LETTERS

Axonal site of spike initiation enhances auditory coincidence detection

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Neurons initiate spikes in the axon initial segment or at the first node in the axon¹⁻⁴. However, it is not vet understood how the site of spike initiation affects neuronal activity and function. In nucleus laminaris of birds, neurons behave as coincidence detectors for sound source localization and encode interaural time differences (ITDs) separately at each characteristic frequency (CF)⁵⁻⁷. Here we show, in nucleus laminaris of the chick, that the site of spike initiation in the axon is arranged at a distance from the soma, so as to achieve the highest ITD sensitivity at each CF. Na⁺ channels were not found in the soma of high-CF (2.5-3.3 kHz) and middle-CF (1.0-2.5 kHz) neurons but were clustered within a short segment of the axon separated by 20-50 µm from the soma; in low-CF (0.4-1.0 kHz) neurons they were clustered in a longer stretch of the axon closer to the soma. Thus, neurons initiate spikes at a more remote site as the CF of neurons increases. Consequently, the somatic amplitudes of both orthodromic and antidromic spikes were small in high-CF and middle-CF neurons and were large in low-CF neurons. Computer simulation showed that the geometry of the initiation site was optimized to reduce the threshold of spike generation and to increase the ITD sensitivity at each CF. Especially in high-CF neurons, a distant localization of the spike initiation site improved the ITD sensitivity because of electrical isolation of the initiation site from the soma and dendrites, and because of reduction of Na⁺-channel inactivation by attenuating the temporal summation of synaptic potentials through the low-pass filtering along the axon.

ITD is an important cue for localizing sound sources in the horizontal plane⁶. Nucleus laminaris (NL) neurons of birds detect the coincidence of binaural synaptic inputs and calculate ITDs in the microsecond range7. Recent works have revealed that several mechanisms underlie this coincidence detection, including the following: first, acceleration of excitatory postsynaptic potential time course by K⁺ channel activation^{8,9}; second, inhibitory synaptic inputs^{10,11}; third, segregation of binaural inputs by dendrites¹²; and fourth, depression of synaptic transmission^{13,14}. NL neurons are arranged tonotopically in a rostro-medial (high CF) to caudo-lateral (low CF) direction within the nucleus¹⁵ (Supplementary Fig. 1a), and show several CF-dependent specializations such as morphology of dendrites¹⁶ and K⁺-channel expression¹⁷. Axon hillock and initial segment are myelinated in the high-CF neuron, implying that spikes may be generated at a different site of neurons depending on the CF18. Here we examined the site of action potential initiation in NL neurons along the tonotopic axis and explored how the location contributes to the coincidence detection.

In the high-CF and middle-CF neurons, both orthodromic (thick traces in Fig. 1a) and antidromic (thin traces) spikes were smaller than those in low-CF neurons. Moreover, the antidromic spike of the high-CF and middle-CF neurons was far smaller than the orthodromic one (P < 0.01, n = 11 cells), although the maximum rate of

rise of these two spikes was not different (see Supplementary Fig. 1b). The small antidromic spike in the high-CF and middle-CF neurons was not an abortive response for the following reasons. First, although antidromic spikes during somatic subthreshold depolarization exceeded the threshold of orthodromic spikes, further regenerative responses were not induced (Fig. 1b, left, and insets for expanded time course, observed in eight cells). Second, the antidromic and the orthodromic spikes blocked each other when evoked at time intervals shorter than 0.5 ms (Fig. 1c, filled circles, seven cells),



Figure 1 | **The site of action potential generation is remote from the cell soma in high-CF and middle-CF neurons. a**, Antidromic (thin black lines) and orthdromic (thick grey lines) spikes at each CF. Left, high CF; middle, middle CF; right, low CF. Broken lines indicate threshold. Orthodromic spikes were elicited by somatic current injection (lower traces), and antidromic spikes by stimulating the axon (arrowheads). b, Antidromic spikes during somatic depolarization. Left, high CF and middle CF; right, low CF. Inset, spikes before and during depolarization recorded at the corresponding symbols below. c, Antidromic spike paired with orthodromic spike; high CF and middle CF. **d**, Voltage-clamp recordings under cell-attached (upper) and whole-cell (lower) conditions. Left, high CF and middle CF; right, low CF.

