\text{m}^2$ ) non-bistable neurons became  
611 bistable during 5-HT. \*\*\*  $p < 0.001$  (Fisher's exact test for **B**).

612

### 613 **DISCLOSURES**

614

615 No conflicts of interest, financial or otherwise, are declared by the authors.

616

### 617 **AUTHOR CONTRIBUTIONS:**

618

619 R. H-W, R.B. and F.B. generated the hypotheses for the paper. R. H-W, B.D. and R.B. performed  
620 electrophysiological experiments and analysis; E.P. performed immuno-histochemical  
621 experiments and analysis. R. H-W drafted the manuscript, and R. H-W, R.B. and F.B. revised the  
622 manuscript and approved the final version of the manuscript.

623

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629



630 **AUTHOR NOTES**

631

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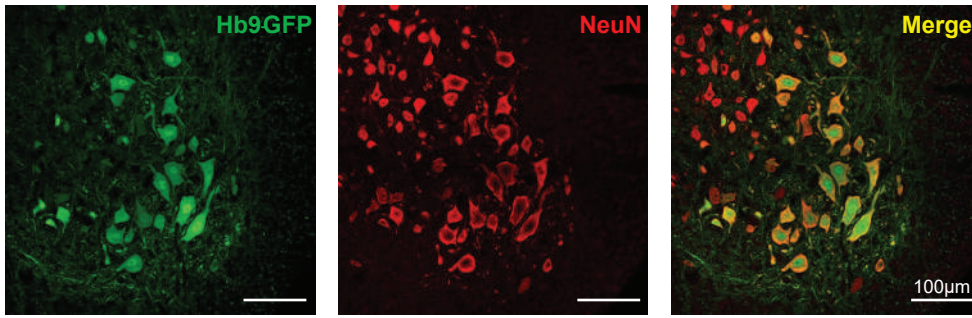
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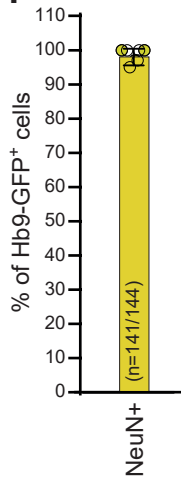
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# Figure 1

