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Somatic Proximity of the Axon Initial Segment Predicts Motoneuron Excitability

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Abstract

As the neuronal site where voltage gated channel density is highest, the axon initial segment (AIS) plays a key role in establishing a neuron's action potential threshold, i.e. excitability. Among the properties of AIS that gain attention are length (AIS_l) and distance from the soma (AIS_d), which are variously found to change together with neuronal excitability following experimentally-induced perturbations in neural activity. The present study was designed to test the possibility that variation in AIS structural parameters regulates the native range in intrinsic excitability for one class of mature neurons. Spinal motoneurons were selected for their naturally large range in excitability and for their experimental accessibility to *in vivo* study. We began by determining whether AIS length or distance differed for motoneurons in motor pools that exhibit different activity profiles. Motoneurons sampled from the medial gastrocnemius (MG) motor pool exhibited values for average AIS_d that were significantly more than for motoneurons from the soleus (SOL) motor pool, which is more readily activated in low-level movements. Next, we tested whether AIS_d covaried with intrinsic excitability of individual motoneurons. Using anesthetized rats, we measured rheobase current intracellularly from MG motoneurons before labeling them for later immunohistochemical study of AIS. This combinatory approach revealed a significant correlation between AIS_d and rheobase, for 16 motoneurons sampled within the MG motor pool. Among multiple electrophysiological and morphological parameters measured here, AIS_d stood out as the dominant predictor of motoneuron excitability. These findings suggest an important role for AIS_d in setting the intrinsic excitability of spinal motoneurons.

Introduction

Diversity in the intrinsic excitability of neurons is attributed in part to heterogeneity in biophysical and structural properties of the axon initial segment (AIS) ¹. By aggregating voltage-gated channels in high density, this specialized structure sets the threshold for action potential initiation in the proximal axon ²⁻⁵. Action potential threshold can be regulated through the AIS not only by varying the mix, density or properties of ion channels, but also by varying AIS length (AIS_l) and/or distance from the soma (AIS_d) ⁶⁻¹². Roles for AIS morphology and location are supported by computational modeling and by biological experiments, which demonstrate that these AIS parameters change together with measures of neuronal excitability, e.g., threshold or firing behavior, in response to experimental manipulation of neural activity ^{4,9,13}. Indirect evidence for AIS regulation of neural excitability may also be reflected in the association between heterogeneity in AIS and diversity in the characteristic firing behavior of neurons belonging to either the same or different populations of cell types ^{14,15}. These observations promote inclusion of AIS_l and/or AIS_d among the candidate factors determining the intrinsic excitability of neurons.

Biophysical determinants of intrinsic excitability are well documented for alpha motoneurons in the mammalian spinal cord. Detailed examination of these neurons was motivated in part by interest in the mechanisms underlying reliable rank ordering in the recruitment of motoneurons within a motor pool, i.e., motoneurons supplying the same muscle. The 20-30-fold range in rheobase current observed among motoneurons in the same motor pool establishes a prominent role for mechanisms intrinsic to motoneurons in determining excitability and rank ordered recruitment ¹⁶⁻²⁰. Among those mechanisms, input conductance, subthreshold voltage-sensitive currents, and

properties of the channels underlying spike generation all contribute in determining motoneuron excitability ²¹. The possibility that AIS morphology and/or location might contribute in setting motoneuron excitability as it does for other classes of neurons is suggested by the substantial variation in these AIS parameters recently reported for rodent motoneurons ^{6,22,23}. Our findings advance this possibility by demonstrating an inverse relationship between AIS_d and intrinsic excitability measured *in vivo* from rat spinal motoneurons.

Results

AIS_d differs in motor pools exhibiting different activity levels

Motoneurons from SOL and MG motor pools were examined for differences in AIS_l and AIS_d. These motor pools express distinct activity patterns in rats as they do in other species ²⁴⁻²⁹. Motor pool activity assessed by EMG or force production of the associated muscle is prominent in SOL during quiet standing and slow locomotor speed in rats, while activity in MG develops progressively with movement intensity, approaching maximum only during rapid and more vigorous movements ³⁰. The differences in activity patterns parallel differences in intrinsic excitability of motoneurons, being greater for the type S motoneurons that dominate the SOL motor pool than for the preponderance of type F motoneurons that populate the MG pool ^{16,31-33}. We examined these two motor pools in attempt to find preliminary support for the idea that AIS dimensions or location contribute to excitability of motoneurons as shown for some other neuron types ¹³.

