Properties of the axial current of retinal ganglion cells at spike initiation

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Abstract

The action potential of most vertebrate neurons initiates in the axon initial segment (AIS), and is then transmitted to the soma where it is regenerated by somatodendritic sodium channels. For successful transmission, the AIS must produce a strong axial current, so as to depolarize the soma to the threshold for somatic regeneration. Theoretically, this axial current depends on AIS geometry and Na^+ conductance density. We measured the axial current of mouse RGCs using whole-cell recordings with post-hoc AIS labeling. We found that this current is large, implying high Na^+ conductance density, and carries a charge that co-varies with capacitance so as to depolarize the soma by ~30 mV. Additionally, we observed that the axial current attenuates strongly with depolarization, consistent with sodium channel inactivation, but temporally broadens so as to preserve the transmitted charge. Thus, the AIS appears to be organized so as to reliably backpropagate the axonal action potential.

Introduction

In most vertebrate neurons, action potentials (APs) initiate in the axon initial segment (AIS), a highly organized structure near the soma (Bender and Trussell, 2012), then propagate forward to the axon terminals and backward to the soma and dendrites (Debanne et al., 2011). This backward transmission is functionally important for synaptic plasticity, which requires a precisely timed signal of firing activity at the synapse (Caporale and Dan, 2008). It is also important for long-term intrinsic plasticity since the soma holds the genetic material (Daoudal and Debanne, 2003), and presumably also for structural plasticity of the AIS, which depends on somatic voltage-gated calcium channels (Evans et al., 2013).

At spike initiation, the soma receives an axial current from the AIS, which depolarizes the membrane. When the somatic membrane potential is depolarized about 30 mV above firing threshold, the AP is regenerated by somatic sodium channels (Kole and Stuart, 2008). Hamada et al. (2016) found indirect evidence that the axial current matches the capacitance of the somatodendritic compartment, as they observed that larger cortical pyramidal cells tend to have a more proximal AIS, which should theoretically produce a stronger current. However, the axial current was not directly measured.

Such a measurement could also allow estimating the conductance density of AIS sodium channels, in particular using resistive coupling theory (Brette, 2013; Kole and Brette, 2018). The fact that the AIS, a small structure, must produce a current able to charge a much larger piece of membrane (soma and proximal dendrites), suggests that conductance density is high, in agreement with immunochemical observations (Lorincz and Nusser, 2010). However, this has remained a somewhat contentious issue (Fleidervish et al., 2010) because direct patch-clamp measurements in the intact AIS indicate low conductance density (Colbert and Pan, 2002), which could be an artifact due to the anchoring of channels to the cytoskeleton (Kole et al., 2008).

Finally, it is known that sodium channels can inactivate substantially below threshold, resulting in spike threshold adaptation (Azouz and Gray, 2000; Fontaine et al., 2014; Platkiewicz and Brette, 2011). This suggests that the axial current at spike initiation may also vary substantially. If this is the case, then how can spikes be reliably transmitted to the soma?

To address these questions, we measured the axial current and spontaneous action potentials in ganglion cells of isolated mouse retina (n = 20) followed by ankyrin-G-antibody labeling to measure AIS geometry. We examined the axial current at spike initiation, just below threshold, and with threshold adaptation, and compared these results to theoretical predictions.

Results

The axial current at spike initiation

Action potentials of retinal ganglion cells

Early work on vertebrate motoneurons showed that APs recorded in the soma typically consist of two components (Coombs et al., 1957; Fatt, 1957): an abrupt depolarization due to the axial current originating from the axon initial segment, followed by a regeneration of the AP at a higher potential, by the opening of somatic sodium channels. These two components are clearly distinguished in recordings of spontaneous APs of retinal ganglion cells (RGC) (Fig. 1). Figure 1A illustrates a typical AP, rising abruptly from threshold. The voltage derivative dV/dt shows two distinct components (Fig. 1B), which appear most clearly when plotted against the membrane potential V (Fig. 1C). We define

the regeneration threshold as the potential when the acceleration d^2V/dt^2 is maximal (Fig. 1D). In n = 10 cells with a stable reference potential, we observed that the spike threshold of spontaneous APs was -49 ± 3.8 mV (s.d.) while the regeneration threshold is -16 ± 4.6 mV (s.d.) (Fig. 1F), about 33 ± 5 mV (s.d.) higher (Fig. 1F). This is similar to previous measurements in layer 5 cortical pyramidal cells (Kole and Stuart, 2008).

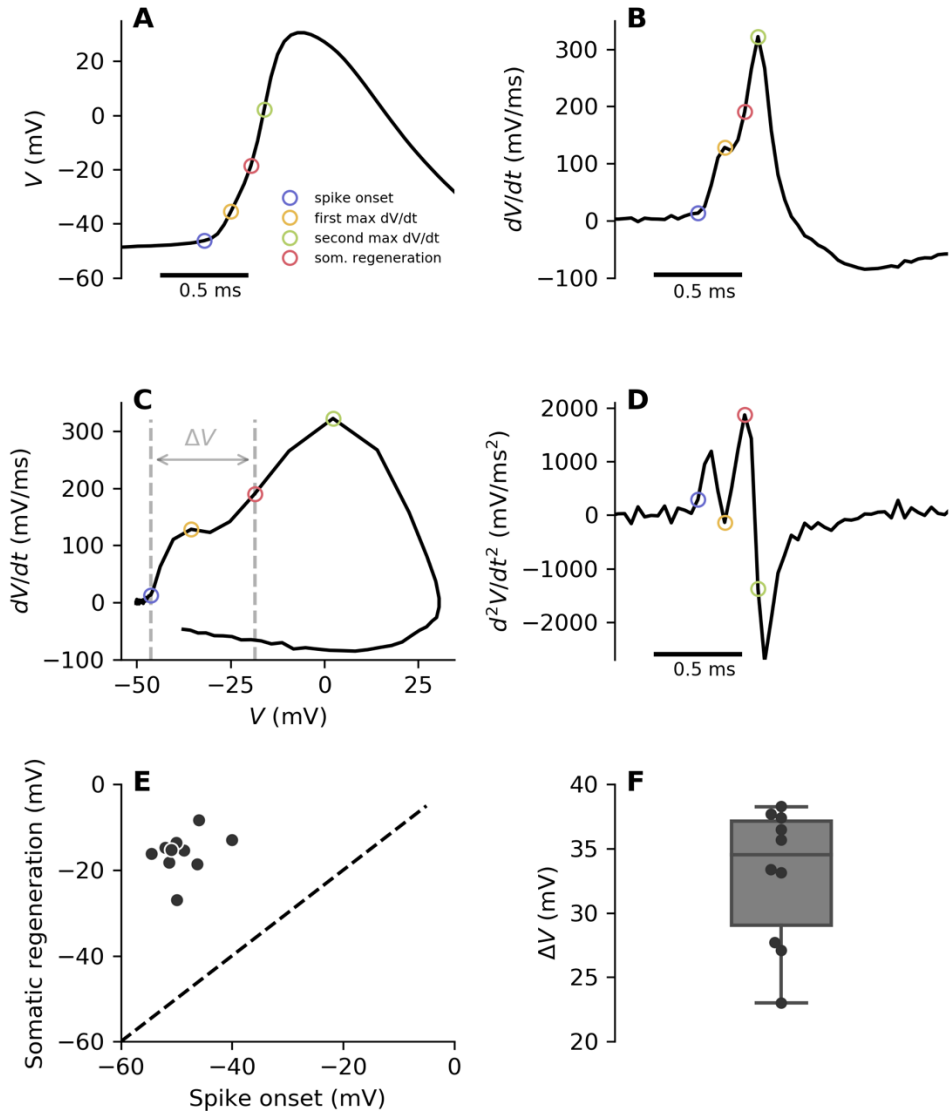

Figure 1. *Spontaneous APs of retinal ganglion cells consist of two components. A, Spontaneous AP of a RGC, highlighting spike onset (blue), point of maximum dV/dt of the first component (yellow), somatic regeneration threshold (red), point of maximum dV/dt of the second component (green). B, Time derivative of the AP trace shown in A. C, Phase plot showing dV/dt vs. V. The double arrow shows the difference ΔV between spike onset and regeneration threshold. D, Second derivative of the AP trace. Somatic regeneration threshold is defined as the point of maximum acceleration. E, Somatic regeneration threshold vs. spike onset over all measured APs (n = 10). F, Statistics of. F, Statistics of ΔV.*

Measuring the axial current

82 We measured the axial current with whole-cell voltage clamp by stepping the command potential from
83 V_0 = -60 mV to a variable potential *V* (Fig. 2). Voltage steps above a threshold value evoke large spikes
84 of inward current (Fig. 2A). When the peak current is plotted against voltage, a sharp discontinuity is
85 seen (Fig. 2B). Similar recordings have been reported in several cell types in whole-cell patch (Diwakar
86 et al., 2009; Magistretti et al., 2006; Milescu et al., 2010), and also in two-electrode voltage clamp
87 recordings of cat motoneurons (Barrett and Crill, 1980). As argued by Milescu et al. (2010), this abrupt
88 increase in current most likely reflects the axial current produced by the AIS AP. Indeed, the current-
89 voltage curve shows a plateau reflecting the all-or-none axonal spike, followed by an increase at higher
90 potential, most likely reflecting the somatic sodium current. These currents were eliminated by 1 μM
91 tetrodotoxin, a potent sodium channel blocker (2 ±0.4 % current remaining, n = 4 cells, paired t_3 = 4.5,
92 p = 0.02).

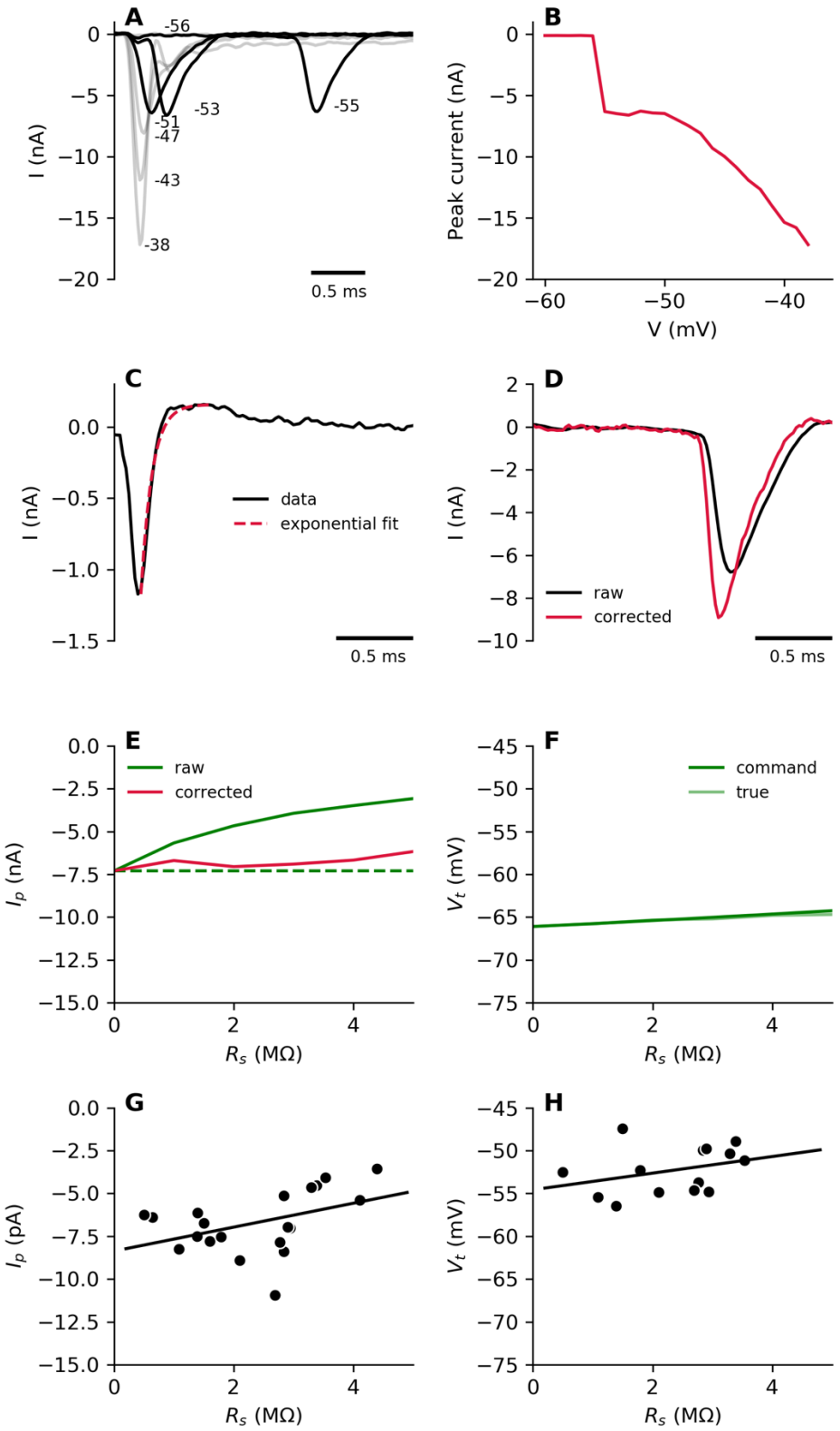

Figure 2. *Measuring the axial current. A, Raw current recordings (corrected for leak current but not series resistance) in response to voltage steps from -60 mV to different target potentials (-55 mV to -38 mV). B, Peak current vs. step command potential. The peak current increases abruptly when the potential exceeds some threshold. C, Passive response to a +5 mV voltage step. An exponential function is fitted on the first 0.5 ms, starting from the peak, to estimate the decay time constant (dashed red). D, A recorded axial current (black) is corrected by defiltering (red), with residual* R_s *= 2.1 MΩ and* τ *= 70 µs. E, Peak current correction in a*