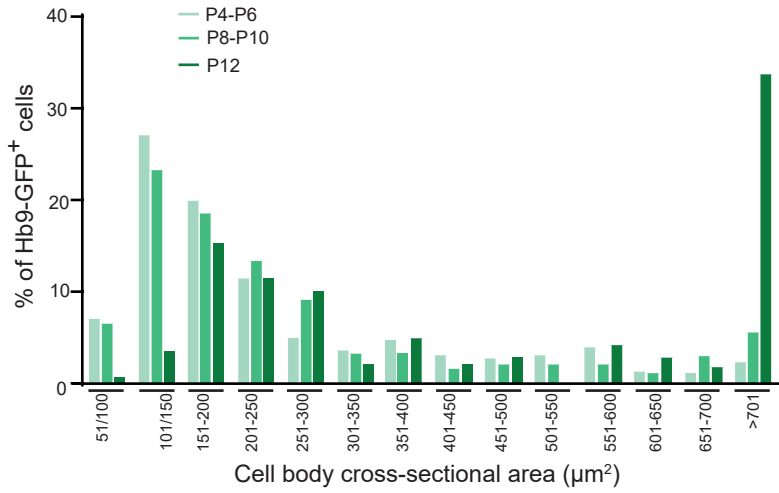
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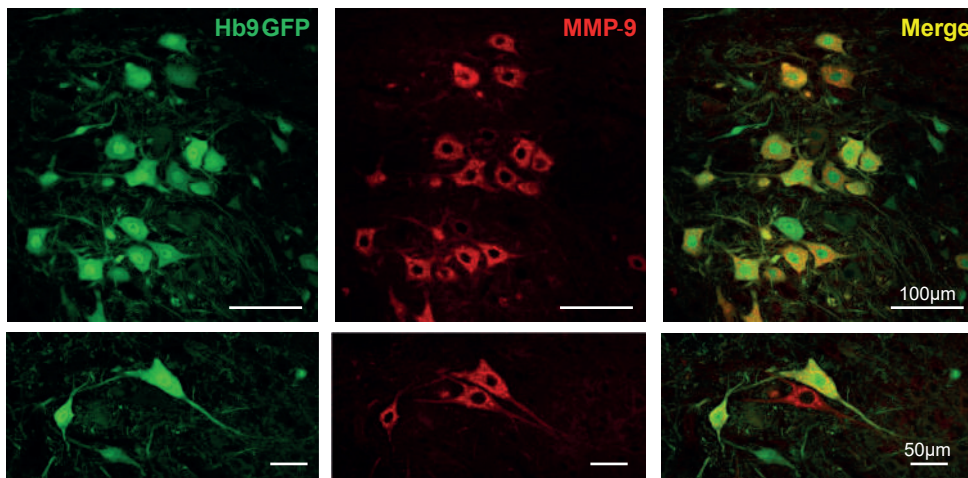
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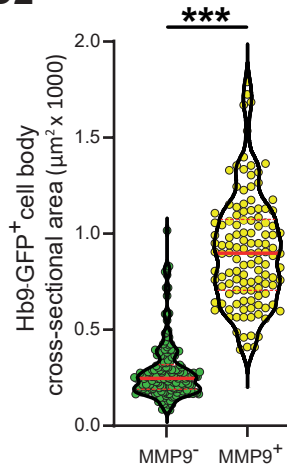