Retrograde CTB labeling revealed the motor pool identities for an unbiased sample of motoneurons (65 MG and 82 SOL) from 5 rats. Expression of AnkG reactivity^{34,35} in 2-dimensional images of motoneurons delineated AIS_l and AIS_d (Fig. 1a,b). Both parameters reported in Table 1 covered ranges similar to those provided in the only published reports on motoneurons, which were obtained from mice^{6,22} and rats²³. Segregating motoneurons by motor pool exposed a significant difference in the AIS_d for the functionally distinct SOL and MG motor pools. A tendency for shorter AIS_d in the SOL vs MG motor pool is represented in comparison of images from two motoneurons shown in Fig. 1a,b. While the AIS_d distributions overlapped considerably over short distances (<10μm), very few SOL motoneurons exhibited AIS_d values extending beyond the mean value, 12.50μm, observed for the sample of MG motoneurons (Fig. 1c). Computational modeling (HMC, Fig. 1d) established significant differences in PPD for both mean and standard deviation of AIS_d (Fig. 1e,f). No significant differences between pools were found for either AIS_l or motoneuron soma cross-sectional maximum diameter. These findings support AIS_d as a candidate contributor to differences in motor pool excitability and possibly also to differences in motor unit type.

AIS distance predicts motoneuron excitability

Next, we tested whether the relation with motor pool activity might emerge from an influence of AIS_d on the intrinsic excitability determined *in vivo* for individual motoneurons (n=18). MG motoneurons were selected for their wide range in rheobase, which would increase the chance of detecting a relationship with AIS_d. Restricting

study to MG motoneurons had the additional benefit of eliminating confounding influences introduced in cross-pool comparisons.

Electrophysiological measures were recorded intracellularly before filling the motoneuron with Neurobiotin for immunohistochemical analysis of AIS morphology. Example images of labeled motoneurons together with selected electrophysiological properties are illustrated in Fig. 2. Data from one motoneuron (Fig. 2a – e3) represent the majority of the sample (16/18 motoneurons) for which the AIS-bearing axons clearly emerged directly from the soma. Dendrite-derived axons were exhibited by the remaining minority (11%) of motoneurons sampled (see Fig. 2f1-3), comparable to the small percent found in mouse motoneurons ⁶. Because the interposition of a dendrite confounded measurement of the distance between soma and AIS, these motoneurons were excluded from further analysis other than to note their low rheobase values (<3nA).

Detailed measurements of multiple morphological properties from confocal image stacks together with various electrophysiological properties are compiled from the 16 remaining MG motoneurons in Tables 2-3. All properties were comparable to those that have been measured in previous studies of rodent motoneurons ^{17,18,36}. The sample also represents a substantial portion of the reported distribution for rheobase among MG motoneurons ¹⁶. Particularly pertinent was the range in AIS_d for our sample of MG motoneurons, which at 27μm covers the span associated with meaningful differences in excitability of other neuron types ⁹.

We focused on correlations between motoneuron excitability and AIS properties. Figure 3a shows that motoneurons with relatively short AIS_d tended to have lower rheobase, i.e., higher excitability than motoneurons with more distant AIS. Differences in AIS_d accounted for 63.1% of the variance in rheobase (R^2). This finding is qualitatively consistent with our indirect population comparisons (Fig. 1). Furthermore, through our multivariant model, we determined for every 0.846 μ m (β_1) increase in distance, there was a 1nA increase in rheobase. By contrast, AIS_I had no statistically significant correlation with either motoneuron excitability (Fig. 3b) or with AIS_d (Fig. 3c).

Motoneuron rheobase correlates stronger with AIS_d than with input conductance

Biophysical principles and observed correlation with rheobase support consensus that input conductance (G_{in}) is a primary determinant of motoneuron intrinsic excitability^{16,17,19}. Reliable measurement of input conductance in a subset of 10 motoneurons from our sample made it possible to test the correlation with rheobase. Input conductance explained a significant amount of variance in rheobase, 64.8% (95% HDI:10.9 – 42.2, $\beta_1 = 26.0$), within this sample. In order to validate our findings, we then calculated the PPD between rheobase and input conductance for a large MG motoneuron database obtained in our lab (n=44). For this larger sample, input conductance explained 64.4% of the variance (95% HDI:15.5 – 25.0, $\beta_1 = 20.2$) in rheobase. Figure 4 illustrates the observed data and predicted slopes for both groups of neurons derived from the probabilistic model. Near complete overlap of slopes indicates strong evidential support

that the small subset of neurons does not differ in their expected relationships (i.e., slope) from neurons previously collected in our laboratory or those described in the literature ^{17,19} or from experimental parameters such as recording with Neurobiotin filled electrodes.

