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Maarten H P Kole & Greg J Stuart

Action potential threshold is thought to be lowest in the axon, but when measured using conventional techniques, we found that action potential voltage threshold of rat cortical pyramidal neurons was higher in the axon than at other neuronal locations. In contrast, both current threshold and voltage threshold of the isolated somato-dendritic spike were substantially higher at the soma. These data indicate that action potential threshold is indeed lowest in the axon.

Cortical neurons process information by encoding spatiotemporal patterns of synaptic inputs into action potentials with millisecond precision *in vitro* and *in vivo*[1,2]. Such fidelity in neural coding requires a spike-generating mechanism that operates with high accuracy. A critical aspect of the spike-generating mechanism is that action potentials are only initiated once the membrane potential reaches a threshold. Pioneering work in motoneurons predicts that the voltage threshold is ~15 mV lower (more hyperpolarized) in the axon initial segment (AIS) than in the soma[3], leading to the widely held belief that action potential voltage threshold is lowest in the axon[4]. Because of the inaccessibility of the axon to direct recording, there is little direct evidence for or against this notion. Furthermore, given that action potentials are initiated in the AIS of most neurons[3,5–10], the measurement of action potential threshold at the soma may not give an accurate measure of threshold in the axon. The inability to measure axonal action potential properties from somatic recordings may also underlie recent alternative and unconventional proposals for Na^+ channel gating during action potential initiation[11,12].

To study action potential threshold properties, we made dual current-clamp whole-cell recordings from the soma and axon (or dendrites) of large layer 5 pyramidal neurons in rat somatosensory cortex, as described previously[9] (see **Supplementary Methods** online). Action potentials were evoked by extracellular synaptic stimulation and threshold was defined as the membrane potential at which the rate-of-rise of voltage crossed 50 V s^{-1} (**Supplementary Fig. 1** online). To our surprise, action potential voltage threshold measured in the axon (–42.0 ± 1.0 mV, $n = 7$, 35 – 55 µm from the axon hillock) was significantly more depolarized than that measured at the soma (–47.9 ± 1.3 mV, $P < 0.01$, $n = 7$; **Fig. 1a**). A similar difference was observed during brief (3 ms) somatic current injection (somatic threshold, –48.1 ± 1.2 mV; AIS threshold, –43.1 ± 0.8 mV; $P < 0.001$, $n = 25$) and over a large range of voltage threshold values and criteria (**Supplementary Fig. 1**). These data suggest that action potential voltage threshold is higher at the site of action potential initiation than at the soma.

We next systematically investigated the dependence of action potential voltage threshold on recording location along the somato-dendritic and axonal axis. Action potentials in these experiments were evoked by somatic current injection. Action potential voltage threshold in the distal AIS was found to be higher (more depolarized) than any other location in the neuron (**Fig. 1b**). A higher voltage threshold for action potentials in the AIS is unexpected, given that action potentials are initiated at this site[3,5–10]. We therefore investigated the current threshold for action potential generation using current injections of different amplitude either at the AIS or soma (**Fig. 1c**). We found that, in contrast with voltage threshold, action potential current threshold was significantly lower in the AIS than the soma ($P < 0.01$; **Fig. 1d**).

What underlies the lower current, but higher voltage, threshold for action potential initiation in the AIS relative to that at the soma? To test the contribution of the high density of voltage-activated Na^+ channels in the AIS[13], we studied the effect of the Na^+ channel blocker tetrodotoxin (TTX). Synaptic input was mimicked by injection of suprathreshold current waveforms at the soma (**Fig. 2a**). Contrary to expectations based on passive attenuation of voltage from the soma to the AIS, before action potential threshold was reached, the membrane potential in the AIS was more depolarized than at the soma. To quantify this membrane potential difference, we subtracted the voltage response at the soma from that recorded in the AIS (**Fig. 2b**). In control conditions, the voltage in the AIS was, on average, 7.3 ± 0.5 mV more depolarized than the soma at action potential threshold ($n = 5$; **Fig. 2b**). In the presence of TTX, the situation was reversed, with the membrane potential in the AIS being more hyperpolarized than the soma by, on average, 1.2 ± 0.3 mV (**Fig. 2b**), which is consistent with passive attenuation of synaptic input as it propagates from the soma to the AIS.

