Serotonin modulates spike probability in the axon initial segment through HCN channels

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The axon initial segment (AIS) serves as the site of action potential initiation in most neurons, but difficulties in isolating the effects of voltage-gated ion channels in the AIS from those of the soma and dendrites have hampered understanding how AIS properties influence neural coding. Here we have combined confocal microscopy, patch-clamp recordings and light-sensitive channel blockers (‘photoswitches’) in binaural auditory gerbil neurons to show that hyperpolarization and cyclic-nucleotide-gated (HCN) channels are expressed in the AIS and decrease spike probability, in a manner distinct from that of HCN channels in the soma and dendrites. Furthermore, the control of spike threshold by HCN channels in the AIS can be altered through serotonergic modulation of 5-hydroxytryptamine 1A (5-HT1A) receptors, which hyperpolarizes the activation range of HCN channels. As release of serotonin signals changes in motivation and attention states, axonal HCN channels provide a mechanism to translate these signals into changes in the threshold for sensory stimuli.

In most neurons in the brain, excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs) interact with different types of voltage-gated channels in the dendrites, and the product of these interactions triggers patterns of action potential output in the axon. From classical studies it was hypothesized on the basis of indirect evidence that the site for action potential generation is the axon initial segment, later supported with simultaneous somatic and axonal recordings. Within this general framework, however, it is clear that the subtypes and density of voltage-gated ion channels in the AIS vary considerably, contributing to the diversity in spike shapes displayed by different neuron classes. Action potential threshold also depends critically on the length and diameter of the AIS, as well as the spatial pattern of expression of voltage-gated ion channels.

The AIS is plastic: sustained changes in the level of synaptic excitation can trigger changes in both the length of the AIS and its distance from the soma, resulting in compensatory alterations in spike threshold that maintain firing frequency within a neuron’s dynamic range. Voltage-gated calcium channels in the AIS have been shown to be important components of the modulatory control of spike initiation. Both T- and R-type voltage-gated calcium channels are expressed in the AIS of several types of neurons, where they may increase the probability of single spikes or contribute to burst firing. In auditory brainstem neurons, dopamine downregulates T-type channels through a protein kinase C pathway. In dentate gyrus granule cells, acetylcholine reduces spike threshold through activation of muscarinic acetylcholine receptors, calcium influx in the AIS via T-type calcium channels and reduction of Kv7 (M-type) potassium currents. Thus, AIS calcium channels and G-protein-coupled receptors provide mechanisms by which the sensitivity of spike generation can be finely tuned.

We have examined action potential initiation in the principal neurons of the medial superior olive (MSO), where control of spike threshold critically influences the processing of cues used for horizontal sound localization. Here we show that HCN channels are expressed in the AIS of MSO principal neurons and that the primary role of these axonal channels is to alter action potential threshold, in contrast to the roles of somatic and dendritic channels in shaping the timing and summation of synaptic potentials. We also show that axonal HCN channels and their influence on spike threshold are subject to long-lasting modulation by 5-HT through 5-HT1A receptors. As the activity of the serotonergic system reports changes in motivational state or attention, axonal HCN channels may provide a way for neurons to translate such state changes into adjustments in firing sensitivity.

RESULTS

Immunostaining for HCN1 subunits in the AIS

To explore the structural bases underlying action potential initiation and control of threshold in MSO principal neurons, we immunostained sections of gerbil brainstem using antibodies against βIV spectrin, a prominent cytoskeletal scaffold restricted to the AIS and nodes of Ranvier, and HCN1 subunits. Although the highest densities of HCN1-containing channels were in the soma and dendrites of MSO neurons (Fig. 1a), we detected low densities of HCN1 channels in 37 of 91 (41%) AIS neurons as well (Fig. 1b). In some instances, HCN1 channels extended beyond the end of the βIV spectrin-labeled AIS (Fig. 1b). In MSO neurons the AIS emanated directly from the soma, rather than a primary dendrite (Fig. 1a). Furthermore, immunostaining for βIV spectrin or panspecific Nav channel antibody (PanNav, which detects all sodium channel isoforms) began immediately adjacent to the AIS.
Figure 1 | Expression of HCN1 subunits in the AIS of MSO principal neurons. (a) Immunostaining of MSO neurons using antibodies against HCN1 (purple) and βIV spectrin (green). Scale bar, 10 µm. (b) Immunostaining of MSO neuron AIS using antibodies against HCN1 (purple) and βIV spectrin (green). Arrowheads indicate HCN1 immunoreactivity colocalizing with βIV spectrin. The arrow indicates the end of the βIV-spectrin-labeled AIS. HCN1 immunoreactivity frequently extended beyond the end of the AIS into the more distal axon. Scale bar, 5 µm. (c) Immunostaining of MSO neurons using antibodies against HCN1 (purple) and Nav1.6. Nav1.6 immunoreactivity frequently decreased in intensity or was absent immediately adjacent to the cell body (arrow). Scale bar, 10 µm. (d) MSO neuron AIS immunostained using PanNav. This immunoreactivity began at the transition from the cell body to the axon. Dotted lines outline the cell body. Scale bar, 10 µm. (e) Immunostaining of MSO using antibodies against Caspr (purple) and βIV spectrin (green). The arrow indicates the end of the AIS and the start of the myelin sheath. Scale bar, 10 µm. (f) Lengths of βIV spectrin, PanNav and Nav1.6 labeling along the AIS of MSO neurons. Nav1.6 labeling was significantly shorter than either βIV spectrin or PanNav staining, reflecting the reduced staining near the cell body. βIV spectrin, n = 46; PanNav, n = 12; Nav1.6, n = 22. βIV spectrin versus PanNav, P = 0.2; βIV spectrin versus Nav1.6, P = 0.006; PanNav versus Nav1.6, P = 0.001 (unpaired t-test with Welch’s correction). Scatterplots show mean ± s.e.m.; NS, non-significant; **P < 0.01.