Next, we developed a set of candidate Bayesian models to test the independent and combinatorial influence of AIS_d and/or G_{in} in determining motoneuron rheobase. This allowed us to identify which, if any, set(s) of parameters were most predictive. Pareto smoothed importance sampling, leave-one-out cross-validation (PSIS-LOO) was used to evaluate the four fitted models ³⁷. We focused on the ELPD (expected log pointwise predictive density) as an unbiased estimate of each model's predictive performance. We find clear evidence that the model containing AIS_d alone contained more predictive information than the model containing G_{in} alone (ELPD diff = -7.2, SE = 4.8). There was, however, no evidence that additive (ELPD diff = -0.8, SE = 1.5) or multiplicative (ELPD diff = -2.2, SE = 1.5) models, including both parameters, improved performance (see Methods) over the AIS_d model alone. We conclude that when considering single parameters, AIS_d provides superior predictive information to motoneuron excitability when compared to G_{in} alone.

Morphological and biophysical parameter comparison

A comprehensive pairwise comparison of all biophysical and morphological parameters is presented in figure 5. Several significant relationships emerged by conducting a classic frequentist correlation approach. Most notable for this report, not previously

discussed here, is the relationship between rheobase and muscle twitch contraction time ($R^2 = -0.50$) and twitch force ($R^2 = 0.23$), which has been used as a predictor of recruitment order^{18,38}. We provide these comparisons to help put our findings in the context of previous studies of the mechanisms of orderly recruitment of motoneurons according to various motoneuron and muscle unit properties.

Discussion

The present study provides, to our knowledge, the first direct examination of the relationship between AIS_d and intrinsic excitability for individual neurons of any type. We selected motoneurons for their wide range in excitability and asked whether this range might relate to AIS location and/or length, both of which have been shown to covary with homeostatic changes in excitability for other types of neurons. Our findings suggest that AIS_d plays a role in establishing the native excitability of motoneurons in healthy adult mammals. Results show that AIS_d co-varied with rheobase measured *in vivo*, such that motoneurons with the greatest rheobase (lowest excitability) tended to have AIS located further away from the soma. Moreover, AIS_d emerged as the best predictor of motoneuron excitability among all physiological and anatomical parameters measured in this study. These findings suggest an important role for AIS_d in establishing diversity in intrinsic excitability among spinal motoneurons.

AIS location and dimensions in relation to neuronal excitability

Our data fit well with other studies establishing AIS_d as a predictor of excitability. Early experimental evidence obtained from cultured hippocampal neurons by Grubb and Burrone demonstrated that chronic depolarization resulted in a distal relocation of the AIS while having little impact on the AIS length⁹. This shift was associated with a compensatory decrease in excitability as indicated by an increase in current threshold compared to controls. Another example comes from the more recent work of Lee et al., who demonstrated that KO of proteasome adapter protein Ecm29 produced a hyper-susceptibility phenotype to drug induced seizures in mutant mice. Pyramidal neurons in these animals exhibited proximal relocation of the AIS together with increased spiking probability, number, and frequency measured in brain slice¹². Together, these studies suggest a homeostatic tuning, whereby excitability increases or decreases inversely with AIS_d consistent with the relationship we find for motoneurons.

AIS_l has also been shown to be associated with neuronal excitability. Meza et al. report that spontaneous firing rate had a significant positive correlation with AIS_l and a significant negative correlation with distance in nigral dopaminergic interneurons¹⁵. However, through computational modeling the authors demonstrated that changes in distance may be secondary to length in determining excitability. In fact, AIS_l has been shown to be the dominate factor over distance in establishing excitability in other neuron types as well^{8,39,40}. So, it appears that both distance and length are related to excitability but biased towards one or the other in different systems.

Modeling the impact of AIS on excitability.

Biophysical models also demonstrate that AIS_l and/or AIS_d influence excitability. AIS_l has a strong and consistent effect with greater length producing an increase in excitability by increasing sodium conductance. While we did not find a strong correlation between excitability and AIS length in our study, we did find a small, but significant, negative correlation between rheobase and AIS surface area ($R = -0.51$). Lower rheobase motoneurons tended to have the largest AIS surface area which could allow for an increase in sodium conductance through a higher density of Nav 1.1 and 1.6 channels, both of which are known to be expressed at the AIS in motoneurons⁶. This would allow motoneurons to initiate and backpropagate action potentials at lower input currents.

By contrast, simulations yield effects of AIS_d that are small and, in most studies, opposite to our experimental findings. Specifically, excitability increases as the AIS moves away from the large capacitive load, or “current sink,” introduced by the soma. The discrepancy between observation and simulation remains unresolved but possible explanations have been considered. Simulated data move more in line with biological observations by increasing outward current activated by low-threshold voltage-gated potassium channels, such as those from the Kv1 and Kv7 family at the AIS⁴¹⁻⁴⁵. These channels could provide a resting hyperpolarizing current at the axon that results in higher rheobase as a function of distance from the soma¹³. This is a plausible mechanism given the high expression of these channels in the motoneuron AIS^{6,46}.

Further support derives from simulations showing that higher Kv conductance at the AIS results in more current needed to reach threshold for distal AIS ⁴⁴.