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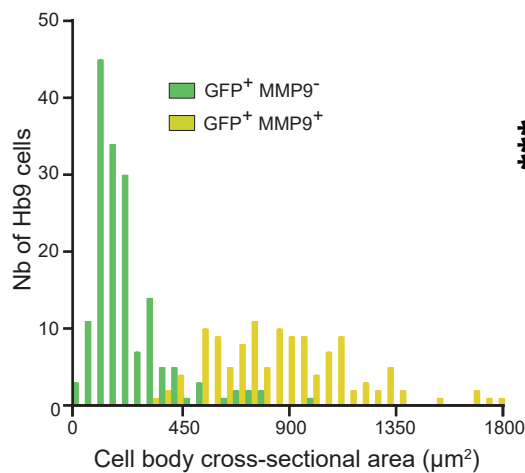
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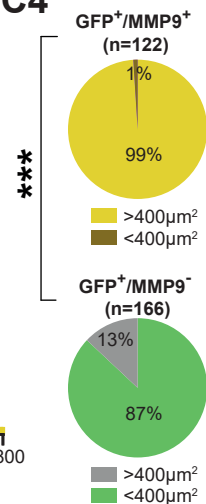
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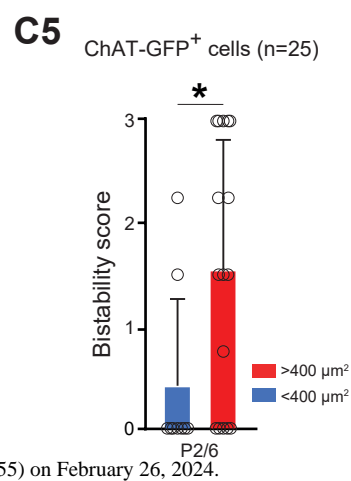