We further investigated the role of Na^+ channels in a computational model of a layer 5 pyramidal neuron (**Fig. 2c,d**; see **Supplementary Methods**). The Na^+ channel model used in these simulations had standard Hodgkin and Huxley properties with densities and voltage dependence determined by recent experimental findings[13], including the observed hyperpolarized shift in voltage dependence of activation and inactivation of axonal Na^+ channels[13,14]. Consistent with the experimental data, the model had a more depolarized voltage threshold in the AIS compared with the soma (voltage difference, 4.4 mV; **Fig. 2c**). This finding was the result of the high density of Na^+ channels in the AIS rather than the different voltage-dependent properties of Na^+ channels in the axon, as it was maintained in models with identical Na^+ channel voltage dependence in the axon and soma (**Fig. 2d**). These results indicate that the membrane potential in the AIS is boosted prior to action potential initiation by the same Na^+ channels that underlie action potential generation. This membrane potential depolarization

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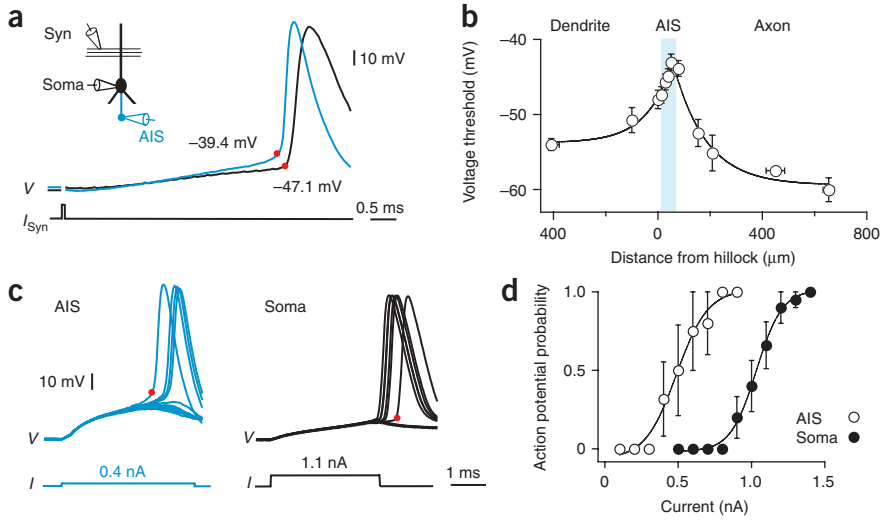

Figure 1 Action potential voltage threshold appears highest in the axon. All experiments were carried out according to guidelines approved by the Animal Ethics Committee of the Australian National University. (**a**) Action potentials recorded at the soma (black) and AIS (blue) of a rat neocortical pyramidal neuron in response to synaptic stimulation (bottom). Action potential threshold values are indicated (red dots, 50 V s^{-1}). Inset, schematic of the recording configuration showing apical dendritic synaptic stimulation (Syn) and whole-cell recording from the soma (black) and AIS (blue). (**b**) Spatial distribution of action potential voltage threshold (generated by current injection into the soma). Data were fit by two single exponentials with distance constants of 98 and 125 μm for the somato-dendritic and axonal regions, respectively. (**c**) Overlaid voltage responses (ten consecutive sweeps) during current injection (bottom) into the AIS (blue, left) or soma (black, right) at action potential threshold. Note the depolarized voltage threshold in the AIS compared with the soma (–34.2 versus –47.3 mV, respectively; red dots). (**d**) Average amplitude of injected current versus action potential probability for action potentials evoked by current injection in the AIS (open circles, $n = 5$) or soma (closed circles, $n = 10$). Data were fit with a sigmoid function. Note the lower current threshold for action potential generation during current injection into AIS ($P < 0.02$). Data are shown as mean ± s.e.m.

prior to action potential generation attenuates as it propagates to more distal sites, elevating the voltage threshold in the axon relative to the soma. As a consequence, action potential threshold measured at the soma using conventional techniques appears lower (more hyperpolarized) than in the axon.