Primary determinants of intrinsic motoneuron excitability

Rheobase is commonly used to measure neuronal excitability. By establishing action potential threshold in response to injected current, rheobase emphasizes the intrinsic as opposed to synaptic determinants of neuronal excitability. However, rheobase is also thought to emerge from the interaction of multiple properties, including somatic leak and inward current conductances ¹⁹. For more accurate determination of the AIS contribution to neuronal excitability, Goethals and Brette recommend measuring somatic voltage threshold, defined as the maximum change in membrane potential that does not elicit an action potential ¹³. In their computer simulation, this parameter is not impacted by input conductance or dendritic morphology. However, measurement of voltage threshold is subjective and sometimes affected by membrane potential fluctuations arising during *in vivo* recording. All considered, we selected rheobase for its common use and practical utility in representing neuronal excitability.

Our main objective was to identify which parameters from our data set were best at predicting rheobase. For that purpose, we applied a multivariate model based on a Bayesian framework. AIS_d proved to be the strongest predictor of excitability.

Surprisingly, input conductance was not a strong predictor of rheobase, and neither an additive nor multiplicative model integrating both AIS_d and G_{in} provided any significant

improvement over AIS_d alone. However, the influence of G_{in} as a predictor of rheobase may have been confounded by various factors. One such factor arises from the possible expression of subthreshold conductances having the potential to influence somatic leak conductance. Nonetheless, data extracted from our sample of 16 motoneurons factor firmly establish AIS_d as a prominent predictor of intrinsic excitability.

Conclusion:

These findings provide direct evidence that at least for motoneurons that AIS_d has a prominent role in establishing the intrinsic excitability. Our data demonstrate that AIS_d co-varies with intrinsic excitability in healthy adult motoneurons, i.e., those with the lowest excitability (highest rheobase) tended to have larger AIS distance from the soma. We also demonstrate using multivariate modeling, that AIS_d is the strongest predictor of rheobase among various morphological and electrophysiological parameters, including input conductance. These findings suggest an important role for AIS_d in setting the intrinsic excitability of spinal motoneurons.

Methods

All procedures were performed using adult Wistar rats (M/F, 250-350 g; Charles River Laboratories, Wilmington, MA) as approved by the Georgia Institute of Technology

Institutional Animal Care and Use Committee. All experimental methods were all performed in accordance with the relevant guidelines and regulations, including the ARRIVE guidelines⁵⁷.

Experimental design:

Rats were divided into two study groups. In one group, retrograde labeling was used to identify multiple motoneurons belonging to either the medial gastrocnemius (MG) or the soleus (SOL) motor pool. These motoneurons were measured for various morphological parameters, including AIS_l and AIS_d. In the second group, both morphological and electrophysiological measures were obtained from individual MG motoneurons isolated by intracellular penetration with glass micropipettes. During all *in vivo* experimental procedures, including terminal and survival surgeries and experimental data collection, rats were deeply anesthetized (verified by the absence of withdrawal reflex) by isoflurane inhalation (5% induction, 1-3% maintenance in 100% O₂). At the end of experiments, rats were euthanized via an overdose of isoflurane inhalation (5%).

Collecting and processing tissue samples from designated motor pools

Rats (n=5) were anesthetized and surgically prepared for a brief survival surgery for the purpose of retrograde labeling of designated motor pools. A midline incision was made along the popliteal fossa on the left hindlimb to expose the MG and SOL muscles. Each muscle was injected with 0.1% Cholera Toxin Subunit B (CTB) conjugated to an Alexa-Fluor 647 or 488 (Invitrogen, Thermo Fisher Scientific, Waltham, MA). The total volume

(MG 40 μ l, SOL 20 μ l) was distributed throughout the muscles by 5 μ l injections via a Hamilton syringe. Following surgical closure of the wound, rats were given a subcutaneous injection of slow-release buprenorphine (0.1mg/kg) and returned to their home cage for approximately 72 hrs before being euthanized via an overdose of isoflurane inhalation (5%) followed immediately by transcardial perfusion with chilled vascular rinse (0.01 M phosphate buffered saline with heparin) followed by a fixative solution (2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Spinal cords were removed and post-fixed for one hour then stored in 30% sucrose at 4°C.

Spinal cords were cut into 30-50 μ m thick cross-sections using a Leica cryostat or microtome (Leica Microsystems, Buffalo Grove, IL) and mounted on glass microscope slides (Superfrost Plus, Fisher Scientific). Slides were incubated for 7-10 mins in cold acetone to reveal ankyrin-G (AnkG) epitopes⁴⁷. Sections were washed in 0.01M phosphate buffered saline with 0.3% triton (PBS-T) and incubated in blocking solution (10% normal goat serum mixed in PBS-T) for 1 hr. Serum was aspirated and replaced with a primary antibody solution containing mouse anti-AnkG (IgG2a ,1:400, Neuromab, RRID:AB_10673030), and sections were then incubated overnight at room temperature (RT) with gentle agitation. Immunoreactive regions were revealed with a mouse-specific secondary antibody raised in goat conjugated to a Cy3 anti-mouse IgG, Fcy subclass 2a specific secondary antibody (1:100, Jackson ImmunoResearch, RRID:AB_2338695) mixed in PBS-T. The sections were incubated in the secondary antibody mixture for 2 hours at RT with gentle agitation. Slides were then washed in PBS, mounted, and coverslipped with Vectashield.