to the cell body and averaged 17.4 ± 0.3 µm (n = 46 axons from 3 gerbils) and 18.2 ± 0.5 µm (n = 12 axons from 3 gerbils) in length, respectively (Fig. 1a,d,f; P = 0.2). Intriguingly, immunostaining with antibodies against Nav1.6 Na channels revealed a prominent gap or reduction in immunoreactivity at the proximal end of the AIS, with the highest levels of Nav1.6 at the distal end (Fig. 1c); this distribution of Nav1.6 is reminiscent of that found in cortical pyramidal neurons and retinal ganglion cells5–16. Consistent with this reduction in Nav1.6 immunoreactivity at the proximal AIS, we measured a shorter AIS length when defined by Nav1.6 immunoreactivity (Fig. 1f; 15.6 ± 0.5 µm, n = 22; P = 0.0062 for βIV spectrin length versus Nav1.6 length and P = 0.0016 for PanNav length versus Nav1.6 length; unpaired t-test with Welch’s correction). In some neurons, the distal end of the AIS also corresponded to the start of myelination, as indicated by immunostaining for the paranodal axogal junction marker Caspr (Fig. 1e). Together, these results reveal molecular and structural details of the AIS of MSO neurons, including the presence of HCN1 channels.

Function of HCN channels in the AIS using photoswitches

To investigate the expression and functional roles of HCN channels in the AIS, we combined confocal microscopy, electrophysiology, and optical manipulation of photoswitches, photoisomerizable compounds that can block the pores of certain voltage-gated ion channels in a wavelength-dependent fashion (Fig. 2a)17. To directly measure the biophysical properties of HCN channels in the AIS, we made whole-cell voltage-clamp recordings in MSO neurons from gerbils 18–23 d old and isolated the hyperpolarization-activated current (Ih) pharmacologically (Fig. 2b and Online Methods). In these experiments we used DENAQ, which photoswitches to the cis (unblocking) configuration with 488-nm light and spontaneously reverts to the trans (blocking) configuration in darkness (Supplementary Fig. 1). In the dark DENAQ does not block Ih with 100% efficiency, yielding reduced currents in response to voltage steps (1 s duration) from −30 mV to −110 mV in 10 mV increments (Fig. 2c). When the AIS was scanned with 488 nm light, DENAQ block of voltage-gated channels was relieved and the Ih in the AIS was augmented (Ih,Dark; Fig. 2c). Subtraction of the Ih,488nm from Ih,Dark yielded Ih isolated from the AIS (Ih,AIS; Fig. 2d). Tail current analysis of Ih,AIS yielded a half-maximal activation voltage (V1/2) of −64.4 ± 2.6 mV, with a slope of 10.0 ± 0.8 (n = 6; Fig. 2e). These values were not significantly different from those obtained with somatic block (Fig. 2e; Ih,Soma: V1/2 = −65.7 ± 2.9 mV, slope = 8.7 ± 0.9, V1/2, slope, P = 0.74; slope, P = 0.27; n = 6). Finally, the activation kinetics of Ih,AIS and Ih,Soma (which exhibited both fast and slow time constants, τfast and τslow) were not significantly different from one another, nor was the relative contribution of each of these components as a proportion of the total current amplitude (Fig. 2f). These findings confirm the presence of Ih in the AIS of MSO cells and also show that these channels display many of the same biophysical properties as those found throughout the rest of the cell.