biophysical model with an extended AIS (5 to 30 µm from the soma), as a function of series resistance R_s. Dashed line: peak current in the ideal situation with no series resistance. F, Change in measured and actual voltage threshold as a function of R_s in a biophysical model. G, Corrected peak axial current at spike initiation vs. series resistance in RGCs (n = 20), showing a weak correlation (Pearson correlation r =0.43, p = 0.06). H, Corrected voltage threshold vs. R_s in RGCs (n = 14; 6 cells excluded because the reference potential drifted), with the regression line (p = 0.25, Pearson test).

Accurate measurement of the axial current is complicated by the presence of the series resistance R_s. Together with the membrane capacitance C, the series resistance forms a RC circuit that low-pass filters the current with a characteristic time constant $\tau = R_s C$. Thus, part of the axial current is lost as a capacitive current that charges the somatic capacitance. This capacitive current should equal $-\tau\, dI/dt$. We correct the recorded current by subtracting this current, as described by Traynelis (1998), with the time constant directly estimated from a passive response to a small voltage step (Fig. 2C) (see Methods). Figure 2D shows a recording from a retinal ganglion cell (black). After correction, the peak current is larger (red).

We tested the effect of series resistance and the correction in a simple biophysical model of a RGC with an extended AIS, with the electrode modeled as a resistance (see Methods). Figure 2E shows that the peak recorded current decreases substantially when increasing R_s, but this error is well corrected by the method described above. The spike threshold is only marginally affected by the series resistance (Fig. 2F; the exact value of the threshold depends on model parameters). We selected cells with residual series resistance smaller than 5 MΩ (n = 20). There was a correlation between measured peak axial current I_p and R_s (Fig. 2G, Pearson correlation r = 0.43, p = 0.06), but it remained small. We observed no correlation between spike threshold and R_s (Fig. 2H, p = 0.25, Pearson test). Therefore, the impact of R_s on our measurements should be moderate.

Transmission of the axial current to the soma

When the soma is not voltage-clamped, the axial current at spike initiation charges the somatic capacitance (and proximal dendrites). Therefore, we expect that the axial current measured in voltage clamp is approximately equal to the capacitive current $C.dV/dt$ during the initial rise of an AP recorded in current clamp (more precisely, $C.dV/dt = -I_p$, as I_p is the current from the amplifier to the cell). This equality assumes that the other currents are negligible, which is plausible given the typical amplitude of axial currents (about 6.7 ± 1.8 nA). Another caveat is that the capacitance is only well defined for an isopotential cell. We estimated the effective capacitance of cells on the first ms of the response to a small current pulse, and measured dV/dt in the initial phase of a spontaneous AP (see Methods).

We found that for most cells, the axial current measured in voltage clamp was indeed close to the capacitive current of a spontaneous AP (Fig.3A; 5 cells were excluded because spontaneous APs or capacitance were not measured). Two cells had a substantially larger capacitive current than expected. As dV/dt was also high in these cells (162 and 201 V/s compared to an average of 142 ± 34 V/s, s.d.), it is plausible that AIS Na^+ channels were less inactivated in these spontaneous APs than in the voltage clamp measurement at -60 mV (see last section). The fact that the magnitude of the axial current is generally consistent with the depolarization observed in current clamp suggests that our axial current measurements are reasonably accurate.

From a functional viewpoint, since the axial current must charge the somatodendritic capacitance, we may expect that I_p and C are linearly correlated. Such a correlation was inferred in layer 5 pyramidal

cortical cells by Hamada et al. (2016), who observed that the placement of the AIS was consistent with the axial current being proportional to the capacitance. In our electrophysiological data, we observed a correlation between axial current and capacitance, but it was not very strong statistically (Fig. 3B; Pearson correlation r= 0.47, p = 0.06). A more robust way to assess the transmission of the AP to the soma is to examine the total charge Q transmitted to the soma, i.e., the integral of the axial current, since the somatic depolarization due to the axial current is $\Delta V = Q/C$. Technically, the measurement of Q is more reliable than that of I_p because the integrated current is not affected by the filtering issue discussed above, and because integration reduces noise.

We found a linear correlation between Q and C (Fig. 3C; Pearson correlation r = 0.56, p = 0.02), with a slope $\Delta V = 31$ mV. This is remarkably close to the difference between spike threshold and regeneration threshold we observed on spontaneous APs (33 mV; Fig. 1G). This correlation was not due to a correlation between current duration and C (current duration at 50% of the peak was t_{50} = 0.36 ± 0.05 ms, excluding one clear outlier; Fig. 3D). Thus, the transmitted charge appears to be just enough to bring the somatic potential to the voltage where somatic sodium channels open and regenerate the AP.

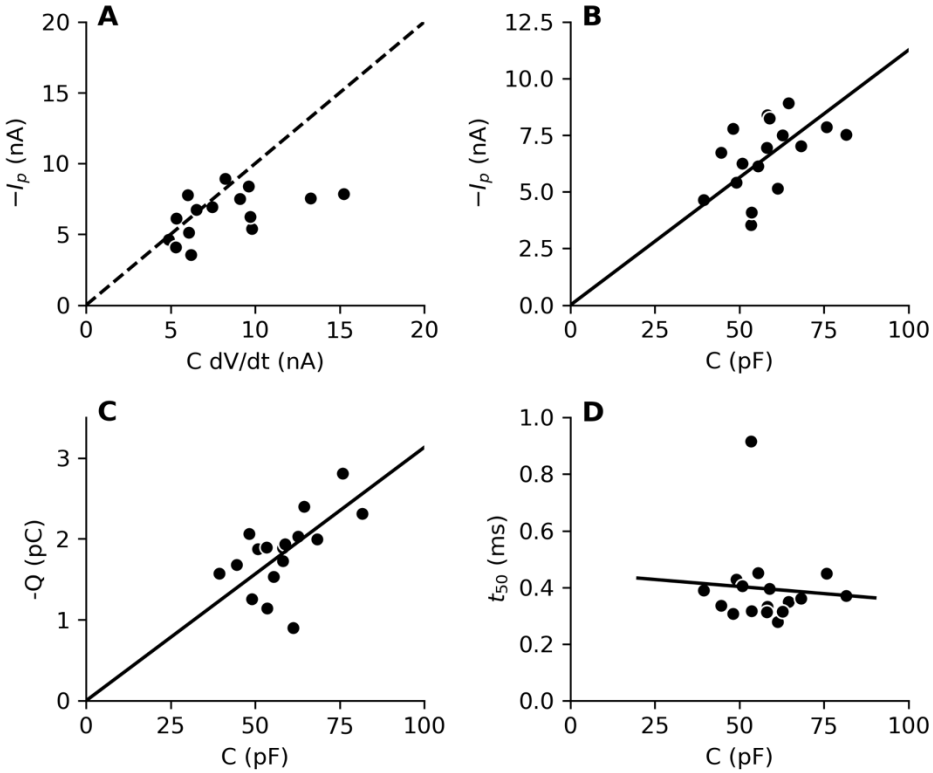

Figure 3. *Transmission of the axial current to the soma. A, Corrected peak axial current at spike initiation measured in voltage clamp vs. capacitive current measured in current clamp (n = 15). The dashed diagonal line represents the identity -I_p = CdV/dt (not a regression line). B, Peak axial current measured in voltage clamp vs. somatic capacitance measured in current clamp (n = 17). The solid line is the best linear fit (not affine; i.e., of the form $-I_p = a.C$). C, Total transmitted charge vs. somatic capacitance (n = 17). The solid line is the best linear fit, with slope 31 mV. D, Axial current duration measured at 50% of peak current, vs. capacitance, with regression line (n= 17).*

Nav conductance density

The axial current was on average I_p = -6.7 nA (s.d. 1.8 nA). From this value, we deduce a lower bound on Na^+ conductance density g_{min} in the AIS by three methods. The first method uses the fact that the axial current is not greater than the maximum current that can pass through each Na^+ channel. Thus, it produces a lower bound that depends only on axial current and AIS area. The second method consists in calculating the maximum current that a cylindrical axon with uniform conductance density can pass to the soma. Thus, it produces a lower bound that depends on axial current, axon diameter and intracellular resistivity. The third method calculates the axial current based on all biophysical parameters using resistive coupling theory. It is more precise but depends on accurate measurements of AIS geometry and intracellular resistivity.

First, the axial current cannot be greater than the maximum current that all Na^+ channels can pass. This maximum Na^+ current is $G(E_{Na} - V)$, where $E_{Na} \approx 70$ mV is the reversal potential of Na^+, G is the total Na^+ conductance and $V \approx$ -15 mV is the local membrane potential at which the current through a Na^+ channel is maximal (based on Na^+ channel properties measured at the AIS of cortical pyramidal cells (Kole et al., 2008)). Therefore, a lower bound for the total Na^+ conductance is $G_{min} = -I_p/(E_{Na} - V) \approx 79$ nS.

To estimate the corresponding minimum conductance density, we measured the geometry of the AIS of n = 14 cells by immunolabeling ankyrin-G, while identifying recorded cells using biocytin in the patch pipette (see Methods) (Fig. 4A). The AIS was on average 31 µm long (± 6 µm s.d.) and started at 8.6 ± 3.3 µm from the soma (Fig. 4B), with no statistically significant correlation between the two measurements (p = 0.59, Pearson test). We also measured the axon diameter at the proximal and distal ends of the AIS. However, it should be kept in mind that these measurements cannot be accurate because of the limitations of conventional light microscopy, and therefore must be considered as rough estimates. The proximal and distal diameters were 0.9 ± 0.3 µm and 0.5 ± 0.2 µm, respectively. For comparison, Raghuram et al. (2019) found by a similar method 1 µm and 0.6 µm on average in α S RGCs of mice, with substantial variability.