Collecting and processing both in vivo electrophysiology and tissue samples from individual motoneurons

Rats (n=8) were prepared for *in vivo* study in terminal experiments as described previously^{48,49}. Briefly, each rat was deeply anesthetized for the duration of the experiment, which typically lasted 8 hours and ended with euthanasia and spinal cord extraction as described above. Experimental preparation began with monitoring and regulating vital signs including heart rate (300-500 beats/min), oxygen saturation (>90%), end-tidal CO₂ (2-5%), respiration rate (40-60 breaths/min), and core body temperature (37-38°C). Next, surgical procedures were used to expose the spinal cord (L4-S1) and the MG muscle and nerve in the left hindlimb. All other peripheral nerves in the left hindlimb were crushed. Finally, each rat was secured in a stereotaxic frame configured to support recording and stimulation devices applied to exposed tissues bathed in warm mineral oil.

Individual MG motoneurons were studied via intracellular penetration within the spinal cord by glass microelectrodes coupled to an electrometer (Axoclamp). Glass micropipettes were filled with 10% Neurobiotin (Vector Laboratories, Burlingame, CA, USA) in 0.1 M Tris-OH and 1.0 M potassium acetate and had electrical resistances ranging between ~5–10MΩ. Motoneurons were selected for study when antidromic action potentials evoked by electrical stimulation of the MG nerve and tested repeatedly during recording, exceeded 60mV in amplitude. Next, a series of biophysical properties were recorded from motoneurons having stable membrane potentials. Voltage responses to intracellular current injection (square-pulses repeated at 1pps) were

recorded to obtain conventional measures of the motoneuron's biophysical properties^{16,20}. Rheobase current, referred to as rheobase from this point forward, was our designated measure of the motoneuron intrinsic excitability and was recorded as the first among progressively incrementing current pulse amplitudes (50ms duration) to produce an action potential. Input conductance (G_{in}) was calculated as the steady-state voltage response elicited and normalized by 1 or 3 nA hyperpolarizing current pulses (50ms duration) averaged over several repeated trials. Inadequate bridge balance eliminated G_{in} measurement for some motoneurons. Afterhyperpolarization (ahp) was measured from action potentials elicited by suprathreshold current pulses (0.5ms duration). Action potentials elicited in the motoneuron during the ahp test evoked isometric motor unit twitch contractions, which were measured by a force transducer attached to the MG muscle tendon. For 2 animals, it was necessary to administer the paralytic drug, pancuronium bromide (0.2mg/kg i.p.), in order to minimize respiratory movement as needed to obtain stable intracellular records of membrane potential. Motor unit contractile properties were not measurable in these cases. Finally, current injection through the micropipette (5nA square pulses, 1ms duration delivered continuously at 2 Hz for 5mins) was used to fill the motoneuron with Neurobiotin (Fig. 2).

Terminal experiments concluded with rat perfusion and extraction of lumbosacral spinal segments for processing and sectioning as described above. Spinal cord sections were incubated with streptavidin conjugated to an Alexa Fluor 488 (1:50; Invitrogen, RRID:AB_2315383) mixed with the secondary antibody solution for purposes of

identifying Neurobiotin-filled motoneurons. Sections were also processed for AnkG immunoreactivity as described above.

All recorded data (electrode current and membrane voltage together with muscle force) were digitized (20kHz; Cambridge Electronic Design Power 1401), stored and later analyzed with Spike2 software.

Image Analysis

Sections containing motoneurons labeled retrogradely with CTB or injected intracellularly with Neurobiotin were imaged at high magnification using confocal microscopy (Zeiss LSM 700). Image stacks (0.5 μ m steps) were captured with a 63X oil immersion objective (N.A 1.4) at 0.5 digital zoom.

Morphological analysis of motoneurons neurons identified by retrograde labeling

Image stacks of retrogradely labeled motoneurons with clear AnkG labeling were analyzed using Imaris (Bitplane, Zurich, Switzerland). Confocal image stacks were uploaded and the soma max cross-sectional diameter, AIS metrics were obtained using the polygon measurement tool. AIS_d was measured from the axon hillock to the proximal end of AnkG immunoreactivity (Figure 1B, between white and red arrows), while AIS_i was measured between the proximal to the distal ends of AnkG immunoreactivity (region between two red arrows).