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suggesting that both spikes have the same origin. These observations indicate that spikes are generated at a remote site from the soma in the high-CF and middle-CF neurons. In contrast, in the low-CF neuron, antidromic spikes were affected and eventually interrupted by the somatic depolarization (Fig. 1b, right, filled circle, and insets, five cells), indicating that spikes are generated at a closer site.

Localization of voltage-gated Na⁺ (Nav) channels was studied electrophysiologically (Fig. 1d). In the low-CF neurons, cell-attached patch recordings from the soma occasionally showed Na⁺ currents (5 of 22 cells, 13.3 ± 1.4 pA at -30 mV, ranging from 10.7 to 18.1 pA; upper right), but not in the high-CF and middle-CF neurons (26 cells; upper left). Under the whole-cell recording condition, depolarizing steps evoked rapidly inactivating tetrodotoxin-sensitive Na⁺ currents in both high-CF and middle-CF neurons and low-CF neurons, and the amplitude at -30 mV was fivefold to sixfold smaller in the high-CF and middle-CF neurons (2.9 ± 0.2 nA, n = 8 cells; lower left) than in the low-CF neuron (16.8 ± 1.4 nA, n = 9 cells; lower right). These observations may indicate that Nav channels are absent from the cell soma in the high-CF and middle-CF neurons. Even in the low-CF neurons, Nav channel density in the soma was small; the Na⁺ current in the patch (0.67 ± 0.07 pA µm⁻², n = 5 cells, 20 µm²;



Figure 2 | **Nav channels cluster in the axon at some distance from the soma in high-CF and middle-CF neurons. a**, Fibrous representation of Nav (hot spot, arrowheads) in NL. Left, high CF; middle, middle CF; right, low CF. **b**, Double immunostaining with Pan-Nav (red, left) and Nav1.6 (green, middle). Right, merged images. **c**, Colocalization of Nav (red, left) with Kv1.2 (green, middle). Right, merged images. **d**, **e**, Nav channels (red) are present on an axon of retrogradely labelled NL neurons (green) in both high-CF and middle-CF neurons (**d**) and low-CF neurons (**e**). **f**, **g**, Distance *D* (**f**) and length *L* (**g**) of Nav hot spot. Error bars indicate standard errors. Asterisk, P < 0.01 compared with middle CF. Numbers in parentheses are the number of cells. **h**, Negative correlation between length and distance: y = -0.29x + 24.0, r = 0.66, P < 0.01, n = 108 cells from **f** and **g**. Yellow symbols are the averages.

see Supplementary Methods) was about 30-fold smaller than that expected from the somatic surface area $(21.0 \pm 1.8 \text{ pA} \,\mu\text{m}^{-2}, n = 9 \text{ cells}, 800 \,\mu\text{m}^2)^{17}$.

These electrophysiological observations were consistent with the following immunohistological localization of Nav channels (Fig. 2). An antibody for the neuronal Nav channels (Pan-Nav) showed fibrous clusters (hot spots; red arrowheads in Fig. 2a) and many puncta presumably representing the nodes of Ranvier. The length of the hot spot increased towards the low-CF region (Fig. 2g). The hot spot was also immunopositive against Nav1.6 (Fig. 2b), which is known to be expressed in the myelinated axon in the brain¹⁹. Nav channels were also co-localized with voltage-gated K⁺ channels (Kv1.2) (Fig. 2c). Thus, the hot spot may correspond to the axon initial segment rather than the node of Ranvier, because Kv channels are confined to perinodal regions at the node²⁰. Subcellular localization of the hot spot was examined by immunostaining after the identification of NL neurons with a retrograde tracer. Nav channels were labelled in the axon in both high-CF and middle-CF neurons and low-CF neurons (Fig. 2d, e, and Supplementary Fig. 2). In the high-CF and middle-CF neurons, the hot spot started at a more distal site from the soma (the separation of the hot spot from the soma is defined as distance, Fig. 2f) and was shorter in length than in the low-CF neurons (the stretch of the hot spot is defined as length, Fig. 2g). The length of hot spot had a clear negative correlation with the distance (Fig. 2h).