To understand the functional roles of HCN channels in the AIS, we performed experiments using whole-cell current-clamp recordings with AAQ (acrylamide-azobenzene-quaternary ammonium). AAQ was maintained in its cis conformation with field illumination at 380 nm, which maintains channels in an unblocked state (Fig. 3a). Local channel blockade was achieved by scanning the AIS, soma or whole cell with the confocal microscope’s 488-nm laser line (Fig. 3b). A family of simulated excitatory postsynaptic currents (EPSCs) was injected through the patch pipette (0 to 4,000 pA, 800 pA increments), giving rise to simulated postsynaptic potentials and typically to action potentials in response to stronger stimuli (Fig. 3b). Light-regulated channel blockade of the AIS, soma and whole cell reversibly hyperpolarized the membrane resting potential (Fig. 3b,c). However, changes in resting
potential were blocked completely when ZD7288 (20 μM), a non-photosensitive blocker of HCN channels, was included in the patch pipette solution (Fig. 3c). AAQ is known to block multiple channel types, raising the possibility that nonspecific actions contribute to light-induced changes in resting potential. Bath application of 10 μM ZD7288 or 2 mM CsCl hyperpolarized MSO cells by an average of 6–7 mV when we maintained AAQ in the unblocked configuration under 380 nm illumination (Fig. 3c). However, after we restored the resting potential to control values via direct current injected through the pipette, no significant changes in resting potential occurred with 488 nm scanning of either the whole cell or AIS (Fig. 3c). Finally, 5 μM XE-991, a blocker of K<sub>s</sub> (KCNQ) potassium channels that are present in the AIS of some cell types, did not significantly alter

**Figure 2** Activation properties of axonal h<sub>c</sub>, as revealed by laser scanning of the photoswitch DENAQ. (a) The cis (unblocking) conformation of DENAQ is maintained at 488 nm light (green) while the trans (blocking) conformation is maintained in the dark (black). (b) Confocal image of an MSO principal neuron. Arrow, axon bleb; scale bar, 30 μm. (c) Measurement of h<sub>c</sub> in the AIS using DENAQ and whole-cell voltage-clamp recordings. Pharmacologically isolated h<sub>c</sub> was partially blocked in the dark (black traces) and then focally unblocked in the AIS at 488 nm (green traces). Voltage steps −30 mV to −110 mV in −10 mV steps, 1 s duration. (d) h<sub>AIS</sub> yielded from subtraction of h<sub>Dark</sub> from h<sub>488nm</sub> (h<sub>AIS</sub> = h<sub>488nm</sub> − h<sub>Dark</sub>). Instantaneous tail currents measured at −100 mV (colors are for clarity of visualization). (e) The voltage dependence and slope of h<sub>c</sub> in the AIS are not significantly different from those in the soma (V<sub>UC</sub>; P = 0.74; slope, P = 0.27; n = 6; two-tailed unpaired t-test; G/G<sub>max</sub>, ratio of conductance to maximum conductance). (f) h<sub>c</sub> values in the axon and soma exhibit fast and slow exponential components, and each component is statistically indistinguishable between the two locations, as is the relative proportion of their current amplitudes (A<sub>fast</sub> and A<sub>slow</sub>) ([<sub>fast</sub>, P = 0.56; t<sub>slow</sub>, P = 0.33; [A<sub>fast</sub> + A<sub>slow</sub>] = 0.08; two-tailed, unpaired t-test; NS, not significant). Error bars indicate s.e.m.