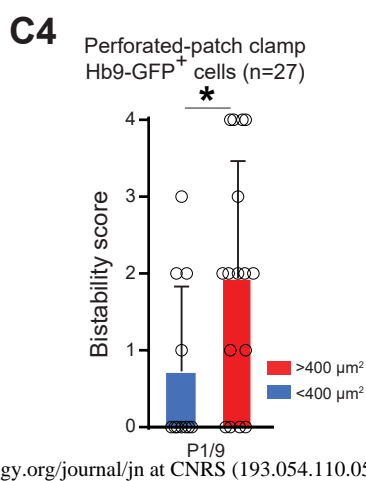
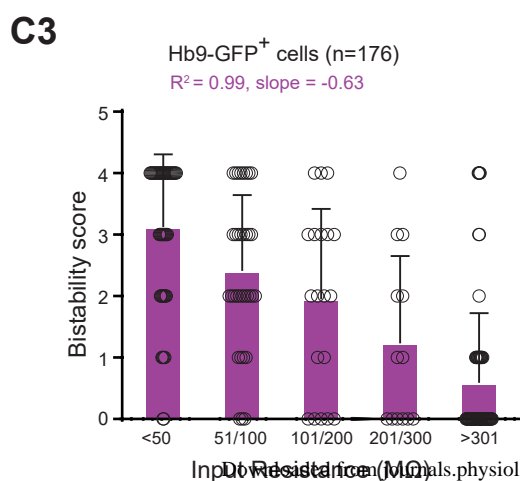
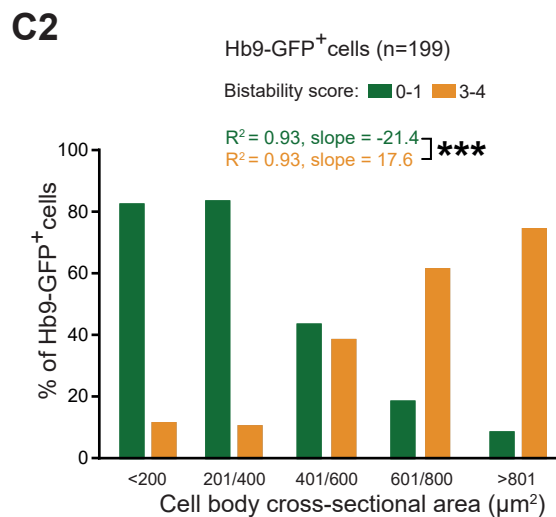
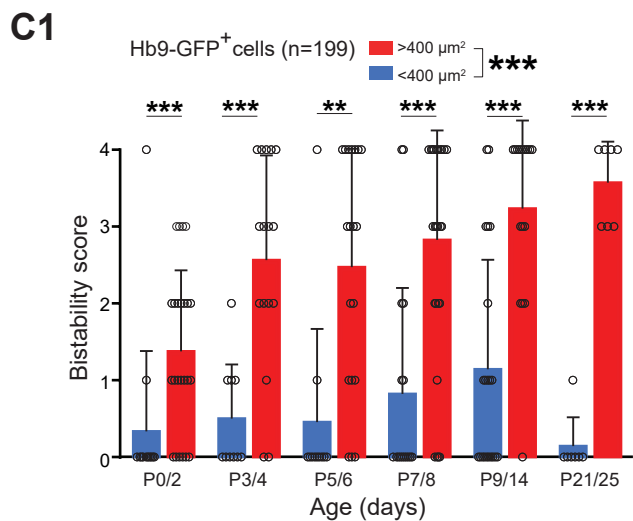
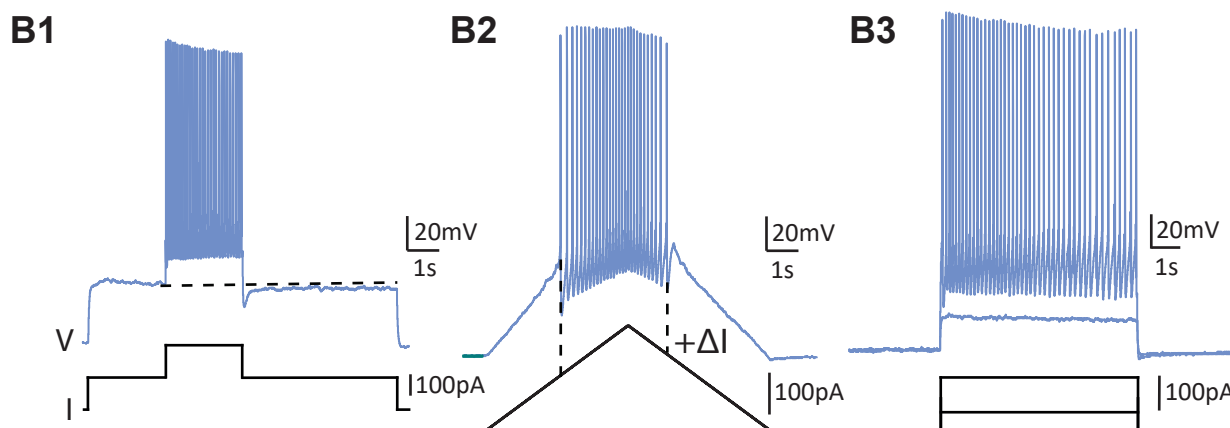