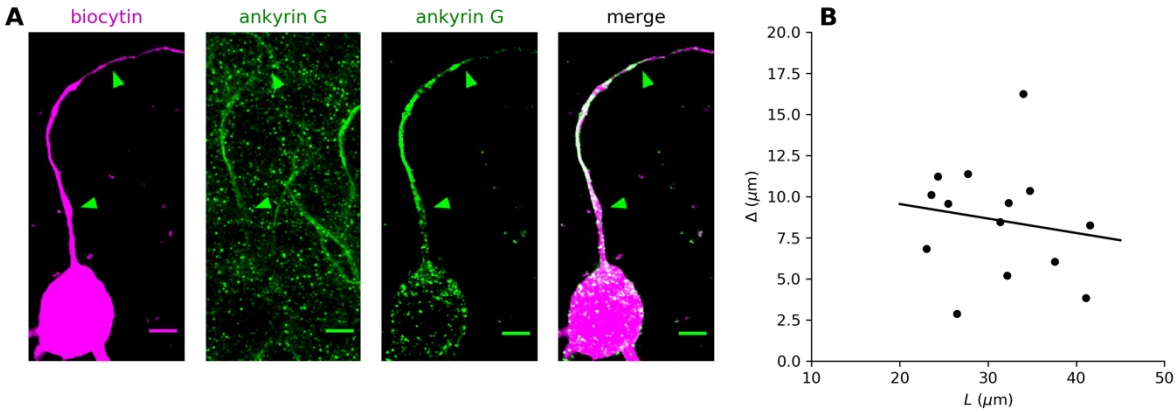

Figure 4. *Geometry of the AIS. A left, Fluorescence image of a RGC labelled with biocytin (pink). The start and end position of the AIS are indicated by green arrowheads. Note that the biocytin staining extends beyond the AIS. Scale bar is 5 µm. A middle left, Fluorescence image of AISs labeled with ankyrin-G antibodies (green). A middle right, Fluorescence image of the AIS masked by the neuron morphology. A right, Merge of the first and third panels. B, Distance Δ of the AIS from the soma vs. AIS length L, with the regression line (p = 0.59, Pearson test).*

Using the high estimate of 1 µm for the diameter, the average AIS area was therefore 97 µm² (± 19 µm² s.d.), which implies a minimum conductance density of 814 S/m². With a diameter of 0.7 µm, we find a minimum of 1159 S/m².

This lower bound holds independently of the conditions of propagation of the action potential. In particular, it makes no assumption on intracellular resistivity R_i, which is challenging to measure. We now consider a different constraint, the geometrical mismatch between the small axon and the large soma. Resistive coupling theory provides a quantitative estimate of the axial current as a function of g, by assuming that current entering the axonal membrane flows resistively towards the soma, which acts as a current sink (Brette, 2013; Goethals and Brette, 2020; Kole and Brette, 2018). We obtain an upper estimate of the axial current by considering open Na^+ channels uniformly distributed along the axon. We then deduce the minimum conductance density necessary to produce an axial current I (see Methods):

$$g_{\min} = \frac{4R_i I^2}{\pi^2 \Delta V^2 d_{AIS}^3}$$

where $\Delta V = E_{Na} - V_t$, with V_t the spike threshold ($\Delta V \approx 120$ mV). This lower bound is independent of AIS geometry, but depends on intracellular resistivity R_i. Intracellular resistivity has not been measured directly in the RGC axons. In dendrites of cortical pyramidal cells, it was estimated to be R_i = 70-100 Ω.cm (Stuart and Spruston, 1998). Modeling studies in RGCs assume somewhat higher values, up to about 150 Ω.cm (Fohlmeister et al., 2010; Sheasby and Fohlmeister, 1999), but these are based on model optimization. Taking R_i = 100 Ω.cm, we find g_{min} = 1263 S/m² for d = 1 µm and g_{min} = 2467 S/m² for d = 0.8 µm (as shown below, it is mostly the geometry of the proximal side that matters for this calculation). With a higher value for R_i, the lower bound on conductance density would be proportionally higher.

Finally, a more precise relation between axial current and conductance density can be estimated using the measured AIS geometry (Fig. 5) (Hamada et al., 2016). Suppose first that conductance density is very high, such that the AIS is clamped at E_{Na} when sodium channels open. Then by Ohm's law, the AIS will produce an axial current $I_p = (E_{Na}-V_t)/R_a$, where R_a is axial resistance between the soma and the proximal end of the AIS. Thus, we obtain an inverse relation between axial current and AIS position, independent of AIS length. However, conductance density is finite, which implies that the proximal side of the AIS is pulled towards the somatic potential (Fig. 5A). This is equivalent to shifting the AIS distally by an amount δ:

$$I_p = \frac{1}{r_a}\frac{E_{Na} - V_t}{\Delta + \delta}$$

where Δ is the distance of the AIS from the soma, r_a is the axial resistance per unit length, and

$$\delta = \sqrt{\frac{d}{4R_i g}}$$

Here, AIS length L can be neglected provided that it is substantially larger than δ (see Methods). This is clearly the case because L was 31 µm on average, while a higher estimate of δ using d = 1.2 µm and g = 1000 S/m² is 17 µm. Thus, in our cells, AIS length should have no impact on axial current. The formula above agrees well with simulations of a simplified model with non-inactivating Na^+ channels (Fig. 5B), except when the AIS is very proximal, where it gives an overestimation.

This analysis shows that it is the proximal geometry of the AIS that matters for the calculation of the axial current. Using d = 1 µm, we find that the error between the predicted and the measured current varies with g, with a broad minimum at about 5500 S/m² (Fig. 5C). This is close to the value that Guo et al. (2013) obtained by model optimization on current-clamp recordings (5000 S/m²). Figure 5D shows the axial current measured in our cells as a function of AIS position, together with the theoretical

predictions using g = 5500 S/m² with diameters d = 0.8 µm, d = 1 µm and d = 1.2 µm. There is no significant correlation between axial current and AIS position (p = 0.66, Pearson test), but this may simply reflect the variability of AIS diameter, which has a strong impact on this relation.

Overall, this analysis shows that the strong axial current produced at spike initiation requires a Na^+ conductance density in the AIS of at least about 1000 S/m² using the most conservative estimates, and plausibly several thousand S/m² based on our measurements of AIS location and standard values of R_i.

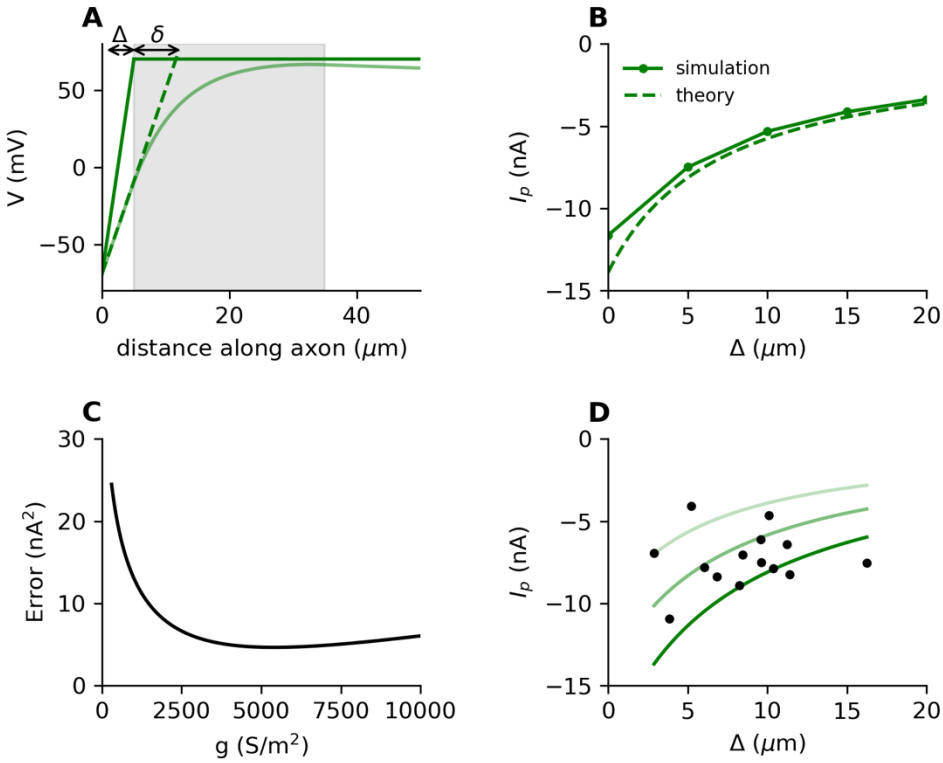

Figure 5. *Predictions of axial current with resistive coupling theory. A, Light green curve: membrane potential along the axon of a simple model when all Na^+ channels are open along the AIS, starting at distance Δ (gray shading), with a voltage clamp of the soma. Solid dark green line: idealized voltage profile for an AIS clamped at E_{Na}. The axial current produced by the model is the same as the current produced by an AIS clamped at E_{Na} and starting at distance $\Delta + \delta$ (dashed line). B, Axial current at spike initiation vs. AIS position Δ in a simple model (solid) compared to theory (dashed). C, Mean squared error between predicted and measured current, as a function of conductance density g (with d = 1 µm). D, Measured axial current vs. Δ, with the theoretical relations for d = 0.8 µm, d = 1 µm and d = 1.2 µm (using g = 5400 S/m², the minimum in C; n = 14).*

The threshold axial current

Variation of axial current near threshold

In a model where the AIS is reduced to a single point, theory predicts that spikes initiate when the sodium current, and therefore the axial current, reaches a threshold $I_t = k/R_a$, where k is the activation slope factor of sodium channels ($k \approx 5$ mV) (Brette, 2013). This makes spike initiation distal from the soma efficient because the Na^+ flux below threshold is low. We show in the Methods that the formula is approximately correct in an extended AIS model, if R_a is measured between soma and the

middle of the AIS. Thus, the threshold axial current is determined by AIS geometry. We tried to estimate I_t in our cells.

To give an order of magnitude, with d = 1 µm and given that the middle position of the AIS is 24 µm on average, we obtain $R_a \approx 31$ MΩ, which gives $I_t \approx 160$ pA (assuming k = 5 mV), a small current. Figure 6A shows a recording of the axial current at threshold, which is noisy. We measure the peak current after smoothing. In addition, theory predicts that the axial current increases very steeply near threshold (dI/dV is infinite at threshold, see Methods), as shown in Figure 6B. This makes the threshold current difficult to measure, and likely leads to an underestimation of the threshold current. We measured the current at different step voltages in steps of 0.5 mV (n = 12). In the example shown in Fig. 6C, a small but noticeable current appears at 3 mV below threshold, which increases at higher subthreshold potentials. More precisely, theory predicts that V-V_t is proportional to $(I/I_t - 1)^2$. This relation is shown in a biophysical model in Figure 6D. In a simplified model (no sodium channel inactivation or potassium channels), the slope β is predicted to be equal to $k/2$ (see Methods). This slope is found to be larger in the more realistic model shown in Figure 6D, $\beta \approx 4.7$ mV, close to k. Our data fitted this quadratic relation well (Fig. 6E), with slopes $\beta \approx 4.2$ mV (± 1.7 mV), in the expected range (Fig. 6F). Thus, the axial current increases steeply just below threshold, in agreement with theory.

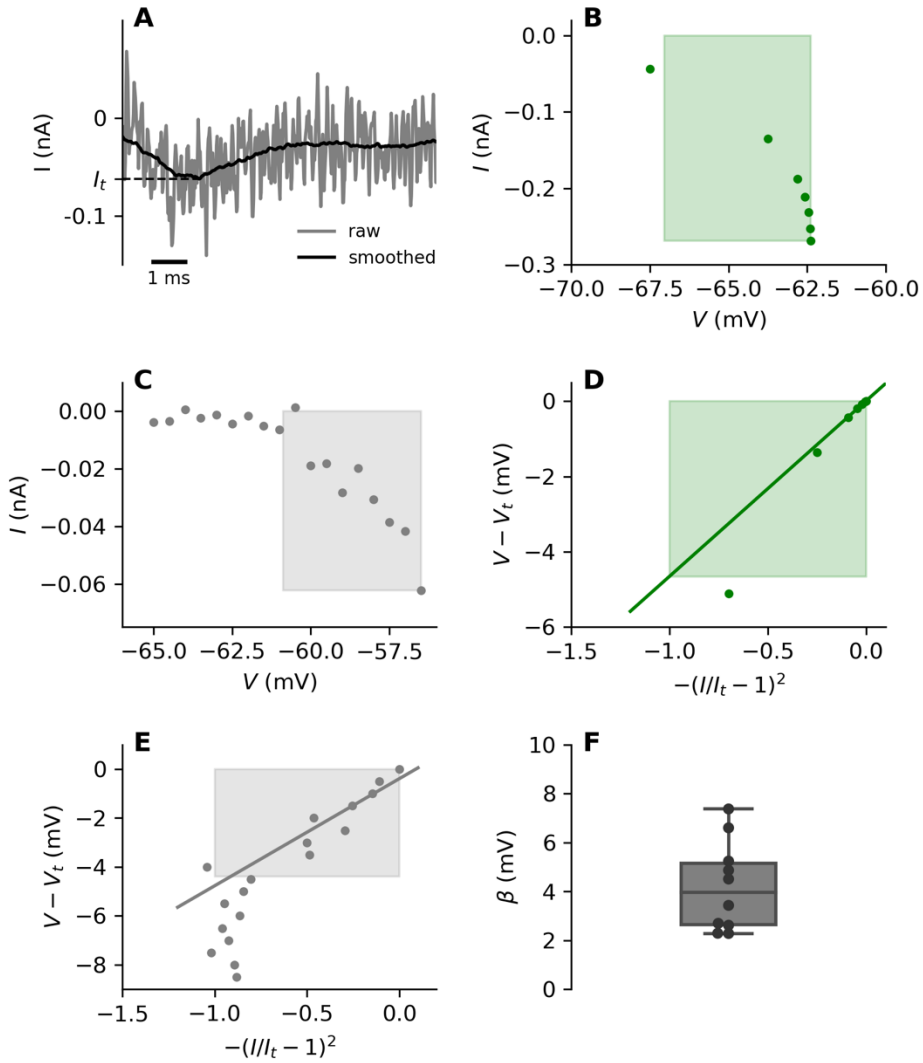

Figure 6. *Axial current near threshold. A, Current recorded at threshold (gray). After smoothing (black), I_t is the peak value. B, Peak current vs. V in a biophysical model with extended AIS. Threshold was measured by bisection for better precision. The shaded box represents the region 3 mV below threshold. C, Peak current vs. V measured below threshold in a RGC. D, Difference between membrane potential and voltage threshold vs. quadratic normalized current in the biophysical model, with the regression line (slope β = 4.7 mV). E, Same as D in the RGC (slope of regression line: β = 4.4 mV). F, Slope of linear regressions shown in E over all cells.*

