ONLINE METHODS

Slice preparation and whole-cell recording. Most experiments were performed on L5 pyramidal neurons from somatosensory neocortical coronal slices prepared from 2–4-week-old Sprague-Dawley rats[6,35]. A few experiments were made from cerebellar Purkinje cells in sagittal slices from the same animals. Rats were anesthetized with isoflurane and decapitated using procedures approved by the Institutional Animal Care and Use Committee of the Marine Biological Laboratory. Recordings were made from slices submerged in a plastic chamber with a glass bottom. The slices were superfused with warmed and oxygenated artificial cerebrospinal fluid (ACSF) at 32–34 °C except where noted. The ACSF contained 124 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$ and 10 mM glucose; pH was 7.4 when bubbled with 95% O_2/CO_2.

The chamber with submerged and superfused slices was attached to a stage rigidly bolted to an air table and cells were viewed with a 20×, 40× or 60× water-immersion lens (Olympus) in an Olympus BX50WI microscope mounted on an X-Y translation stage. Somatic whole-cell recordings were made using patch pipettes pulled from 1.5-mm outer diameter thick-walled borosilicate glass tubing (1511-M, Friderick and Dimmock). Tight seals were made with the 'blow and seal' technique using video-enhanced differential interference contrast optics to visualize the cells[36]. For current clamp experiments the pipette solution contained (in mM): 140 mM potassium gluconate, 4 mM NaCl, 4 mM Mg-ATP, 0.3 mM Na-GTP and 10 mM HEPES, pH adjusted to 7.2–7.4 with KOH. This solution was supplemented with 2 mM SBFI and 2–4 μM Alexa-488 (Molecular Probes). The pipette solution for whole-cell voltage-clamp experiments contained 135 mM CsCl, 4 mM NaCl, 2 mM $MgCl_2$ and 10 mM HEPES (cesium salt), pH 7.25, also supplemented with 2 mM SBFI and 2–4 μM Alexa-488; Ca^{2+} currents were blocked by adding 200 μM Cd^{2+} to the bath. For some experiments measuring dynamic $[Na^+]_i$ and $[Ca^{2+}]_i$ changes in the same cell, Alexa-488 was replaced with 50–100 μM of the high affinity Ca^{2+} indicator OGB-1 (Molecular Probes). Current clamp recordings were made with a Dagan BVC-700 amplifier; data were low-pass filtered at 10 kHz (−3 dB, single-pole Bessel filter) and digitized at 20 kHz. Voltage-clamp recordings were made with an Axopatch 200B amplifier; data were low-pass filtered at 2 kHz (−3 dB, four-pole Bessel filter) and sampled at 10 kHz. Care was taken to maintain membrane access resistance as low as possible (usually 3–4 MΩ and always less than 7 MΩ); series resistance was 80% compensated using the built-in circuitry of the amplifier.

Electrophysiological data analysis was accomplished using pCLAMP 10.0 (Axon Instruments) and Origin 6.0 (Origin Lab). If not otherwise noted, values are given as mean ± s.d. Student's *t* test was used for statistical analysis.

Dynamic sodium and calcium measurements. SBFI fluorescence was excited with a 75W Xenon arc lamp using a Semrock fura-2 filter set (excitation = 387(11) nm, dichroic = 409 nm, emission = 510(84) nm). For most experiments, changes in fluorescence were acquired using a back-illuminated 80 × 80 pixel cooled CCD camera (NeuroCCD-SMQ, RedShirt Imaging), controlled with Neuroplex software (RedShirtImaging). Images were acquired at 500 frames per s. Fluorescence changes from Purkinje cells (**Supplementary Fig. 1**) were detected with a Photometrics AT300 camera at 40 frames per s. To improve the signal-to-noise ratio of the traces, we typically averaged five to ten trials and smoothed the 500-Hz signals with a five point moving average. Occasionally, more trials were averaged with no temporal filtering to improve the time resolution of the measurements. Signal averaging was limited by photodynamic damage to the preparation. To reduce this damage, all positioning and focusing was done by monitoring the fluorescence from the co-injected Alexa-488 using an Olympus U-MWIBA filter set (excitation = 475(30) nm, dichroic = 510 nm, emission = 532(35) nm). In addition, the illumination intensity during sodium measurements sometimes was reduced with a 10× neutral density filter. Indicator bleaching was corrected by subtracting an equivalent trace without stimulation.

Fluorescence of the high-affinity calcium indicator OGB-1 was examined with the same U-MWIBA filter set using the same apparatus and camera. Images were acquired at 500 frames per s and responses were often averaged. Photodynamic damage was not a substantial problem with this indicator. Data for both sodium and calcium measurements were analyzed using Neuroplex and SCAN, a custom software package written in our laboratory.