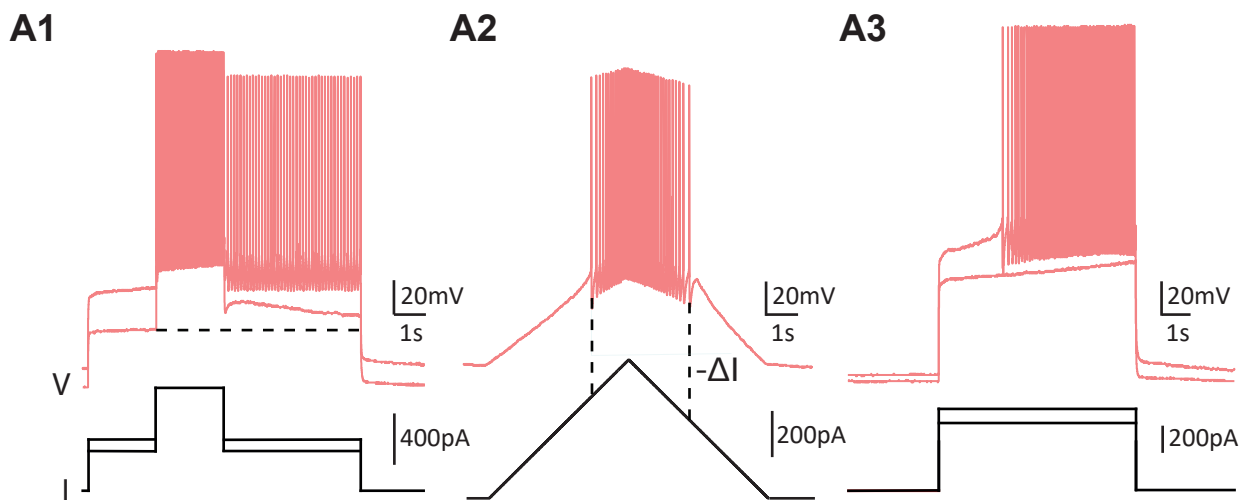
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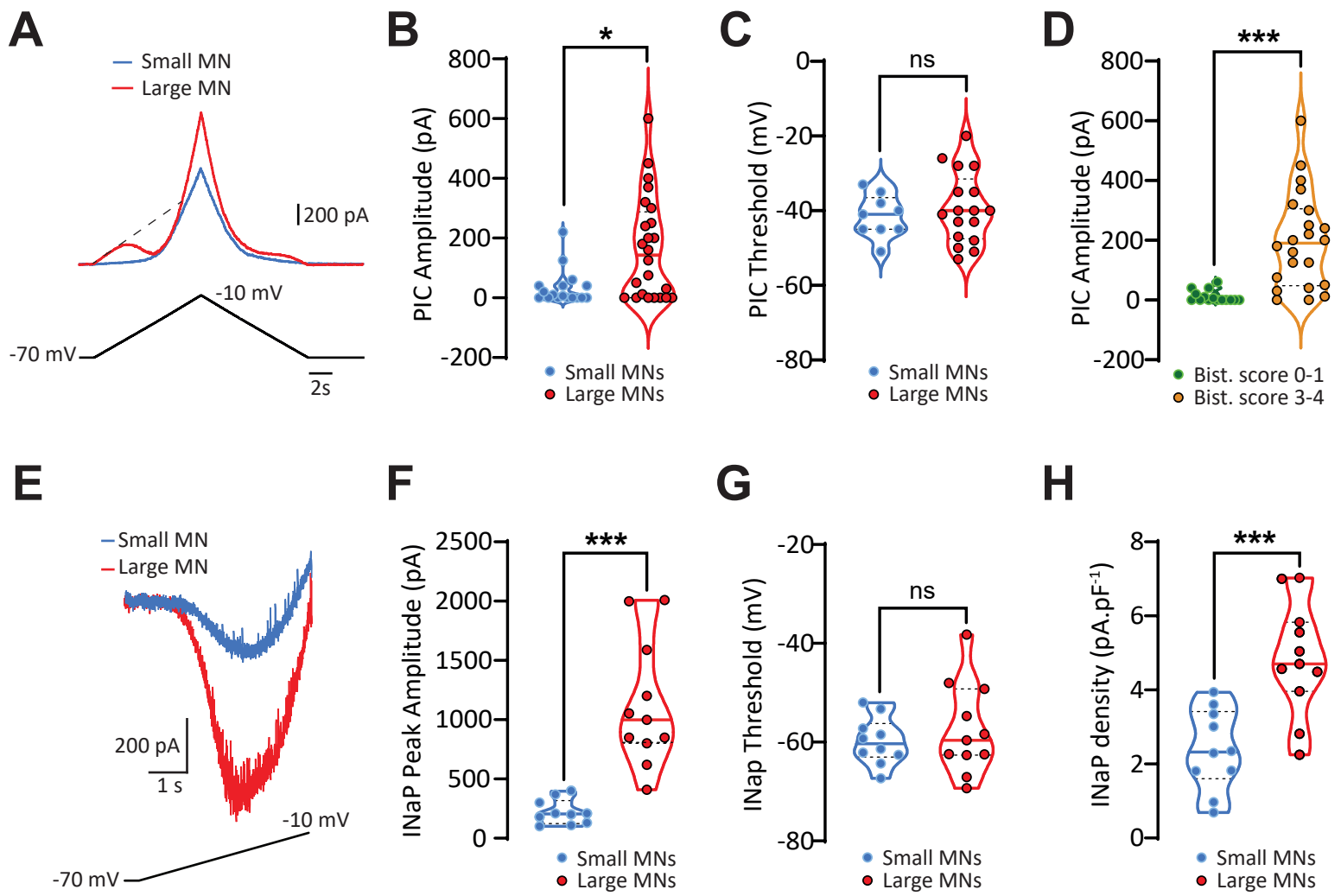
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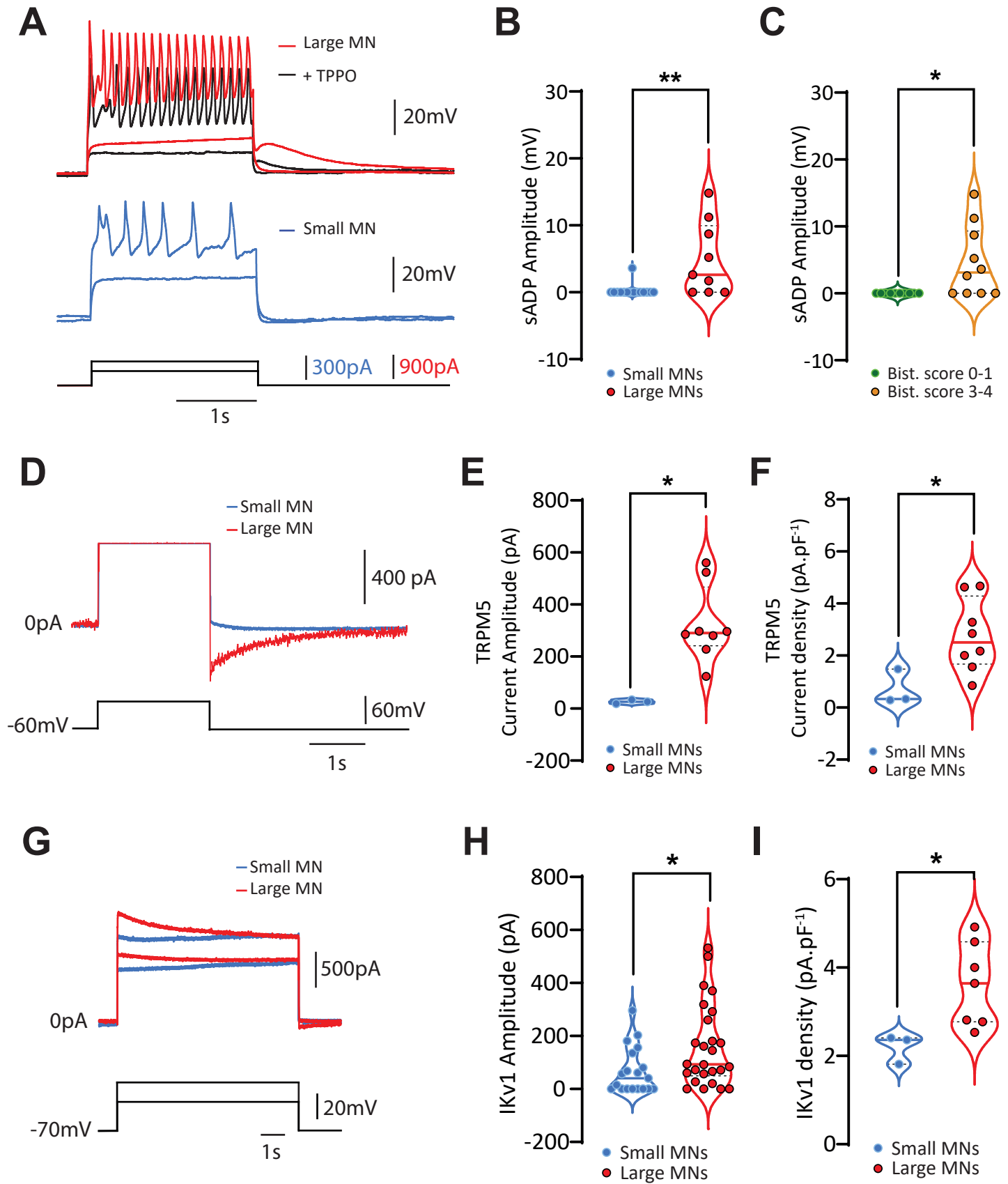
# Figure 2