Morphological analysis of motoneurons examined electrophysiologically

Motoneurons filled with Neurobiotin and immunolabeled with AnkG were analyzed using Neurolucida (Microbrightfield, Williston, VT). Motoneuron cell bodies were reconstructed in 3D through a series of contours traced in each optical plane. AIS_d and AIS_l were traced in 3D following their tortuosity through each optical plane to accurately measure both AIS distance and length. These reconstructions were also used to measure motoneuron soma surface and volume. All morphological measures were performed blind in regard to biophysical properties including rheobase.

Statistical analysis and Bayesian modeling.

Analyses focused on answering four central questions: (1) are distinct differences in excitability between motor pools predicted by AIS distance from soma? (2) what morphological features of motoneurons predict rheobase? (3) are input conductance and AIS distance co-equal predictors of rheobase? (4) what other biophysical and morphological features of the motor unit covary with proximal axon morphology?

Mixed-effects statistical models are preferred for answering these questions, because they have the power to reduce type I error rates. However, implementation of these models in a traditional frequentist framework relies on maximum likelihood estimation, which generally requires large sample sizes for model convergence and to mitigate type II errors. For small sample sizes, such as those typical *in vivo* electrophysiological

studies of single neurons, mixed-effects models implemented in a Bayesian framework can overcome convergence issues, will more accurately reflect the uncertainty in effects that are based on small sample data, and are more robust to guard against the over-interpretation of unlikely results ⁵⁰. Thus, models were fit in a fully Bayesian inferential framework to empirically derive the full joint posterior probability distributions (PPD) of model parameters simultaneously (e.g., means, standard deviations, and effect sizes) ⁵⁴.

Our models describe uncertainty in the response variable, e.g., rheobase, AIS distance, y , conditional on unknown parameters θ (e.g., regression coefficients) and predictors (e.g., biophysical and morphological parameters or motor pool membership), x , as well as the *a priori* uncertainty about these parameters and predictors ⁵⁶. Bayes theorem describes the proportional relationship ($\propto$) between our prior knowledge about the parameters (before observing the data) and our posterior beliefs about the parameters (after observing the data) as

$$p(\theta|y, x) \propto p(y|\theta, x) * p(\theta|x) \text{ }^{56}$$

The first probability on the right-hand side of the equation is the likelihood - the joint probability of the data for all possible θ values given the observed predictors x ⁵⁶. The second term on the right is the prior which describes uncertainty in θ before observing data y . Finally, the left-hand side is the posterior - the joint probability distribution of all parameters θ after we observed data y ⁵⁶. It serves as a compromise between the likelihood and the prior and describes the chance of all parameter values conditional on

the probability model. Evaluation of the population level AIS proximity (μ m) distribution (question 1) revealed a positive continuous variable, that is peaked, and has non-normally distributed heavy tails (overdispersion), so we fit a bespoke shifted log-normal regression model (Stan and rstan) with motor pool membership as the only population-level predictor to always positive predictions for the response variable as expected. For all remaining modeling analyses, we used R's *rstanarm* package, specifically the *stan_glm* function, to construct a hierarchical Bayesian model ⁵⁴. Prior to modeling, parameters were standardized.

Direct examination of the PPD enabled intuitive statistical judgments regarding the strength of the evidence, thus reallocating credibility across answers to our central questions calculating the slopes (β_i) and correlation coefficients (R^2). From the PPD, we derived the Bayesian equivalent of R^2 . As defined in the standard regression model notation of:

$$y_j \sim N(u_i, \sigma) \text{ Eq. 1}$$

$$\text{where } u_j = X\beta$$

the R^2 is formulated as ⁵¹:

$$R^2 = \frac{\sigma_f^2}{\sigma_f^2 + \sigma_e^2} \text{ Eq. 2}$$

$$\text{where } \sigma_f^2 = \text{var}(\mu), (u_j = X\beta), \text{ and for Gaussian models } \sigma_e^2 = \text{var}(y - \mu)$$

Marginally informative priors were applied to model parameters and variance components such that inferences were driven predominantly by the experimental data

to explicitly answer our central questions. Prior specifications were based on results from previous preliminary data and published studies by our group⁵² and others^{17,19}.

All models were fit using Hamiltonian Markov Chain Monte Carlo sampling to compute credible parameter values (θ), e.g., means, standard deviations, regression coefficients, effect sizes. Each model was run with four independent chains for 400 warm-up and 4,000 sampling steps. Steps to perform model evaluation and validation have been extensively described in our previous work⁵³⁻⁵⁵. Briefly, for all parameters, the number of effective samples was >2000 , convergence was assessed and assumed to have reached the stationary distribution by ensuring that the Gelman–Rubin shrinkage statistic for all reported parameters was <1.05 . Results are presented as posterior means and 95% Bayesian credible intervals - specifically, highest posterior density intervals, which denote the 95% most plausible values of the parameter being estimated. Briefly, trace plots were examined and indicated clear stationarity and good mixing, and numerical checks of sampling quality indicated convergence (i.e., $\hat{R}=1.0$, Monte Carlo standard error = 0.0, and effective sample size > 2000).