So what is the voltage threshold at the soma? To determine this, we examined the properties of the somato-dendritic spike in isolation. Recordings were made from pyramidal neurons with clear termination of the axon at the distal AIS (~45 μm) and action potentials evoked by somatic current injection (**Fig. 3a**). Taking the first time derivative (dV/dt) of the somatic voltage allowed us to temporally separate the AIS and somato-dendritic spikes (**Fig. 3b**)[3]. The first inflection point reflects invasion of the soma by the AIS spike, whereas the second inflection is thought to coincide with the onset of the somato-dendritic spike[3]. To test whether the second inflection point reliably reflects somato-dendritic spike threshold, we isolated the somato-dendritic spike by blocking the AIS spike using local TTX application (**Fig. 3c**). Three observations confirmed the local block of axonal Na^+ channels. First, action potential onset shifted to the somatic region during block of Na^+ channels in the AIS (AIS to soma latency difference in control, –64 ± 10 μs; TTX, +57 ± 12 μs; $P < 0.01$, $n = 4$; **Fig. 3c**). Second, the first time derivative of the somatic voltage showed only a single small amplitude peak (**Fig. 3d**). Third, current threshold measured at the soma increased substantially from, on average, 1.0 ± 0.1 nA in control to 5.3 ± 0.7 nA after local TTX application in the AIS ($P < 0.001$, $n = 6$). Voltage threshold of the isolated somato-dendritic spike was, on average, –20.0 ± 1.9 mV ($n = 4$; **Fig. 3d**), which is essentially identical to the membrane potential reached at the second inflection point in the

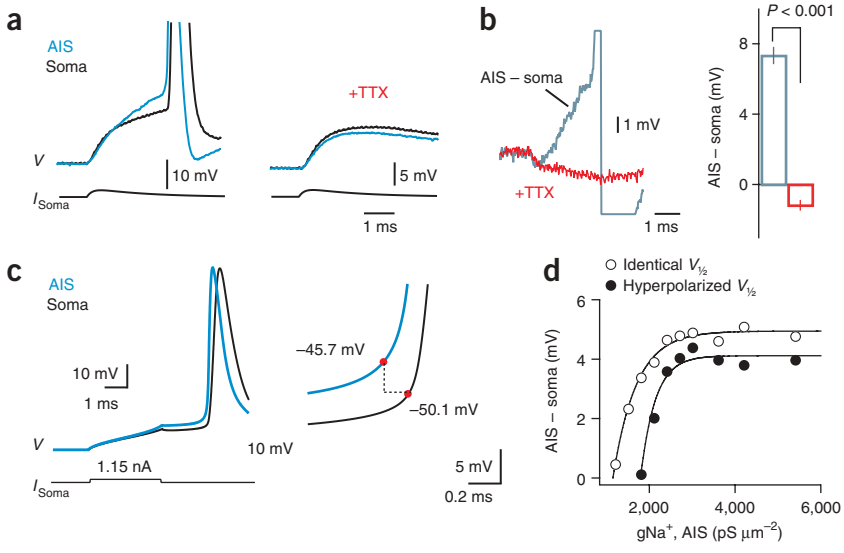

Figure 2 Role of AIS Na^+ channels in setting action potential voltage threshold. (**a**) Left, action potentials generated by simulated excitatory postsynaptic potential current injection at the soma and recorded at the soma (black) and AIS (blue). Right, same recording in the presence of TTX (1 μM). (**b**) Left, voltage difference (AIS – soma) in control (gray) and TTX (red), revealing the depolarizing ramp in the AIS before action potential initiation. Action potentials were truncated. Right, bar plot of the maximal potential difference before action potential threshold in control (gray) and at the same time point in TTX (red, $n = 5$). (**c**) Action potentials evoked by somatic current injection in a model of a layer 5 pyramidal neuron. Na^+ channel density in this model was 3,000 pS $μm^{-2}$ in the AIS. Right, expanded voltage traces with action potential threshold indicated (red dots, 50 V s^{-1}) for the AIS (blue) and soma (black). (**d**) Action potential threshold difference (AIS – soma) plotted versus the density of Na^+ channels in the AIS in models with identical Na^+ channel voltage dependence at all locations (open circles, identical $V_{1/2}$) compared with models with the observed more hyperpolarized voltage-dependence of Na^+ channels in the axon (closed circles, hyperpolarized $V_{1/2}$). Data fit with single exponential functions.