Two-photon microscope reconstruction of cell morphology. At the end of many experiments, we placed the slice that contained the cell(s) injected with SBFI in a beaker containing ACSF and carried it to a nearby Zeiss LSM-510 confocal microscope equipped with a Chameleon pulsed laser for two-photon excitation. We first used a mercury arc lamp and a fura-2 filter set, similar to the one on our setup, to find the cell and place it in the center of the field using a 40× or 63× Zeiss water-immersion lens. We then changed to two-photon excitation at 740 nm and scanned the cell at 1.0-μm intervals. The resulting *z* stack was imported into ImageJ (US National Institutes of Health) for reconstruction and image processing.

Sodium buffering. Intrinsic proteins and exogenous ion indicators can distort the amplitude and time course of ion concentration changes inside neurons and other cells. This is particularly true of measurements of $[Ca^{2+}]_i$ changes in pyramidal and other neurons. This buffering can be understood quantitatively by carefully measuring the amplitude and time course of the $[Ca^{2+}]_i$ change at different indicator concentrations and extrapolating to zero added indicator[37,38]. In this formulation, $\beta = 1 + \kappa_B + \kappa_{dye}$ is the buffering power, where κ_B and κ_{dye} are the ratios of the change in buffer bound Ca^{2+} to the change in free Ca^{2+} for a given Ca^{2+} influx for the cases in which the buffer is an intrinsic protein (B) or an added indicator (dye). In a typical pyramidal neuron, $\kappa_B \approx 100$ and $\kappa_{dye} \approx 10$ when a low affinity Ca^{2+} indicator is used. This means that $\beta \approx 111$, where $\beta = 1$ indicates no buffering. The peak $[Ca^{2+}]_i$ is reduced by factor β and the time constant for Ca^{2+} removal is increased by the same factor compared with the amplitude and rate in cells that have no Ca^{2+} buffers.

For $[Na^+]_i$ changes, the same analysis applies, but the effects of buffering are much different. First, there are almost no intrinsic Na^+ buffering molecules in the cytoplasm; the diffusion constant of Na^+ in cytoplasm (0.6 $\mu m^2\ ms^{-1}$) is almost the same value as in water[13,39]. This means that $\kappa_B \approx 0$ for Na^+. Second, as the resting $[Na^+]_i$ (~4 mM) is much lower than the K_D for the SBFI:Na^+ reaction (~26 mM)[15], $\kappa_{dye} \approx [SBFI]/K_D$ (ref. 37). In our experiments, typical [SBFI] was 2 mM. Therefore, $\kappa_{dye} \leq 0.1$. Combining the two values indicates that $\beta \approx 1.1$ for Na^+ in cytoplasm. Consequently, we expect little distortion of the true amplitude and time course of $[Na^+]_i$ changes by SBFI in our experiments.

Comparison of the Na^+ fluxes in different regions using ΔF. Unlike $\Delta F/F$ measurement, this approach is not dependent on accurately measuring tissue autofluorescence. However, there are additional technical considerations. If we want to determine the charge entry per unit membrane area, then we must relate surface area to the area of a region measured in an imaging experiment (cross section). We simplify this comparison by assuming that the dendrites and axons are all cylinders and the soma is a sphere. Then there is no correction in comparing changes between dendritic regions and the axon. To compare the soma to the axon, we have to correct by $\frac{2\pi rl / 2rl}{4\pi r^2 / \pi r^2} = \frac{\pi}{4} = 0.78$, which is the ratio of the surface of a cylinder to its cross section (what the camera images) divided by the same ratio for a sphere (*r* is the radius of the soma or the axon and *l* is the length of the axon segment).

When the axon or a dendrite is in focus, other elements may be slightly out of focus. Parts of the soma are inevitably out of focus because its thickness is substantially greater than the focal depth of the lens that we used (usually 60×). To estimate whether these out of focus elements affect a quantitative estimate of the fluorescence intensity, we measured the total fluorescence of 4-μm diameter fluorescent beads as we changed focus (**Supplementary Fig. 2**).

We measured the fluorescence in a 10-μm diameter box surrounding the bead and found that the fluorescence changed by less than 10% with a change in focus of ±5 μm, even though the image clearly appeared out of focus. If the box was larger (~30 μm diameter), then the fluorescence did not change 10% for changes in focus of ±15 μm. Therefore, as the soma diameter was 22.9 ± 4.3 μm ($n = 25$, from two-photon microscope reconstructions), it is likely that the error in measuring the cell fluorescence is <10% if a measuring box is chosen appropriately. If there is an error in estimating the signal size from the soma, it is likely to be an underestimate, as the signals from out of focus elements are smaller. In a related series of experiments, we also determined that the combined sensitivity of the camera and the evenness of the fluorescence excitation over the field of view did not vary by more than 5%.