Data Availability All data, models, and code are immediately available upon request.

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Competing Interest The authors declare no competing interests.

Figure Legends

Figure 1. AIS distance differs in motor pools distinguished by their activity patterns. **a**, **b**) MG and SOL motoneurons pre-labeled with the retrograde tracer cholera toxin subunit B conjugated to Alexa fluor 488 (CTB) and immunolabeled for ankyrin-G (AnkG), a marker for the AIS. Our definition of AIS distance (AIS_d) and AIS length (AIS_l) is identified in panel A (green and red arrows). The two red arrowheads in A point to

lipofuscin granules that is common in mature motoneurons. **B)** white arrow indicates the start of axon from soma and red arrow indicates initiation of AIS. **c)** Distribution of the observed AIS distance between the MG (n = 65) and SOL (n = 82) motoneurons. Black vertical line inside the plot represents the median value. MG: avg. 12.50 ± 5.95 (s.d.), SOL: 7.11 ± 2.65 . **d)** Bayesian posterior predictive modeling distributions for AIS_d in MG and SOL motor pools conditioned on the observed data in C). **e)** Average and **f)** standard deviation derived from shifted log-normal regression model (see Methods).

Figure 2. Morphological and *in vivo* electrophysiological parameters measured in individual Neurobiotin filled motoneurons. **a)** Collapsed confocal image (10x) of a Neurobiotin filled motoneuron in the ventral horn of the spinal cord. Solid white line indicates white/gray matter boundary. **b)** Rheobase generated by 50ms square pulse current injection. **c)** Input resistance measured by hyperpolarizing currents (-1/3nA) for 50ms. Input conductance is reported as the inverse of resistance **d)** Muscle twitch elicited by 0.5ms suprathreshold intracellular current injection. Twitch force is defined as the maximum amplitude (vertical dashed red line). Contraction time (CT) is measured between the twitch onset to the maximum amplitude (horizontal dashed red line). **e1-3)** High magnification confocal image of Neurobiotin filled MG motoneuron revealed with streptavidin-488 (e1). White arrow heads indicate motoneuron axon. Sections were immunolabeled with AnkG to identify the AIS (e2). Panels e1 and e2 are merged and displayed in e3. **f1-3)** A small percent (11% in our sample) of axons are of dendritic origin. **f1)** Neurobiotin filled motoneuron with a parent dendrite indicated with the red arrowhead and the protruding axon indicated with the white arrowhead. **f2)** AnkG immunoreactivity along axon. **f3)** Merge Imaris rendition of image displayed in **f2**.

Figure 3: AIS_d but not AIS_l covaries with rheobase. All comparisons were performed using a Bayesian analytical approach to calculate a posterior probability distribution. Plots are based on a generative model conditioned on previous reports and the current data set. Each grey line represents a single trial from 4,000 generative samples and each black dot is an observed data point (n=16). From the 4,000 samples we provide a 95% high density interval (HDI). The median slope from the generative sample is represented as a blue line (β_1). From the slopes and generative model, we compute an R^2 equivalent, and this is presented with an HDI and a median. **a)** Rheobase: AIS distance. As rheobase increases the AIS distance increased from the motoneuron soma. Model slope: 95% HDI 0.463 – 1.21, $\beta_1 = 0.846$; R^2 : 95% HDI 0.364 – 0.732, median **R^2 0.631**. **b)** Rheobase : AIS length. As rheobase decreases the AIS tends to increase in length, though this was not significant (slope crosses 0). Model slope: 95% HDI -1.59 – 0.228, $\beta_1 = -0.680$; R^2 : 95% HDI 0.00 – 0.407, median **R^2 0.161**. **c)** AIS distance: AIS length: There was no relationship detected between these two variables. Model slope: 95% HDI -0.962 – 0.811, $\beta_1 = -0.121$; R^2 : 95% HDI 0.00 – 0.208, median **R^2 0.033**. Plots generated by hierarchical Bayesian model (see Methods). Note similarity in R^2 values presented here in bold in comparison with those computed by classical frequentist statistics presented in figure 5.