Threshold vs. AIS geometry

Both the voltage and axial current at threshold are predicted to depend on AIS geometry, namely to decrease when the AIS is shifted away from the soma, all else being equal. We analyzed these relations in n = 10 cells (cells were excluded either because AIS geometry was not measured or reference potential drifted). There was no significant linear correlation in our data between voltage threshold and either AIS start position (Fig. 7A, p = 0.54, Pearson test) or length (Fig. 7B, p = 0.14, Pearson test). However, voltage threshold varies theoretically with both quantities as $-k\log(x_{1/2}L)$. The correlation was stronger with $\log(x_{1/2}L)$, although still weak (Pearson correlation r = 0.62, p = 0.06). The regression slope was k = 4.3 mV, a plausible value (Fig. 7C). We note that diameter and perhaps conductance density, which both contribute to the voltage threshold, may also vary across cells.

We observed an inverse correlation between axial current threshold and AIS position (Fig. 7D, p = 0.04, Pearson test). Theory makes a quantitative prediction: $I_t = k/R_a$, with R_a measured from the soma to the middle of the AIS. This may differ by a constant factor in a complex biophysical model (Fig. 7E, compare dashed and solid lines). Measured currents are lower than predicted and the inverse correlation is barely significant (Pearson correlation r = -0.65, p = 0.08). As explained above, underestimation and limited precision were expected. Nonetheless, the magnitude of measured currents was reasonably close to theoretical estimations (92 pA vs. 160 pA on average, with all but two cells between 70 and 150 pA).

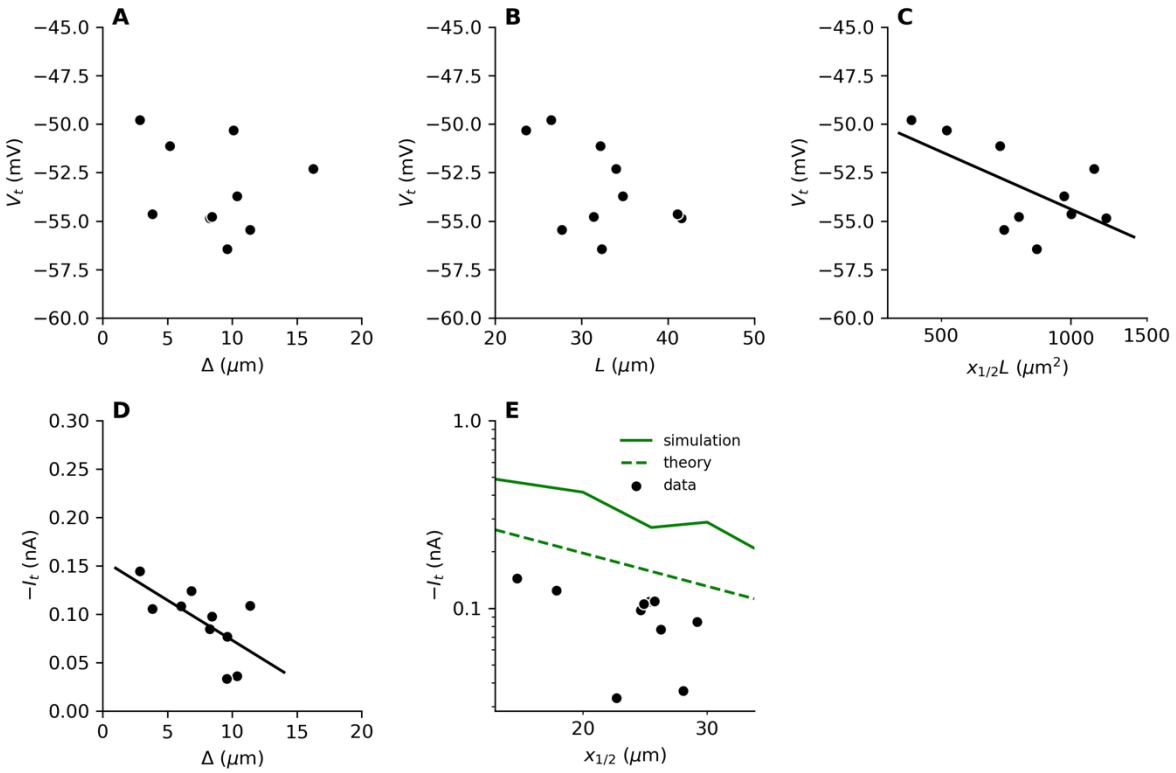

Figure 7. *Threshold vs. AIS geometry. A, Voltage threshold vs. AIS position Δ is RGCs. B, Voltage threshold vs. AIS length L. C, Voltage threshold vs $x_{1/2}L$ in logarithmic space, with logarithmic regression line (slope k = 4.3 mV; p = 0.06, Pearson test). D, Current at threshold vs. AIS position, with regression line (p = 0.04, Pearson test). E, Current at threshold vs. AIS middle position $x_{1/2}$, with theoretical prediction using d = 1 μm (dashed green) and simulation in a biophysical model (solid).*

Adaptation of the axial current

Properties of adaptation

We observed that the axial current at spike initiation has just the right magnitude to depolarize the soma to the somatic regeneration threshold. What would happen if the availability of sodium channels varied? In many neurons, sodium channels can inactivate substantially below threshold, producing voltage threshold adaptation (Azouz and Gray, 2000; Fontaine et al., 2014; Platkiewicz and Brette, 2011). Threshold adaptation has been observed in current-clamp recordings of salamander RGCs (Mitra and Miller, 2007). If this phenomenon reflects the inactivation of AIS sodium channels, it may compromise the transmission of the AIS spike to the soma.

We examined this issue by holding the neuron at different potentials V_0 before measuring the voltage threshold. We observed that the threshold increases substantially with V_0 (Fig. 8A). The relation between V_t and V_0 follows the theoretical expectation for threshold adaptation due to sodium channel inactivation (Fontaine et al., 2014; Platkiewicz and Brette, 2011), where the threshold starts increasing above the baseline V_{min} when V_0 exceeds the half-inactivation voltage V_i of sodium channels, with a slope $k_i/k_a \approx 1$ in the depolarized range (where k_i and k_a are the inactivation and activation slope factors, respectively). By fitting the theoretical relation, we find $V_i \approx -55.8 \pm 3.1$ mV (Fig. 8B), $V_i -$

$V_{min} \approx -0.7 \pm 2.9$ mV (Fig. 8C), $k_a \approx 4.1 \pm 2.2$ mV (Fig. 8D), and $k_i/k_a \approx 0.9 \pm 0.18$ (Fig. 8E). These values are consistent with expectations if threshold adaptation is due to sodium channel inactivation.

We then measured the axial current at spike initiation (just above threshold) as a function of V_0 (note that there are fewer data points because current recordings were discarded when R_s changed by more than 30%). We observed that the current decreased considerably with increasing V_0 (Fig. 8F). On average, it attenuates by a factor 12.3 ± 5.1 when V_0 increases from -60 to -40 mV (Fig. 8G). At V_i, the current is 32 ± 10 % smaller than the maximum current (Fig. 8H).

If adaptation of voltage threshold and axial current are both due to sodium channel inactivation, then axial current and voltage threshold should co-vary with V_0. Theoretically, V_t varies with available conductance g as $-k \log g$ (Platkiewicz and Brette, 2011). For low g, the axial current I_p is proportional to $\sqrt{g}$. Therefore, V_t should vary with I_p as $-k \log I_p^2$, or equivalently, $-2k \log |I_p|$.

We first note that the potential V_i^* at which the axial current is attenuated by $\sqrt{2}$ is indeed close to the half-inactivation voltage V_i estimated from threshold adaptation ($V_i^* = -56.5 \pm 1.9$ mV vs. -55.8 mV) (Fig. 8I). Then when we compare V_t with I_p, we find a logarithmic relation (Fig. 8J, K) with half-slope $k_a = 2.4 \pm 2.7$ mV (Fig. 8L). Note that this average includes one outlier; the median k_a is 3.4 mV (the smaller number of points is due to the fact that exclusion criteria for both V_t and I_p are applied). This strongly suggests that both threshold and axial current adaptation are due to the same phenomenon, sodium channel inactivation.