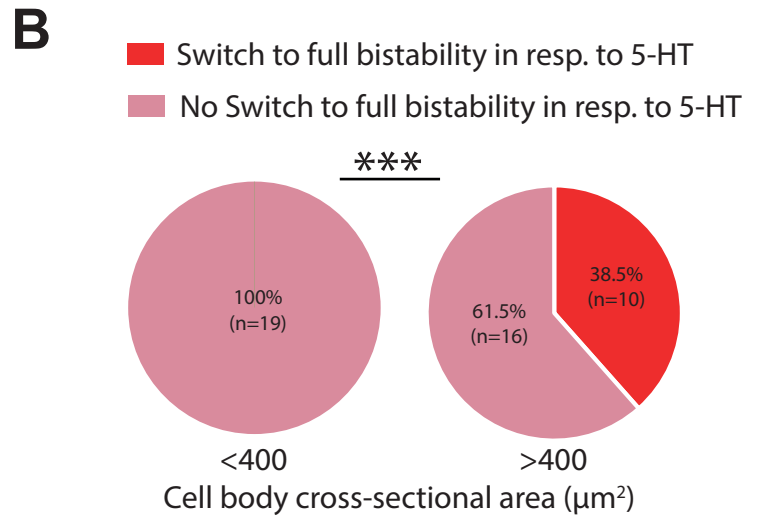
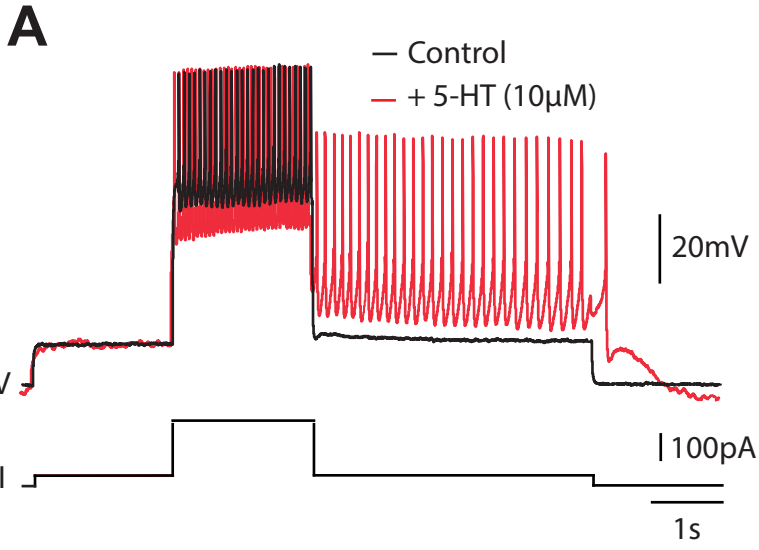
# Figure 3