Figure 4: Close similarity in rheobase and input conductance between two samples of MG motoneurons. A hierarchical Bayesian model (as described in figure 3) was constructed for 44 MG motoneurons (black filled circles, grey lines) pooled from a larger MG motoneuron database produced by the Cope lab and for 10 MG Neurobiotin filled motoneurons (open circles, blue lines) pooled from the 16 cells presented in figure 3. Models were conditioned on previous reports^{22, 43} and datasets from this study. The positive correlation between rheobase and conductance is representative of prior reports^{22, 43} and our small sample of 10 motoneurons fell within the expected range produced from our larger dataset. *44 motoneurons* - Model slope: 95% HDI 15.5 – 25.0, $\beta_1 = 20.2$; R^2 : 95% HDI 0.522 – 0.728, median **R^2 0.644**. *Neurobiotin filled motoneurons* - Model slope: 95% HDI 10.9 – 42.2, $\beta_1 = 26.0$; R^2 : 95% HDI 0.239 – 0.743, median **R^2 0.648**.

Figure 5: A pairwise comparison of all morphology and biophysical properties from filled motoneurons (n=10-16 motoneurons). *Upper left to the bottom right diagonal:* Histogram plots showing distribution of data from motoneurons. Fitted line to the distribution of data is in red. *Left of histogram:* Scatter plots for all comparisons. Each dot represents a single data point. The open white circles represent comparisons that do not reach significance. All black circles represent significant correlations. *Right of the histograms:* Each value listed in the matrix is an r-squared value. The number of red asterisks refer to significance level for Pearson correlation coefficients (*p<0.05, **p<0.01, ***p<0.001). Red squared indicates a relationship between two variables but did not reach statistical significance.

Tables

Population Summary				
MG (n=65)	Mean	Std. Dev.	Min	Max
AIS Distance (AIS _d)	12.50	5.95	4.48	28.10
AIS Length (AIS _l)	28.78	4.96	18.10	43.30
Soma Diameter	37.14	5.15	26.55	48.50

SOL (n=82)				
AIS Distance (AIS _d)	7.11	2.65	2.88	17.80
AIS Length (AIS _l)	28.00	5.62	13.30	39.50
Soma Diameter	36.47	5.37	22.40	51.20

Table 1: Population data measurements from retrogradely labeled MG and SOL motoneurons.

Morphological Measurements						
Variable	Mean	Std. Dev.	SE	N	Min	Max
AIS Distance (μm)	13.38	7.92	1.98	16	5.58	32.61
Prox. Axon Surface (μm²)	245.74	163.96	40.99	16	88.22	617.65
AIS Length (μm)	26.98	5.00	1.25	16	18.23	36.98
AIS Surface (μm²)	245.25	78.03	19.51	16	123.76	394.71
Soma Surface (μm²)	5026.42	861.29	215.32	16	3391.10	6687.74
Soma Volume (μm³)	23305.44	4361.25	1090.31	16	13139.70	30398.90
Max Soma Diam. (μm)	54.15	7.50	1.94	15	41.65	70.19

Table 2: Morphological characteristics of motoneurons and the AIS

Conventional Motoneuron and Motor Unit Measurements						
Variable	Mean	Std. Dev.	SE	N	Min	Max
Rheobase (nA)	13.38	8.46	2.12	16	2.00	28.00
Conduction Delay (ms)	1.44	0.14	0.03	16	1.20	1.64
Action Potential Height (mv)	67.06	4.21	1.12	14	60.00	75.00
Input Conductance (S)	0.51	0.18	0.06	10	0.25	0.87
AHP Amplitude (mV)	1.59	0.81	0.21	15	0.70	3.71
AHP ½ Width (ms)	11.02	4.15	1.07	15	4.04	17.20
Twitch Force (grams)	2.78	2.09	0.56	14	0.50	6.93
Contraction Time (ms)	15.08	4.37	1.17	14	8.60	21.47

Table 3: Biophysical measurements from intracellularly recorded MG motoneurons.

*Two motoneuron heights were excluded. In both instances spikes were blocked and not able to produce a full spike even with positive current injection.

Figures

Figure 1

Figure 2

Motoneuron Excitability & AIS Parameters

Figure 3

Figure 4

Figure 5

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Morphological and in vivo electrophysiological parameters measured in individual Neurobiotin filled motoneurons. a) Collapsed confocal image (10x) of a Neurobiotin filled motoneuron in the ventral horn of the spinal cord. Solid white line indicates white/gray matter boundary. b) Rheobase generated by 50ms square pulse current injection. c) Input resistance measured by hyperpolarizing currents ($-1/3\text{nA}$) for 50ms. Input conductance is reported as the inverse of resistance d) Muscle twitch elicited by 0.5ms suprathreshold intracellular current injection. Twitch force is defined as the maximum amplitude (vertical dashed red line). Contraction time (CT) is measured between the twitch onset to the maximum amplitude (horizontal dashed red line). e1-3) High magnification confocal image of Neurobiotin filled MG motoneuron revealed with streptavidin-488 (e1). White arrow heads indicate motoneuron axon. Sections were immunolabeled with AnkG to identify the AIS (e2). Panels e1 and e2 are merged and displayed in e3.

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