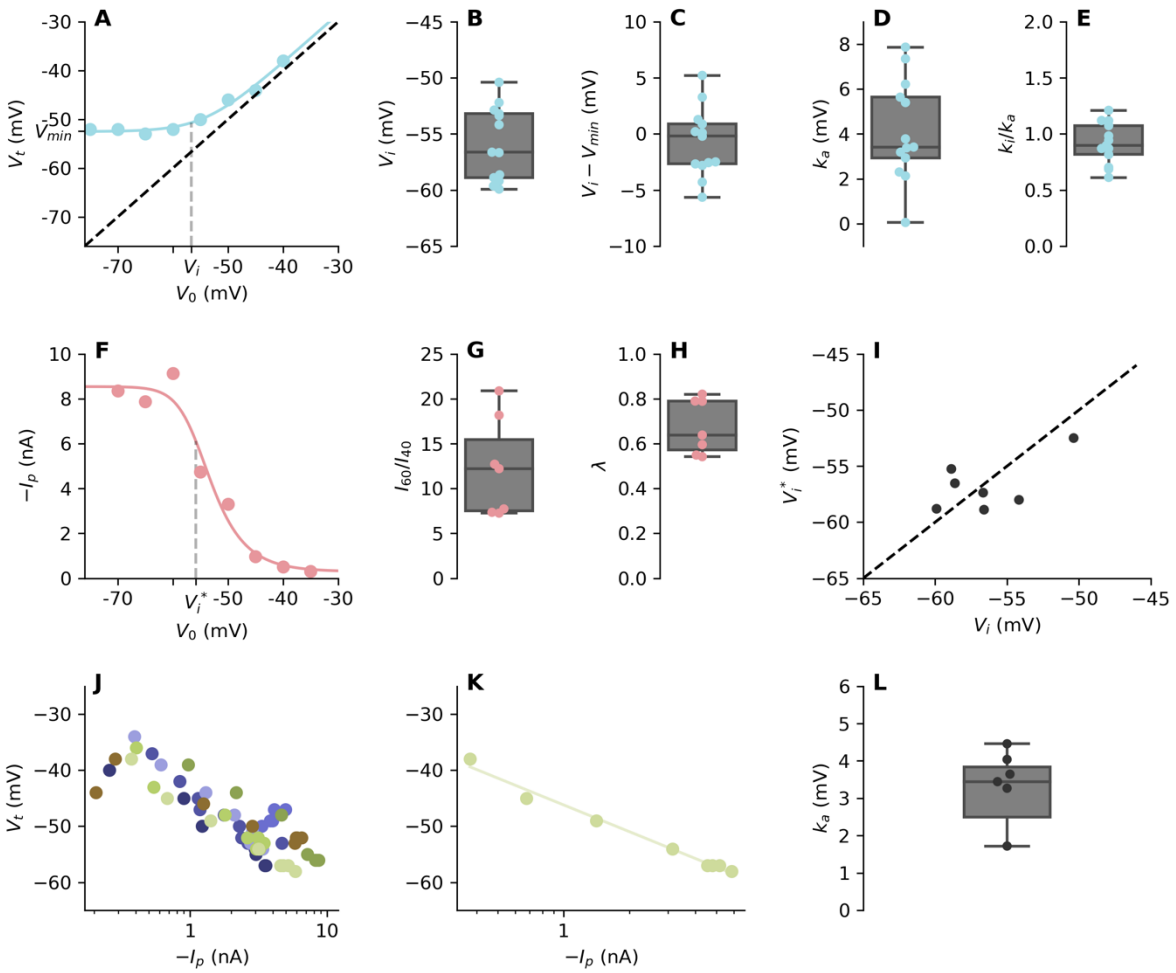

***Figure 8**. Adaptation of the axial current at spike initiation. A, Voltage threshold vs. initial holding potential V_0 in a RGC. The dashed line is the identity $V_t = V_0$, and the solid curve is a fit to the theoretical relation. B, Statistics of half-inactivation voltage V_i from theoretical fits. C, Statistics of V_i-V_{min} from fits. D, Statistics of activation slope k_a. E, Statistics of k_i/k_a. F, Axial current I_p at spike initiation vs. initial holding potential V_0 in a RGC. The dashed line shows the potential V_i* where I_p is attenuated by $\sqrt{2}$. G, Current attenuation I_{60}/I_{40} from -60 to -40 mV, over all cells. H, Current at half-inactivation voltage V_i, relative to the maximum current. I, Comparison between V_i* obtained from current attenuation and V_i obtained from voltage threshold adaptation (the diagonal line is the identity). J, Voltage threshold V_t vs. axial current in logarithmic space, over all RGCs (n = 9; each color corresponds to one cell) K, Voltage threshold V_t vs. axial current for one RGC, with logarithmic regression line (half-slope k = 3.4 mV, r = 0.99). L, Statistics of k_a from logarithmic regressions over all cells.*

While the axial current above threshold is strongly modulated by the available Na^+ conductance, resistive coupling theory predicts that the threshold axial current depends on AIS geometry but not on sodium conductance ($I_t = k/R_a$). Figure 9A shows current-voltage relations for different V_0 in the same cell. The curves appear to shift horizontally when V_0 is changed, so that the voltage threshold increases with V_0 but the axial current at threshold does not, as shown specifically on Figure 9B. Over all measured cells (n = 6; voltage threshold and current threshold were only measurable with a stable R_s in a few cells), I_t varied by a factor smaller than 2.5 (1.2 ± 0.6) between -60 and -40 mV (Fig. 9C), whereas I_p varied by a factor 12.3 on average.

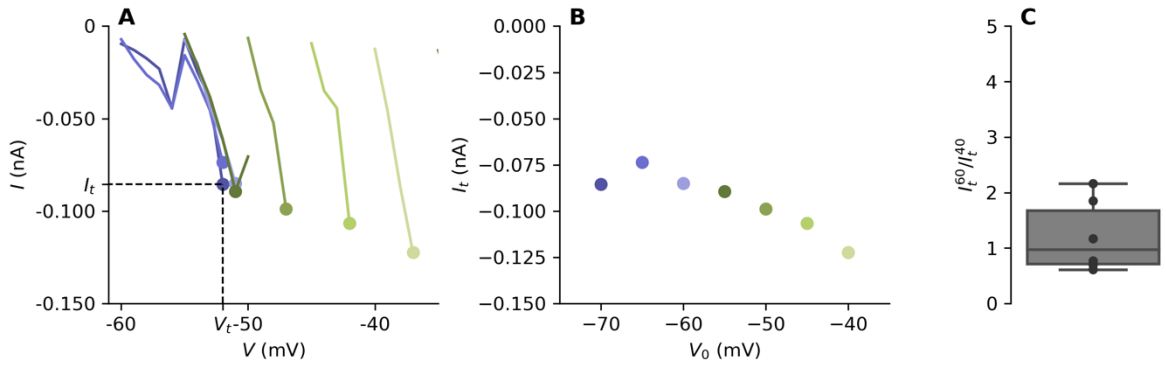

Figure 9. *Adaptation of the axial current at threshold. A, Current vs. somatic potential V a few mV below threshold, shown for different initial holding potentials V_0 (-70, dark purple to -40 mV, light green) in the same RGC. B, Current at threshold vs. V_0 in the same cell. C, Attenuation of threshold current I_t^{60}/I_t^{40}.*

Compensation of axial current attenuation

Figure 10A shows the attenuation of I_p as a function of V_0 in one cell. Here I_p attenuates by a factor 7.3 between -60 and -40 mV. If the axial current at spike initiation attenuates by a factor 7, then we expect the induced somatic depolarization to also attenuate by a factor 7, to about 4 mV, which seems insufficient to reach the threshold for somatic regeneration. However, this is not what we found. In this cell, the total transmitted charge, obtained by integrating the current, attenuates only by a factor 1.7 (Fig. 10B). This occurs because current duration increases at high V_0 (Fig. 10C). Over all measured cells (n = 7), transmitted charge attenuated by a factor 3.1 $\pm$ 1.4 from -60 to -40 mV, compared to 12.3 $\pm$ 5.1 for the axial current (Fig. 10D). The increase in current duration was observed consistently above -50 mV (Fig. 10E).

Indeed, we could occasionally observe spontaneous bursts on a top of a depolarizing wave, with APs triggered at potentials up to -40 mV, with no sign of transmission failure. An example is shown in Figure 10F, with phase plots from the first 31 APs shown in Figure 10G (only one in 3 APs is plotted for readability). During this burst, spike onset increased up to about -40 mV while the somatic regeneration threshold was stable (Fig. 10H; for the APs initiated at the highest potentials, the recordings were too noisy for accurate measurement of the regeneration threshold - by eye, between -30 and -20 mV).

Thus, detailed properties of the axial current appear to be such as to ensure reliable AP transmission to the soma in changing conditions.

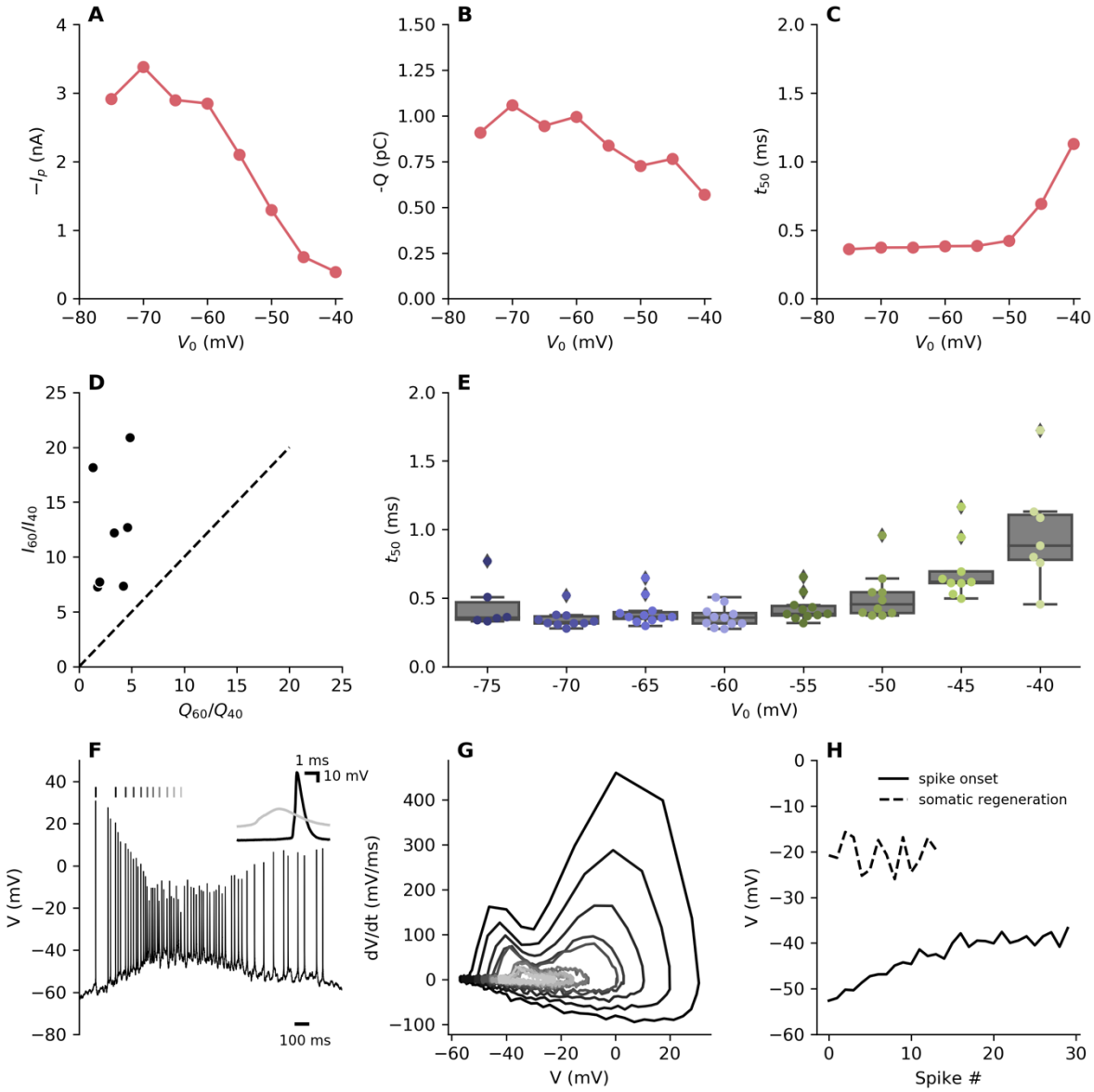

Figure 10. *Compensation of axial current attenuation. A, Axial current at spike initiation vs. initial potential V_0 in a RGC. B, Total transmitted charge Q vs. V_0 in the same cell. C, Current duration t_{50} vs. V_0 in the same cell. D, Current attenuation vs. charge attenuation between -60 and -40 mV, over all cells. E, Current duration t_{50} vs. V_0 in all cells. F, Spontaneous burst of APs in a RGC. The inset shows the first AP of the burst (black) and the smallest AP (gray). G, Phase plot of action potentials marked in F. H, Spike onset and somatic regeneration threshold of the successive APs in F. Somatic regeneration threshold could only be accurately measured for the first 12 spikes because of noise.*

Discussion

Summary

In summary, we have observed that the AIS of RGCs produces a large axial current at spike initiation (about 7 nA), which requires a high Na^+ conductance density (most likely several thousand S/m^2). The charge that this current transmits to the soma co-varies with somatic capacitance, in such a way as to

410 produce a depolarization of about 30 mV, the amount necessary to bring the somatic potential to spike
411 regeneration threshold. Theory shows that the axial current is mainly determined by AIS position and
412 diameter, and to some extent by Na^+ conductance density, but perhaps counter-intuitively not by AIS
413 length.

414 In agreement with resistive coupling theory (Brette, 2013; Kole and Brette, 2018), the axial current is
415 small below threshold (on the order of 100 pA at threshold, and undetectable a few mV below) and
416 decreases when the AIS is further away from the soma, which reduces energy consumption.

417 We have also observed that the voltage threshold for spike initiation adapts to depolarization, in a way
418 compatible with Na^+ channel inactivation. Consistently, the axial current at spike initiation also
419 decreases when the threshold adapts. This attenuation can reach a factor of 10 or more for large
420 depolarizations, which could potentially compromise spike transmission to the soma. However, we
421 found that this attenuation is compensated by a broadening of the axial current.

422 Overall, our results are in good agreement with predictions of resistive coupling theory. The inferred
423 Na^+ channel activation slope factor, which was found consistently to be $k \approx 4$ mV in several distinct
424 data sets, may seem to be on the low end of Boltzmann fits to patch-clamp recordings, typically 4-8 mV
425 (Angelino and Brenner, 2007). However, this is likely because this parameter is generally obtained
426 from fits on a broad voltage range, while an exponential fit around the spike initiation voltage yields
427 lower values (Platkiewicz and Brette, 2010, Fig. 10). For example, Hodgkin and Huxley (1952) found
428 that the Na^+ current-voltage curve of the squid axon was well fitted by an exponential of slope 4 mV;
429 Baranauskas and Martina (2006) also noted that in cortical pyramidal cells, the slope was lower when
430 estimated around spike threshold than on a broader range (5.4 mV vs. 6.4 mV).