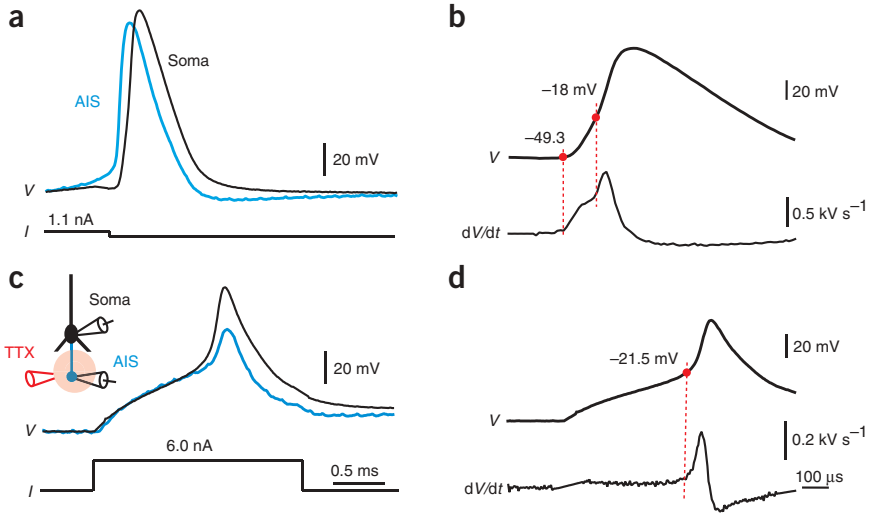

Figure 3 Identification of somato-dendritic action potential threshold. (**a**) Action potentials recorded at the soma (black) and AIS (blue) at the end of a somatic current injection (lower trace). (**b**) Somatic voltage (top) and differentiated voltage (d*V*/d*t*, bottom). Dotted lines indicate the two phases in the somatic voltage arising from the AIS and somato-dendritic spike and occurring at absolute membrane potentials of –49.3 and –18.0 mV, respectively. (**c**) Voltage response at the soma (black) and AIS (blue) in response to suprathreshold current injection following local application of TTX to the AIS. Note the increased current threshold for action potential generation (6.0 nA, bottom). Inset, schematic of the recording configuration. (**d**) Somatic voltage (top) and differentiated voltage (d*V*/d*t*, bottom). Dotted lines indicate the onset of the isolated somato-dendritic spike at an absolute membrane potential of –21.5 mV.

d*V*/d*t* of the somatic action potential under control conditions (–19.9 ± 1.0 mV, $n = 11$, $P > 0.5$; **Fig. 3b**). A similar ~20 mV difference in action potential threshold for the somato-dendritic spike compared to the AIS spike was observed in the model (**Supplementary Fig. 2** online).

In summary, we have directly investigated the mechanisms underlying action potential voltage and current threshold in cortical pyramidal neurons. We found that the same Na^+ channels in the AIS that underlie action potential generation drive a local depolarizing ramp just before action potential initiation. This voltage attenuates as it propagates from the site of action potential initiation to more distal recording sites, such as the soma, giving the impression that the voltage threshold is higher in the axon when measured using conventional techniques. In contrast, isolation of the somato-dendritic spike revealed that the voltage threshold at the soma is ~20 mV more depolarized than that of the action potential in the AIS. These data provide direct experimental evidence that voltage threshold is indeed lowest in the axon. Furthermore, consistent with recent estimates of somatic action potential threshold variability[15], these data show that estimations of action potential voltage threshold are only accurate when measured at the site of initiation.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

M.H.P.K. carried out, analyzed and designed the experiments. G.J.S. provided support and helped with planning the experiments. M.H.P.K. and G.J.S. wrote the manuscript.

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