We tried to minimize the effects of diffusion on these measurements by using short trains of five spikes at 100 Hz to generate the $[Na^+]_i$ increase. In this case,

the rising phase of the signal is much faster than the decay rate and there is little effect of diffusion on the peak amplitude except at locations very close to the soma (**Figs. 1** and **3**). In other experiments, we measured the fluorescence signals from trains of ten action potentials evoked at 30-ms intervals to improve the signal-to-noise ratio of the measurements. In this case, the removal rate in the axon is comparable to the influx rate and the amplitude of the fluorescence change is not proportional to the total Na^+ entry. In the soma and dendrites, the removal rate is much slower and the peak amplitude should be proportional to Na^+ entry.

To correct for the distortion in the peak amplitude resulting from the rapid removal of Na^+ in the axon, we constructed a simple model of Na^+ entry in the AIS and soma and removal by diffusion and compared the peak fluorescence change in the case of normal Na^+ diffusion (which accurately replicates the $[Na^+]_i$ changes in these regions; **Fig. 3**) with the results of a model in which the diffusion constant was set to zero. The latter case corresponds to the situation with no removal and the peak amplitude should be proportional to Na^+ entry. The model showed that, in the center of the AIS, the peak amplitude with normal diffusion is about 70% of the amplitude without diffusion in the case where ten action potentials are generated at 30-ms intervals (total stimulation time of 300 ms); closer to the ends of the AIS, the differences are greater because Na^+ in those regions is rapidly diffusing into the soma or the myelinated region of the axon. As expected, the difference is much less for a short train of 50 ms (except next to the soma) and greater for a longer train. These differences are substantial for the longer trains and are the largest corrections that must be applied to estimate the true relative Na^+ entry in different regions during a slower spike train.

Modeling Na^+ diffusion and electrophysiological properties. Numerical simulations were performed in the NEURON simulation environment[40]. Unless otherwise stated, electrophysiological parameters and dynamic $[Na^+]_i$ changes were studied in a simplified compartmental model that encompassed the fundamental morphological and electrical features of layer 5 pyramidal and Purkinje neurons. In the model, the 1.2–2-μm-thick AIS extended over the first 40–50 μm of the axon in L5 pyramidal cells and 15 μm in Purkinje cells. The subsequent segment (length, 50 μm; diameter, 1.2 μm) was myelinated. The first node of Ranvier (length, 1 μm; diameter, 1.2 μm) was positioned at 100 μm from the soma, which approximately corresponded with the location of the experimentally observed localized $[Na^+]_i$ increases (**Fig. 6**). The soma (length, 35 μm; diameter, 23 μm) gave rise to a single apical dendrite (length, 700 μm; diameter, 3.5 μm) and to two basal dendrites (length, 200 μm; diameter, 1.2 μm). For computational precision, all compartments were divided into many segments with the length of individual segments usually less than 1 μm. The passive electrical properties R_m, C_m and R_i were set to 15,000 Ω cm^2, 0.9 μF cm^{-2} and 125 Ω cm, respectively, uniformly throughout all compartments. Myelination was simulated by reducing C_m to 0.02 μF cm^{-2}. The resting membrane potential at the soma was set to −75 mV. All simulations were run with 5-μs time steps and the nominal temperature of simulations was 32 °C.

The model incorporated a Hodgkin-Huxley–based Na^+ conductance as previously described[2]. The activation time constant was given by $\tau_m = k/(\alpha_m(V_m) + \beta_m(V_m))$; unless otherwise stated, the scaling factor k was 0.2 (**Fig. 8a**). The Na^+ conductance was 250 pS μm^{-2} in the soma, 200 pS μm^{-2} in the apical dendrite, 40 pS μm^{-2} in the basal dendrites, 750 pS μm^{-2} in the AIS and 1,200 pS μm^{-2} in the nodes of Ranvier. Myelinated internodes possessed no Na^+ channels. These densities were tuned to account for previously published action potential characteristics[1,19,26,41] and for the measured Na^+ fluxes in the different neuronal compartments. Thus, the somatic Na^+ conductance was set to 250 pS μm^{-2} (maximal m^3h of ~220 pS μm^{-2}) to achieve a maximal spike upstroke velocity of ~500 V s^{-1} and Q_{Na} of ~12 fC μm^{-2}. This value is consistent with the channel density as calculated from the data on isolated cortical pyramidal neurons[22]. In the model, persistent Na^+ conductance differed from the transient Na^+ conductance in that there was no inactivation variable and voltage dependence of activation was shifted to the left by 10 mV. The model included K_v and K_v1-like K^+ channels with kinetics and density as previously described[3]. The K^+ equilibrium potential was set to −85 mV.

Diffusion of Na^+ was modeled as the exchange of Na^+ ions between adjacent neuronal compartments using the intrinsic protocols in NEURON assuming a diffusion coefficient of 0.6 μm^2 ms^{-1} (ref. 13). The resting intracellular and the extracellular Na^+ concentrations were set to 4 and 151 mmol l^{-1}, respectively.

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