431

432 **Limitations**

433 One of the main technical limitations to interpret the results of this study is that axonal diameter d
434 cannot be measured precisely with conventional optical microscopy. This is an important limitation
435 because theory shows that key properties are very sensitive to diameter. Specifically, axial resistance
436 is inversely proportional to d^2. This results in an error in resistance estimation of around 50% for a
437 200 nm error in axon diameter estimation (assuming $d \approx 1$ µm). This translates to comparable errors
438 in axial current predictions. This limitation should also be kept in mind when interpreting other
439 studies where changes in AIS geometry are observed (see below). The best way to overcome this
440 limitation would be to measure axonal diameter precisely using either electron microscopy or super-
441 resolution microscopy.

442 Axial resistance is proportional to intracellular resistivity R_i, but this parameter is difficult to estimate.
443 Ideally, it should be measured by simultaneous recordings in the axon and soma, and a precise estimate
444 requires a precise measurement of axon diameter. Stuart and Spruston (1998) estimated R_i = 70-100
445 Ω.cm in dendrites of cortical pyramidal cells, based on simultaneous recordings in soma and apical
446 dendrite. Although this value is mainly determined by the concentration of the most mobile ions (i.e.,
447 mainly K^+), which is not expected to vary widely across the cell, it is conceivable that it is higher in thin
448 crowded structures such as the proximal axon. Higher values, up to 150 Ω.cm, have been used in
449 modeling studies of RGCs (Fohlmeister et al., 2010; Sheasby and Fohlmeister, 1999), but these are
450 based on model optimization using somatic recordings.

451 In the theory, we did not take into account axonal tapering. However, in RGCs, axon diameter decreases
452 from soma to AIS, then decreases again along the AIS (Raghuram et al., 2019). The theory assumes a

uniform diameter, because general analytical solutions do not exist with variable diameter. For the calculation of the axial current at spike initiation, taking into account tapering would tend to reduce the axial resistance between soma and AIS, as if the AIS were closer to the soma. The diameter in the calculation of δ should be the diameter of the proximal AIS.

We were not able to confirm the inverse relation between AIS position and axial current at spike initiation that theory predicts. A plausible reason is that the axial current is very sensitive to diameter, which might have varied substantially across cells. It is also possible that the available Na^+ conductance density varied across cells. Indeed, our data on adaptation show that half-inactivation voltage (about -57 mV) is close to the initial potential used in our voltage clamp measurements of axial current (-60 mV). Another potential source of variability is that we have studied mice at an age where there are developmental changes in the expression of Na^+ channel subtypes (Boiko et al., 2003; Van Wart et al., 2007), which might have contributed some variability. Finally, series resistance introduces errors that may have been incompletely corrected offline. This issue could be addressed by measuring currents with two electrode voltage-clamp (Barrett and Crill, 1980).

Na^+ conductance density

Whether the AIS has high Na^+ conductance density has been controversial (Colbert and Pan, 2002; Fleidervish et al., 2010; Kole et al., 2008). This question has been typically considered from the viewpoint of excitability: high conductance density has been proposed to account for the fact that the AIS spike initiates about 30 mV below the somatic regeneration threshold. Other contributing factors are the lower activation threshold of axonal sodium channels (Hu et al., 2009; Kole and Stuart, 2008) and the axial resistance between soma and AIS (Brette, 2013). Here we examined another empirical constraint on Na^+ conductance density, the axial current that the AIS generates at spike initiation.

By just considering the area of the AIS, to produce a current of 6.7 nA requires a conductance density of about 1150 S/m^2 with d = 0.7 µm (average diameter across the AIS in our data) or 800 S/m^2 with an upper estimate of d = 1 µm. This is a lower bound that neglects considerations of cable theory, namely the fact that the axial current flows from the distal end of the AIS to the soma.

It is possible to calculate the maximum axial current produced by an axon of diameter d and conductance density g. This calculation shows that, to account for a current of 6.7 nA, g must be at least 1200 S/m^2 if d = 1 µm, and about 2500 S/m^2 if d = 0.8 µm, independently of AIS position. Here the relevant diameter is the diameter of the proximal AIS (about 0.9 µm in our data). Finally, taking into account measured AIS position, the data are consistent with g around 5000 S/m^2 (with d = 1 µm), although the minimum is broad. Overall, this analysis indicates that g should be several thousand S/m^2. This estimate is independent of Na^+ channel kinetics, and in particular it holds even if Na^+ channels cooperate (Naundorf et al., 2006).

Lorincz and Nusser (2010) counted 187 Nav1.6 channels per µm^2 in the AIS of CA1 pyramidal cells. Assuming a unitary conductance of 10-20 pS per channel (Hille, 2001), this amounts to 1870-3740 S/m^2. However, this is an estimate of the structural density, not necessarily of the functional density. In a computational model of layer 5 pyramidal cells, a density of 2500 S/m^2 was necessary to account for the measured initial depolarization speed of somatic APs (Kole et al., 2008). Similarly, optimization of a model of RGCs for AP shape yielded a conductance density of about 5000 S/m^2 (Guo et al., 2013). Our analysis provides an estimation that is less dependent on model specifics, and confirms these previous studies.

496 The theoretical analysis indicates that a high conductance density is likely a necessary condition to
497 transmit the AIS spike to the soma in a variety of cell types, due to the drastic geometrical variation at
498 the axosomatic boundary. The minimum conductance density to produce an axial current I is
499 proportional to I^2/d_{AIS}^3. If we assume that the current must scale with the area of the soma, then the
500 minimum g is proportional to d_{soma}^4/d_{AIS}^3. This ratio appears to be approximately conserved across
501 cell types (Goethals and Brette, 2020), and therefore most neurons should face the same constraint
502 requiring a similar conductance density in the AIS.

503 It should be noted that, despite a high conductance density at the AIS, the total Na^+ influx through the
504 AIS should theoretically have the same order of magnitude as through the soma and proximal
505 dendrites, as observed (Fleidervish et al., 2010). Indeed, the total Na^+ influx at the AIS should match
506 the charge necessary to depolarize the soma by about 30 mV, while the total Na^+ influx at the soma
507 (and proximal dendrites) should account for a further depolarization of a few tens of mV (about 45 mV
508 in our cells). The AIS influx should occur preferentially in the proximal AIS, even if conductance density
509 is uniform, because the driving force of the Na^+ channel is larger there (see Fig. 5A). This has indeed
510 been observed in cortical pyramidal cells (Baranauskas et al., 2013).

511

512 **Structural tuning of the AIS**

513 In layer 5 cortical pyramidal cells, Hamada et al. (2016) observed that AIS position was inversely
514 related to the diameter of the apical dendrite. Quantitatively, this relation was consistent with a
515 proportionality relation between the axial current produced by the AIS and the somatodendritic
516 capacitance. Here we showed more directly that, in RGCs, the charge transmitted by the AIS covaries
517 with the somatodendritic capacitance, in such a way as to depolarize the soma to the threshold for
518 somatic spike regeneration.

519 Overall, our measurements are in line with quantitative predictions of resistive coupling theory.
520 However, we did not observe a correlation between AIS position and capacitance. Theoretically, the
521 structural parameters that determine the axial current are AIS position and diameter (and not AIS
522 length, at least not in the range of observed lengths). Therefore, one would expect a negative
523 correlation between AIS position and capacitance if diameter were homogeneous across cells, or at
524 least uncorrelated to capacitance. In fact, Raghuram et al. (2019) observed a positive correlation
525 between AIS position and soma size in α S RGCs. Such a correlation would be expected if AIS diameter
526 scaled with soma size. The authors did observe a positive correlation between soma size and the
527 diameter of the proximal axon (we note that observing such correlations for the AIS proper, which is
528 below 1 μm in diameter, may not be feasible). It cannot be excluded that the lack of significant inverse
529 correlation between AIS position and capacitance is due to the limited precision of our measurements,
530 especially as we did observe an inverse correlation between AIS position and threshold axial current.
531 It is possible that the availability of Na^+ channels varied across cells. In any case, we stress that axon
532 diameter is a key structural parameter in setting the axial current as well as excitability, and therefore
533 it must be considered to correctly interpret experimental results.

534 It remains that, in order to produce an axial current of appropriate magnitude from an AIS of a given
535 diameter and conductance density, the AIS must be positioned appropriately. A number of studies have
536 shown that AIS position can vary across cells (Hamada et al., 2016; Höfflin et al., 2017; Kuba et al.,
537 2006), during development (Galiano et al., 2012; Gutzmann et al., 2014; Kuba et al., 2014), and with
538 activity (Evans et al., 2015; Grubb et al., 2011; Grubb and Burrone, 2010; Jamann et al., 2020, 2017;
539 Kuba, 2012; Kuba et al., 2010). These changes have often been suggested to reflect a homeostatic
540 regulation of excitability, but theory predicts a small effect of AIS position on excitability (Goethals and

541 Brette, 2020), and a large effect on axial current. Therefore, it is conceivable that these changes reflect
542 a homeostatic regulation not of excitability *per se*, but of the axial current required to transmit the AIS
543 spike to the soma. For example, Grubb and Burrone (2010) report that when cultured hippocampal
544 neurons are depolarized with 15 mM KCl, capacitance decreases by about 10% while the AIS shifts
545 away from the soma. This distal shift is consistent a decrease in axial current required to match the
546 decrease in capacitance. In the same way, developmental changes in AIS position may also be
547 consecutive of changes in somatic diameter or dendritic area. Finally, in neurons where
548 backpropagation of the AP to the soma may be undesirable, such as some types of auditory neurons
549 (Kuba et al., 2006; Scott et al., 2007), a distal placement of the AIS may be beneficial.

550

551 **Adaptation**

552 Threshold adaptation has been observed in current-clamp recordings of salamander RGCs (Mitra and
553 Miller, 2007), as well as in many other cell types (reviewed in (Platkiewicz and Brette, 2011)). We also
554 observed it in mice RGCs and quantified it precisely. The voltage threshold starts increasing when the
555 membrane is depolarized above ≈ -56 mV, and for large depolarizations the slope of the relation
556 between potential and threshold is close to 1. That is, the threshold tracks the membrane potential so
557 as to remain a few mV above it. These observations are consistent with theoretical expectations based
558 on Na^+ channel inactivation (Platkiewicz and Brette, 2011). In layer 5 pyramidal cells, half-inactivation
559 voltage of AIS Na^+ channels is about -61 mV (Kole and Stuart, 2008), which is in line with our
560 observations.

561 To our knowledge, adaptation of the axial current had not been reported before. Our quantitative
562 analysis shows that the co-variation of axial current and threshold is consistent with AIS Na^+ channel
563 inactivation being the cause of both phenomena. The axial current attenuated by a factor 12 on average
564 over a 20 mV depolarization. This would reduce the charge transmitted to the soma by the same factor
565 if the current spike shape were unchanged, possibly compromising spike transmission to the soma.
566 However, we observed that this attenuation was largely compensated by a broadening of axial
567 currents. This means that the AP at the AIS broadens when the soma is depolarized. In fact, such
568 broadening has been observed in layer 5 pyramidal cells and attributed to the inactivation of Kv1
569 channels (Kole et al., 2007). As Kv1.2 is expressed in the distal AIS of RGCs (Van Wart et al., 2007), this
570 might explain our observations.

571 In conclusion, our observations indicate that structural and channel properties of the AIS are
572 functionally organized in such a way as to ensure reliable transmission of the AP to the soma.

573

574 ## Materials and Methods

575 All data are available at https://zenodo.org/record/4005629#.X00H9y3pP-Z. Code for both data
576 analysis and model simulations is available at https://github.com/romainbrette/AIS-geometry-and-
577 axial-current.

578 **Ethical statement**

579 Timed-pregnant Swiss and C57BL/6NRj mice were purchased from Janvier Labs (Le Genest Saint Isle,
580 France) and housed under controlled conditions (22 ± 1°C, 60 ± 10% relative humidity, 12/12h
581 light/dark cycle, food and water *ad libitum*). All animal procedures were performed in strict
582 accordance with institutional guidelines and approved by local ethics committees (C2EA-05: Comité

583 d'éthique en expérimentation animale Charles Darwin) and by European Communities Council
584 Directive 2010/63/UE.