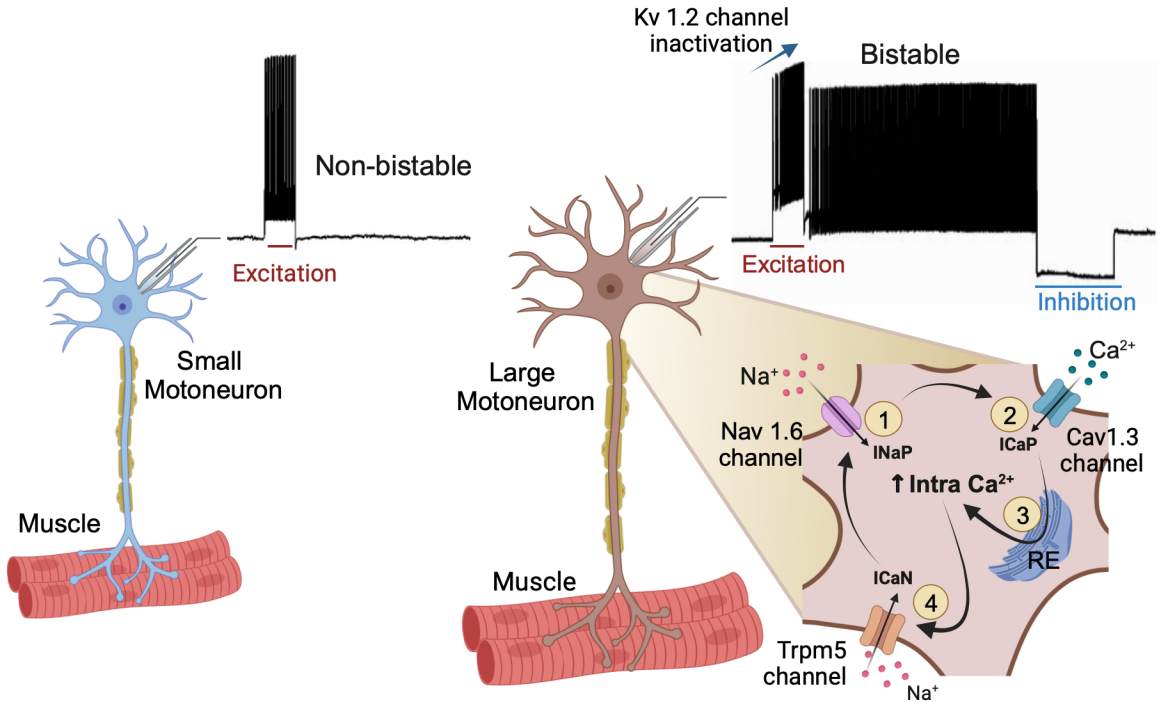
# Figure 4







# Effect of size on expression of bistability in mouse spinal motoneurons



Large mouse spinal motoneurons exhibit sustained firing after stimulation due to expression of ionic currents supporting bistability. Small spinal motoneurons lack such currents and are not bistable.