How these geometries of the hot spot affect firing efficacy and coincidence detection of a neuron was examined by simulation with a multiple-compartment model. For simplicity, the geometry of soma-dendrites was assumed to be independent of CF^{16,17}, whereas the lengths of the hot spot (length, L) and myelinated initial segment (distance, D) were varied depending on the frequency region of the model neuron. The model well reproduced the action potentials observed at each CF when the corresponding distance and length of hot spot were assigned (Fig. 3a, b, and Supplementary Figs 3 and 4). The excitability of the model neuron was evaluated as the threshold current (0.3 ms in duration) required for generating an action potential. The geometry of the hot spot critically affected the threshold current (Fig. 3c, and Supplementary Fig. 5). When the hot spot was short (10 µm in length; arrow f in Fig. 3c), increasing the hot spot distance reduced the threshold current, and the minimum (red, indicating the highest excitability) was found at a distance of 20-30 µm. With a long hot spot (25 µm in length; arrow h in Fig. 3c), however, the minimum was found with a shorter distance (0 µm). The distribution of threshold current almost overlapped with the observed distribution of the hot spot in the NL neuron (dots in Fig. 3c); the observed distribution was slightly more distant in the high-CF neurons (30-80 µm; white dots). Thus, the distance and the length of hot spot were arranged to increase the excitability of NL neurons. Spike generation was significantly affected by the somatic capacitance and K⁺ current. Therefore, either the isolation of the hot spot from the soma or an increase in the hot spot length was required to reduce the threshold current. However, a further increase in the distance or the length of hot spot increased the threshold current, because of the dissipation of charging current across the axonal membrane or the increased axonal K⁺ current (see Supplementary Figs 5 and 6).

Next we tested how the geometry of the hot spot affects the ITD sensitivity by applying trains of binaural synaptic inputs into the soma at various phase differences (Fig. 3d, boxed insets; 180° corresponds to 0.17 ms of ITDs at 3 kHz CF; see Supplementary Methods). The model neuron could respond to ITDs at the maximum firing rate for the synaptic inputs applied in phase (0°) and at the minimum rate for the out-of-phase (180°) inputs (Fig. 3d, e). The spikes were phase-locked to the in-phase inputs (standard deviation of peak times, 41 µs). The firing rate and the ITD sensitivity, defined here as the difference of firing rate between in-phase and out-of-phase inputs, increased with the increase in input conductance (Fig. 3e), as was observed in the barn-owl NL neurons *in vivo*¹⁰.

ITD sensitivity was calculated as a function of the distance of the hot spot, for model neurons having the hot spot of a corresponding length at each frequency input (Fig. 3f-h; see Fig. 2h): Supplementary Fig. 7 complementarily shows the effects of hot spot length on the ITD sensitivity. The input conductance was set to fire the neuron at about 600 Hz (ref. 10) (Supplementary Methods; see effects of input conductance on ITD sensitivity in Supplementary Fig. 8). The ITD sensitivity depended on the distance of the hot spot from the soma (Fig. 3f-h); the maximum (arrowheads) was given at 0–10 µm for the low CF, 20–40 µm for the middle CF, and 50–60 µm for the high CF. This tendency was the same as that observed in the immunohistochemistry (yellow symbols and red broken lines; see Fig. 2h) and the distribution of threshold current (lower panels of Fig. 3f-h; see Fig. 3c). However, there was a dissociation in the distance between the maximum ITD sensitivity and the minimum threshold current (asterisks and black broken lines), particularly at high-CF neurons (Fig. 3f); the maximum ITD sensitivity was given at a more remote site.