585

586 **Whole-cell electrophysiology of RGCs**

587 Mice were taken at postnatal day 10-12 (P10-12). The pup was rapidly decapitated, the eyes were
588 removed and placed in Ringer's medium containing (in mM): 119 NaCl, 2.5 KCl, 1.0 KH_2PO_4, 11 glucose,
589 26.2 $NaHCO_3$, 2 $CaCl_2$ and 1 $MgCl_2$ (290-295 mOsm), bubbled with carbogen (95% O_2/5% CO_2). The
590 retina was dissected and fixed on filter paper over a small hole (N8895, Sigma-Aldrich) with the RGC
591 layer upwards and continuously perfused with Ringer's solution warmed to 32 degrees Celsius.

592 Thick-walled borosilicate pipettes (OD/ID of 1.5/0.87 mm; 30-0060, Harvard Apparatus) were pulled
593 on a P-1000 Flaming/Brown puller (Sutter Instruments). Pipettes were filled with intracellular
594 solution containing (in mM): 128 K-gluconate, 10 HEPES, 16 KCl, 1 EGTA, 2 Mg-ATP, 0.5 Na_2-GTP, pH
595 7.25 with KOH (275 mOsm) and 1-2 mg/mL biocytin (B4261, Sigma-Aldrich). Open tip resistance was
596 2.5-4 MΩ. Reported potentials were corrected for a liquid junction potential of -11 mV. Whole-cell
597 recordings were made with a Multiclamp 700B amplifier (Axon Instruments), filtered at 10 kHz and
598 digitized at 50 kHz using a DigiData 1440A (Axon Instruments) and Clampex 10.7 running on Windows
599 10. High-resistance patch seals (>1 GΩ) were obtained before breaking into the cell. Recordings with
600 a series resistance R_s above 25 MΩ, or with a residual R_s (after compensation) above 5 MΩ, were
601 discarded. The resting membrane potential of the cell was recorded in the first minute after breaking
602 in.

603 Passive cell properties were recorded by stepping from -70 to -80 mV in voltage-clamp mode without
604 whole-cell compensation. Series resistance was electronically compensated 80-95% with a lag of 18
605 µs. Between protocols we repeated the voltage step without compensation to monitor changes in
606 series resistance, and series resistance compensation was adjusted if necessary. Passive currents were
607 subtracted using a P/n protocol (5 steps of 5 mV) that preceded each protocol. The P/n protocol was
608 missing for a few recordings; we then subtracted the passive response using a 10 mV step.

609 Adaptation protocols started with a long adaptation step at V_0 (0.5 s, V_0 varied by steps of 5 mV)
610 followed by an activation step (resolution of 1 mV) to elicit an AIS spike. We ensured that the
611 adaptation step was long enough by varying the step duration in a few cells.

612 In current-clamp mode, bridge balance and pipette capacitance cancellation (6.2-7.1 pF) were used.
613 Hyperpolarizing current pulses were injected to measure the cell's capacitance (see
614 *Electrophysiological data analysis*). Next, for n = 16 cells, 5-20 minutes of spontaneous activity were
615 recorded to analyze spontaneous APs.

616 At the end of the experiment, the pipette was retracted to obtain an outside-out patch. Outside the
617 retina the tip was cleaned with brief, positive pressure to remove the remaining membrane patch and
618 the potential offset was noted to check for any drift in the reference potential.

619 The retina was rinsed in 0.12 M PBS, fixed for 15 minutes in 4% paraformaldehyde in 0.12 M phosphate
620 buffer, rinsed again 2 times with 0.12 M PBS and kept in 0.12 M PBS at 4 degrees Celsius for
621 immunolabeling procedure at a later stage.

622

623 **Immunohistochemistry**

624 A few days after recording, retinas were washed during 5 minutes in PBS and permeabilized overnight
625 at 4°C in 5% normal goat serum (NGS, Sigma Aldrich) and 1% Triton-X100 in PBS. Retinas were then
626 incubated with a solution of 0.1M PBS containing the mouse monoclonal anti-ankyrin-G (clone
627 N106/36; 1:250; NeuroMab; ID: AB_2749806) and 1% normal donkey serum (NDS) for 24h at 4°C.
628 Retinas were washed 4 x 15 minutes in 0.1M PBS and then incubated with a PBS solution containing
629 donkey anti-mouse Alexa Fluor 488 (1:500, Thermo Fisher; ID: AB_141607), Streptavidin Alexa Fluor
630 594 (1:500, Thermo Fisher, ref. S11227) , 5% NGS and 0.1% Triton-X100 overnight at 4°C. Retinas
631 were washed 4x15 minutes in PBS and mounted onto Superfrost™ slides and coverslipped with
632 Fluoromount. Mounted retinas were stored at 4°C until confocal imaging.

633

634 **Confocal imaging**

635 The retinas labelled for biocytin and ankyrin-G were sequentially captured with an inverted laser
636 scanning confocal microscope (FV1000, Olympus) equipped with an Argon (488nm) ion laser and laser
637 diode (555 nm), filter cubes appropriate for Alexa Fluor 488 and Alexa Fluor 594, and a 40x objective
638 (oil, NA 1.3). Acquisition settings were optimized for each cell. Z-stacks were obtained with a step size
639 of 0.5 µm. Confocal x-y resolution was 0.155 µm, except for three cells for which it was 0.207 µm.

640

641 **Electrophysiological data analysis**

642 Data were analyzed with custom Python scripts.

643 *Estimation of passive properties*

644 The raw series resistance R_s^* was measured from responses to a test pulse in voltage clamp: $R_s =$
645 $\Delta V / I_0$, where ΔV is the voltage pulse amplitude and I_0 is the amplitude of the first transient peak. The
646 residual series resistance R_s during a given recording is $R_s = R_s^* - R_{rec}\%_{comp}$ where R_{rec} is the series
647 resistance used for compensation during the experiment and $\%_{comp}$ is the amount of compensation.
648 Effective capacitance is estimated from the response to current pulses, by fitting an exponential to the
649 first ms, which is the time scale of the axial current. This estimation was done in n = 17 cells.

650

651 *Analysis of APs*

652 The first AP recorded during spontaneous activity was used to measure AP features (Fig. 1).
653 Spontaneous activity was recorded in 16 cells; 6 of them were excluded from this analysis because the
654 reference potential drifted by more than 3 mV. To compute the phase plots (dV/dt vs V), we ensured
655 that the plotted points are isochronic, by considering that dV/dt corresponds to the derivative midway
656 between two consecutive points, and interpolating the values of V at that midpoint. Spike onset was
657 defined as the potential when dV/dt crosses 20 mV/ms for the last time before the AP peak. The value
658 of dV/dt for the initial segment component was defined as the first local maximum between spike onset
659 and the global maximum of dV/dt. In a few cells, this was equal to the global maximum. The
660 regeneration threshold is defined as the potential at the point of maximal acceleration d^2V/dt^2 after
661 the initial segment component.

662

663 *Correction of series resistance error*

Axial currents were corrected using a minor adjustment of the method described by Traynelis (1998). The presence of the series resistance results in an error in clamping the somatic potential equal to -$R_s.I_e$, where I_e is the current through the electrode. This produces a capacitive current through the somatic membrane equal to $C.dV/dt = -R_sC\ dI_e/dt$, which results in filtering the axial current through a low-pass filter with time constant $\tau = R_sC$. We correct the recorded current by subtracting this capacitive current:

$$I^* = I_e + \tau \frac{dI_e}{dt}$$

where I* is the corrected current. The time constant is estimated directly by fitting an exponential to the first 0.5 ms of the response to a voltage step (with the same amplifier tunings as for subsequent recordings). We used the steps from the P/n protocol, except for a few cells with no P/n protocol, where we used a -10 mV test pulse before the axial current recording. We then correct for the loss in driving force due to imperfect clamping as in Traynelis (1998):

$$I = I^* \left(\frac{V_c - E_{Na}}{V_c - I_e R_{res} - E_{Na}} \right)$$

where V_c is the command potential. In practice, this was a minor correction.

Threshold

The voltage threshold V_t is defined as the highest command potential where no axonal spike is elicited. The membrane potential at the soma differs slightly from the command potential by $-R_{res}I_e$. As the axial current at threshold is about 100 pA, this error is smaller than 0.5 mV. 6 cells for which the reference potential drifted by more than 3 mV during the recordings were discarded from voltage threshold analyses.

As the current at threshold is small, we used only the recordings with P/n protocol (n = 15) to ensure accurate leak subtraction. Three additional cells were excluded from the analysis of threshold current because the recordings were either too noisy or with unstable baseline current. Thus, n= 12 cells were used. The current traces below threshold were smoothed with a sliding window (half-window size is 50 points, 1 ms) before peak detection (Fig. 6A). The threshold current was then measured as the largest peak current for the data points between V_t - 1mV and V_t.

Charge and current duration

The charge Q transferred to the soma at spike initiation is estimated as the integral of I_e in the time window where the current is greater than 10% of its peak value (to avoid integrating noise). Current duration t_{50} is the duration during which the current is greater than 50% of the peak value.

Adaptation

Relations between V_t and V_0 (Fig. 8) were fitted to the theoretical formula for threshold adaptation with sodium channel inactivation (Fontaine et al., 2014; Platkiewicz and Brette, 2011):

$$V_t = V_{min} + k_a \log\left(1 + \exp\left(\frac{V - V_i}{k_i}\right)\right)$$

For the current adaptation analysis, cells were discarded if R_s increased by more than 30% during the protocol. In a few cells, the threshold could not be clearly measured at -40 mV and therefore I_{40} is missing.

Morphological data analysis

Axon tracing in 3D was performed automatically with Vaa3D (Peng et al., 2010) (version 3.2 64-bit ran on MacOSX10.13.6) based on biocytin Z-stacks. The start and end position of the axon tracing was manually chosen by the researcher to include the entire AIS. Axon coordinates (x, y, z, radius) were stored in SWC files and analyzed with custom Python scripts. Coordinates were interpolated with a finer spacing corresponding to the pixel size (including in the z direction also). Interpolation was performed with B-splines using Scipy (Virtanen et al., 2020) to evaluate the spline at each pixel comprised in the axon profile, and coordinates were rounded at the nearest pixel. The ankyrin-G images were then loaded as a 3D stack to get the fluorescence intensity at the interpolated coordinates along the axon profile. The intensity profile was smoothed with a sliding mean (half-width 15 pixels). The AIS start and end position were manually defined using the normalized intensity profile, the 3D stacks and the maximal intensity projection in Fiji (Schindelin et al., 2012). Several cells for which the start or end position were considered too unclear to be determined accurately, were discarded from the analyses, so that morphological measurements were available for n = 14 cells.

Statistics

The box plots in Fig. 1, 6 and 8 display the distribution of data in the following way. The central bar shows the median. The lower and upper limit of the box shows the first and third quartiles (Q1 and Q3), respectively. The lower and upper whisker bars show Q1-1.5 IQR (interquartile range, the range between Q1 and Q3) and Q3 + 1.5 IQR, respectively. The data points outside the whiskers are outliers and indicated by diamonds marker.

Theory

Axial current at spike initiation

The axial current at spike initiation has been derived previously based on resistive coupling theory (Hamada et al., 2016):

$$I_p = \frac{E_{Na} - V_t}{r_a(\Delta + \delta)}$$

with

$$\delta = \delta' / \tanh(L/\delta')$$

$$\delta' = \sqrt{\frac{d}{4R_i g}}$$

where Δ is AIS distance from the soma, L is AIS length, d is AIS diameter, g is Na^+ conductance density, R_i is intracellular resistivity and

$$r_a = \frac{4R_i}{\pi d^2}$$

is axial resistance per unit length. The derivation makes the following assumptions: all Na^+ channels are open; channel kinetics are neglected; capacitive and leak currents are considered negligible. These assumptions all tend to overestimate the axial current, but the approximation is generally good (see Fig. 5B). When L is much greater than δ' (which was the case in our measurements), $\delta \approx \delta'$ and the axial current is essentially insensitive to L.

The maximum current across all possible AIS geometries can be calculated by setting Δ =0 and $L = \infty$ (AIS of infinite length starting from the soma):

$$I_p^{\max} = \frac{\pi}{2} d^{3/2} \sqrt{\frac{g}{R_i}} (E_{Na} - V_t)$$

Therefore, the minimum conductance density necessary to produce an axial current I is:

$$g_{\min} = \frac{4R_i I^2}{\pi^2 (E_{Na} - V_t)^2 d_{AIS}^3}$$

Axial current at threshold

In a model where the spatial extent of the AIS is neglected (all axonal Na^+ channels clustered at a single point), the axial current at threshold is k/R_a, where k is the Boltzmann activation slope of Na^+ channels and R_a is the axial resistance between soma and AIS (Brette, 2013). It is possible to calculate this current for an AIS of length L starting from the soma.

The axial current at threshold is:

$$I_t = \frac{V'(0)}{r_a}$$

where r_a is resistance per unit length. To obtain $V'(0)$, we solve the cable equation in a simple axon model where only the axial current and the Na^+ current are considered, as in (Goethals and Brette, 2020). We consider a cylindrical axon of diameter d. The AIS has length L and starts from the somatic end. It has a uniform density of Nav channels. The total Nav conductance is

$$G = g \times \pi d L$$

where g is the surface conductance density. We neglect leak and K^+ currents, Nav channel inactivation, as well as all time-varying phenomena. The cable equation then becomes:

$$\frac{d^2V}{dx^2} = -\pi d r_a g (E_{Na} - V) \mathrm{e}^{(\mathrm{V} - V_{1/2})/\mathrm{k}}$$

where $V_{1/2}$ is the half-activation voltage of Nav channels. The boundary conditions are $V(0) = V_s$ (somatic potential) and $V'(L) = 0$ (no axial current flowing towards the distal axon). In units of the AIS length L, this equation reads approximately:

$$\frac{d^2V}{d(x/L)^2} = -\pi d r_a g L^2 (E_{Na} - V_{1/2}) \mathrm{e}^{(V - V_{1/2})/k}$$

Here the driving force $(E_{Na} - V)$ has been approximated by $(E_{Na} - V_{1/2})$ as in (Brette, 2013). We now write the following change of variables:

$$U = (V - V_{1/2})/k + \log(\pi d r_a g L^2 (E_{Na} - V_{1/2}))$$

$$y = x/L$$

and we note $U' = dU/dy$. That is, voltage is in units of k and space is in units of AIS length L. The rescaled cable equation is:

$$U'' + e^U = 0$$

with the boundary conditions:

$$U(0) = U_0 = (V_s - V_{1/2})/k + \log(\pi d r_a g L^2 (E_{Na} - V_{1/2}))$$

$$U'(1) = 0$$

This equation is analytically solvable, with general solution

$$U(y) = \log\frac{c_1}{2} - 2\log\left(\cosh\left(\frac{1}{2}\sqrt{c_1(c_2 + y)^2}\right)\right)$$

From $U'(1) = 0$, it follows that $c_2 = -1$. We then obtain for the boundary condition at 0:

$$U(0) = U_0 = \log\frac{c_1}{2} - 2\log\left(\cosh\left(\frac{\sqrt{c_1}}{2}\right)\right)$$

which defines c_1 as an implicit function of U_0. We look for a bifurcation, that is, a value of U_0 when the number of solutions changes. This is obtained by setting the derivative of the right hand-side to 0, which gives:

$$\frac{\sqrt{c_1}}{2}\tanh\left(\frac{\sqrt{c_1}}{2}\right) = 1$$

The solution can be calculated: $\sqrt{c_1}/2 \approx 1.2$, giving $c_1 \approx 5.8$. We have $V'(0) = \frac{k}{L}\, U'(0)$ and

$$U'(y) = \sqrt{c_1(1 - y)}\tanh\left(\frac{\sqrt{c_1(y-1)^2}}{2}\right)$$

so $U'(0) = 2$ and we obtain:

$$I_t = \frac{2k}{r_a L}$$

A simple extrapolated formula consistent with the formulae for both point AIS away from the soma and extended AIS starting from the soma is:

$$I_t = \frac{k}{r_a x_{1/2}}$$

where $x_{1/2} = \Delta + L/2$ is the middle position of the AIS, relative to the soma. In simulations, we find that this is a good approximation (Fig. 11).

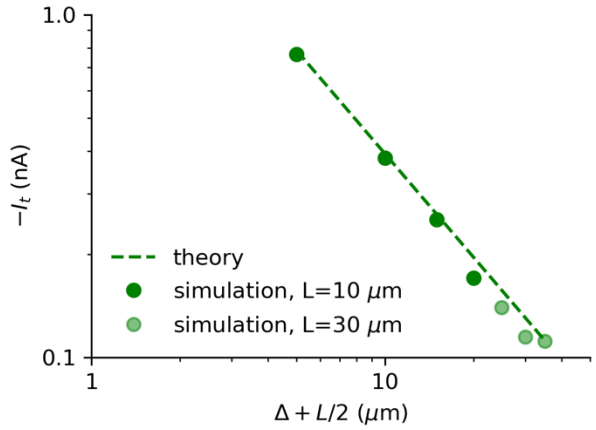

Figure 11*. Theoretical estimation of axial current at threshold compared to simulations. The simple model (no inactivation, no Kv channels) is simulated with L = 10 µm (dark green dots) and L = 30 µm (light green dots), and the start position is varied from 0 to 20 µm. The theoretical curve is shown as a dashed line.*

Axial current near threshold

We calculate the axial current just below threshold as a function of somatic voltage in a point AIS model. We consider a cylindrical axon of diameter d where all the Nav channels are located at a single location. The AIS contains a total Na^+ conductance G. The axial current is

$$I = \frac{V - V_s}{R}$$

where V is the axonal voltage, V_s is the somatic voltage, and R is the axial resistance between the soma and the AIS. It must equal the Na^+ current:

$$I_{Na} = G(E_{Na} - V)\exp\left(\frac{V - V_{\frac{1}{2}}}{k}\right)$$

which is the exponential approximation near threshold. Near threshold, we have $(E_{Na} - V) \approx (E_{Na} - V_s)$. We consider this driving force as a constant ΔV. We then absorb $V_{1/2}$ into G and take k as the units of voltage. Thus, the equation reads:

$$I = I_{Na} = G\Delta V e^V$$

Using $V = V_s + RI$, we obtain:

$$I = G\Delta V \exp(V_s + RI)$$

At the bifurcation (threshold), we have (differentiation with respect to I):

$$1 = GR\Delta V \exp(V_s^* + RI^*)$$

where V_s^* is the somatic voltage threshold and I^* is the axial current at threshold. We divide the two previous equations and obtain:

$$I = \frac{1}{R}\exp(V_s - V_s^* + RI - RI^*)$$

In a point AIS, the axial current at threshold is:

$$I^* = 1/R$$

Therefore:

$$I = I^* \exp\left(V_s - V_s^* + \frac{I}{I^*} - 1\right)$$

This can be rewritten as:

$$V_s = V_s^* + 1 - \frac{I}{I^*} + \log\frac{I}{I^*}$$

A Taylor expansion gives:

$$V_s \approx V_s^* - \frac{1}{2}\left(1 - \frac{I}{I^*}\right)^2$$

In original voltage units, we then obtain:

$$V_s \approx V_s^* - \frac{k}{2}\left(1 - \frac{I}{I^*}\right)^2$$

Relation between voltage threshold and axial current at spike initiation

Theoretically, voltage threshold varies as $-k \log g$, where g is the available Na^+ conductance (Brette, 2013; Goethals and Brette, 2020; Platkiewicz and Brette, 2011). The axial current at spike initiation also depends on g, and therefore voltage threshold and axial current co-vary when g is varied. The general relation is complicated, but a simple approximated relation can be obtained by considering the equation for the maximum current $I_p^{\max}$. Since $I_p^{\max} \propto \sqrt{g}$, it follows that with this approximation the threshold varies as $-k \log I_p^2$, i.e., as $-2k \log I_p$.

Simplified model

In Fig. 5A-B and Fig. 10, we used a simplified model with only non-inactivating Nav channels to check analytical expressions, similar to Brette (2013). A spherical soma of diameter 30 µm is attached to an axonal cylinder of diameter 1 µm and length 500 µm (soma diameter is in fact irrelevant as the soma is voltage-clamped). Specific membrane capacitance is C_m = 0.9 µF/cm²; specific membrane resistance is R_m = 15 000 Ω.cm²; leak reversal potential is E_L = -75 mV; intracellular resistivity is R_i = 100 Ω.cm. If not specified, Nav channels are placed from 5 µm to 35 µm on the axon. In Fig. 11, the length ranges from 10 µm to 30 µm and the start position from 0 µm to 20 µm. We used simple single gate activation dynamics with fixed time constant:

$$I_{Na} = Gm(E_{Na} - V)$$

$$\tau_m \cdot \frac{dm}{dt} = m_\infty(V) - m$$

$$m_\infty(V) = \frac{1}{1 + \exp((V_{1/2} - V)/k)}$$

where E_{Na} = 70 mV, k = 5 mV, $V_{1/2}$ = -35 mV and τ_m = 53.6 µs (corresponding to 150 µs before temperature correction, see (Goethals and Brette, 2020)). For Fig. 5A and B, Na^+ conductance density was g = 5000 S/m². For Fig. 11, the total Na^+ conductance was fixed (G = 350 nS) to keep the total number of Na^+ channels fixed when AIS length is varied. This corresponds to conductance densities of about 11 100 and 3700 S/m² for a 10 and 30 µm long AIS, respectively. The model is simulated in voltage-clamp and the threshold is measured with the bisection method. We used the Brian 2 simulator (Stimberg et al., 2019) with 10 µs time step and 1 µm spatial resolution.

Biophysical model

In Fig. 2E, 2F, 6B, 6D and 7E, we used a biophysical model of an AP with inactivating Nav channels and non-inactivating Kv channels, similar to (Goethals and Brette, 2020). The biophysical model has a simple geometry, consisting of a spherical soma (30 µm diameter), a long dendrite (diameter: 6 µm, length: 1000 µm) and a thin unmyelinated axon (diameter: 1 µm, length; 500 µm). The dendrite is irrelevant to most simulations because the soma is voltage-clamped, electrically isolating the dendrites from the axon. It only contributes an additional somatodendritic capacitance when an electrode model is added (Fig. 2). When not specified, the AIS extends from 5 µm to 35 µm from the soma. Specific membrane capacitance is C_m = 0.9 µF/cm²; specific membrane resistance is R_m = 15 000 Ω.cm²; leak reversal potential is E_L = -75 mV; intracellular resistivity is R_i = 100 Ω.cm.

In Fig. 2E-F, we inserted an electrode model, which consists of a resistance R_s (0 to 5 M Ω) between the amplifier and the soma, such that a current $(V_c-V)/R_s$ is injected into the soma (where V_c is the voltage command). The Na^+ conductance density g was 7400 S/m², to obtain peak axonal currents and thresholds comparable to measurements in RGCs.

In Fig. 6B-D, the AIS start position was 10 µm, close to the mean AIS start position in our cell population. The threshold was approached with 0.01 mV precision using the bisection method. In Fig. 7E, the AIS start position was varied from 0 to 20 µm and the AIS length was 30 µm. In these three panels, the Na^+ conductance density was g = 3700 S/m².

Passive properties	R_m	15 000 Ω.cm²
	E_L	-75 mV
	R_i	100 Ω.cm
	C_m	0.9 µF/cm²
Nav channels	g_{Na}, soma	250 S/m²
	g_{Na}, dendrite and axon (non AIS)	50 S/m²
	g_{Na}, AIS	variable (3500-7400 S/m²)
	E_{Na}	70 mV
	$V_m^{1/2}$, soma	-30 mV
	$V_h^{1/2}$, soma	-60 mV
	$V_m^{1/2}$, AIS	-35 mV
	$V_h^{1/2}$, AIS	-65 mV
	k_m	5 mV
	k_h	5 mV
	τ_m^*	150 µs (corrected: 54 µs)

	τ_h^*	5 ms (corrected: 1.8 ms)
Kv1 channels	g_K, soma and dendrite	100 S/m²
	g_K, AIS	1500 S/m²
	E_K	-90 mV
	$V_n^{1/2}$	-70 mV
	k_n	20 mV
	τ_n^*	1 ms

Table 1. *Parameters values of the biophysical model. Time constants corrected for temperature are indicated in brackets.*

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Competing interests

The authors have no competing interests to declare.

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