To explore the causes of this difference, we examined the effects of a high-frequency burst of synaptic inputs on the membrane potential at the hot spot, under the block of spike generation (Fig. 4a, thick black traces). During high-frequency inputs (3 kHz), synaptic potentials were temporally summated to depolarize the hot spot located near the soma by about 20 mV (Fig. 4a, top trace). This depolarization strongly inactivated Na⁺ channels and reduced the excitability

(Fig. 4b, open triangle). However, when the hot spot was separated from the soma (50 μ m distance), the distance attenuated the depolarization (Fig. 4a, bottom trace) and reduced the extent of inactivation of Na⁺ channels at the hot spot (250% increase in h_{Na} ; Fig. 4b, filled triangle; Fig. 4c, bottom). The distance also decreased the activation of K⁺ current at the hot spot, but the effect was small (less than 25%). As a result, when neurons receive high-frequency synaptic inputs (3 kHz), the hot spot to give the highest excitability (minimum threshold current) was shifted towards a distant location (from *D* 20 μ m without inputs to *D* 40 μ m with inputs; Fig. 4c, middle). Thus, the remote localization of the hot spot was preferable for the detection of ITDs, when a high-frequency burst of inputs arrived (see frequency effects in Supplementary Fig. 9).

We have shown here that the reduction of Na⁺ channel inactivation is crucial in detecting ITDs at high sensitivity during high-frequency inputs. The findings are consistent with the observation that the initial segment is myelinated in high-frequency NL neurons of the barn owl¹⁸. In the medial superior olive, the mammalian homologue of NL, the spike amplitude was small and showed a short latency when recorded near the axon in gerbils²¹, indicating that axonal spike initiation is probably a common feature of binaural coincidence detectors in birds and mammals. Moreover, recent works have suggested that other central neurons initiate spikes at a more distant site than the axon initial segment^{2,3,22}. Although the functional significance of axonal spike initiation in those neurons remains unknown,

> Figure 3 | Nav channel distribution is optimized for ITD detection. a, b, Recorded (a) and simulated (b) action potentials. Broken lines indicate threshold. Thick blue lines, orthodromic; thin black lines, antidromic. c, Threshold current (see the text) as a function of length L and distance D. Dots and yellow symbols are from Fig. 2h. The notation '4.5<' stands for injected current of larger than 4.5 nA. d, Responses to binaural synaptic inputs of 3 kHz. The binaural phase delay was 0° (left) and 180° (right). e, Firing rate as a function of phase and input conductance (triangles, 98 nS; circles, 90 nS; squares, 82 nS; diamonds, 74 nS). f-h, ITD sensitivity (top) and threshold current (bottom) as a function of hot spot distance. f, High CF (L 10 μm; 3 kHz); g, middle CF (*L* 15 μm; 2 kHz); h, low CF (L 25 µm; 1 kHz). Asterisks and black broken lines indicate the threshold minimum calculated in c. Yellow symbols and red broken lines are the observed average distances from Fig. 2h. Arrowheads indicate the maximum ITD sensitivity.





Figure 4 | Cable properties of axon enhance ITD sensitivity during highfrequency inputs. a, Membrane potentials of hot spot located at 0-µm (upper) and 50-µm (lower) distance during binaural 3-kHz inputs (0°, 90 nS) in high-CF neuron (10-µm length). Plateau depolarization was defined as an average of membrane potential (horizontal broken lines and triangles). **b**, Voltage dependence of h_{Na} (inactivation parameter) during depolarization. Filled triangle, 50 µm with inputs; open triangle, 0 µm with inputs. Vertical broken line indicates the rest. **c**, ITD sensitivity (top, from Fig. 3f), threshold current (middle) and h_{Na} (bottom) as a function of hot spot distance. In the middle panel, circles represent with inputs, and diamonds represent without inputs. The plateau depolarization (induced by constant current inputs to soma, 5.5 nA) displaced the threshold minimum to a distant location (middle, with inputs, double asterisk). The single asterisk indicates threshold minimum without inputs.

the present results help our understanding of how the site of action potential generation can fine-tune neuronal computations.

METHODS

Whole-cell or cell-attached recordings were made from NL neurons in brainstem slices (200–250 µm) of chicks at 3–7 days after hatching (Fig. 1)²³. Antibodies against Pan-Nav and Nav1.6 were raised for immunohistochemistry (Fig. 2)^{17,24}. Some immunostainings were made after revealing NL neurons with a retrograde tracer. Neuronal modelling and simulation were performed with NEURON 5.6 (Figs 3 and 4). K⁺ and Na⁺ currents in the model followed the web-accessible Model DB^{25–28}. Statistical significance was tested with Student's *t*-test. Values are presented as means ± s.e.m. Detailed methods are provided in Supplementary Methods.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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