**Figure 3** Compartment-specific block of resting conductances by laser scanning of AAQ. (a) The unblocking and blocking conformations of AAQ are maintained at 380 and 488 nm light, respectively. R, acrylamide group; QA, quaternary ammonium group. (b) Scanning blockade of different cellular compartments reversibly alters the resting potential and duration of responses to simulated EPSCs during whole-cell current-clamp recordings. Purple and green traces: unblocked and blocked conformations of AAQ, respectively. Black traces: simulated EPSCs (t<sub>depol</sub>, 0–4 nA, 0.8 nA steps. (c) Compartment-specific effects of 488 nm illumination of AAQ on resting potentials (gray bars; P = 2.8 × 10<sup>–4</sup> for AIS, P = 4.72 × 10<sup>–7</sup> for soma, and P = 1.3 × 10<sup>–4</sup> for cell; n = 9). The resting potential is not altered by 488-nm scanning in the presence of the HCN channel blocker ZD7288 (20 μM) applied through the recording pipette (blue bars, ZD7288, Internal; P = 0.07 for cell, P = 0.66 for AIS; n = 6). Block of HCN channels with external 10 μM ZD7288 or 2 mM CsCl hyperpolarizes the resting potential when AAQ is under 380 nm illumination, but after restoration of the membrane potential to control values with DC current, no further changes in voltage are induced with 488 nm scanning (blue bars, ZD7288 “Bath”; P = 0.012 for cell at 380 nm, P = 0.08 for AIS and P = 0.86 for cell at 488 nm, n = 5; red bars, Ca<sup>2+</sup> “Bath”; P = 0.0001 for cell at 380 nm, P = 0.24 for AIS and P = 0.13 for cell at 488 nm, n = 6). Spatial dependence of photoswitch-induced changes in resting potential. Three ROIs (30 μm long) were placed adjacent from proximal to distal axonal locations (yellow, blue, and green, respectively). Scale bar, 30 μm. (e) Changes in resting potentials occurred only in the proximal ROI overlapping the AIS (0–30 μm, P = 0.0007; 30–60 μm, P = 0.28; 60–90 μm, P = 0.52; n = 5). Paired two-tailed Student’s t-test in c and e. Error bars indicate s.e.m. *P < 0.05; ***P < 0.01; NS, non-significant.
Figure 5. Local pharmacological blockade of HCN channels in the AIS mimics the effects of photoswitches. (a) Top, first suprathreshold spike in response to a train of somatic simulated EPSCs (100 Hz; 0-6 nA, 0.4 pA steps) in a whole-cell current-clamp recording in control (black) and following brief focal pressure application of a NaCl-based vehicle onto the AIS (gray). Bottom, phase plot of the first spike under each condition. Dotted lines, spike threshold. The vehicle (Veh, gray) altered neither the shape, maximum rate of rise (dV/dt) nor threshold of spikes. Vrest = −59 mV for both control and vehicle. (b) Brief focal pressure application of 50 µM ZD7288 onto the AIS (ZD, blue) hyperpolarized both the resting potential and action potential threshold (phase plot), and increased maximum dV/dt of the spike. Vrest = −52 mV (control, Veh), −54 mV (ZD7288). (c) Focal application of 50 µM ZD7288 to the soma on the opposite side of the AIS hyperpolarized the resting potential but did not change the threshold or shape of the action potential. Vrest = −59 mV and −61 mV for control (Veh) and ZD7288, respectively. (d) Group data quantifying average ∆Vrest, ∆Max dV/dt and ∆Max dV/dt with focal ZD7288 blockade of HCN channels in the AIS (∆Vrest; Veh, P = 0.77, ZD (AIS), P = 0.07, ZD (soma), P = 0.01; ∆Threshold; Veh, P = 0.88, ZD (AIS), P = 0.008, ZD (soma), P = 0.08; ∆Max dV/dt; Veh, P = 0.51, ZD (AIS), P = 0.01, ZD (soma), P = 0.71; Veh, n = 3; ZD, n = 5). Two-tailed paired t test. Error bars indicate s.e.m. ***P < 0.001; NS, non-significant.
9% during somatic blockade (79.8 ± 5.7 mV/ms to 86.9 ± 6.2 mV/ms, P = 0.06, paired two-tailed Student’s t-test). In addition, the voltage threshold for action potentials was significantly more hyperpolarized during AIS blockade (−1.7 ± 0.4 mV, P = 0.03) than for somatic blockade (−0.4 ± 0.3 mV, P = 0.24, paired two-tailed Student’s t-test). The effects of somatic photoswitch blockade on spike probability and shape could be offset completely when light-induced changes in resting potential were compensated for by injecting depolarizing current through the recording pipette (Supplementary Fig. 2). In addition, somatic current injection could induce changes in both spike threshold and maximum dV/dt in the soma but required strong hyperpolarization of the membrane potential (26 mV; Supplementary Fig. 3).

Additional experiments confirmed the involvement of HCN channels in controlling spike probability. In whole-cell current-clamp recordings we generated trains of action potentials at 100 Hz with simulated EPSCs (0 to 6,000 pA in 400 pA increments) and then applied brief puffs of either 50 µM ZD7288 or a NaCl-based vehicle focally to the AIS or soma (Fig. 5a–c). In these experiments, we visualized the axon as before with confocal imaging of Alexa-568 and visualized the spread of ZD7288 by monitoring the spread of fast green (1%), the axon as before with confocal imaging of Alexa-568 and visualized the spread of ZD7288 by monitoring the spread of fast green (1%), and visualized the spread of ZD7288 by monitoring the spread of fast green (1%).

Modulation of HCN channels by 5-HT

HCN channels are known to be the target of numerous modulatory neurotransmitters. Given the strong influence of HCN channels in the AIS on spike initiation, we asked whether modulation of these channels could influence action potential firing. The MSO receives a dense network of serotonergic fibers arising from the dorsal raphe nucleus. To examine whether 5-HT influences the properties of HCN channels, we made whole-cell voltage-clamp recordings from MSO neurons and isolated I_{h} pharmacologically (Fig. 6a and Online Methods). When 5-HT (300 µM) was continuously applied to the axon, soma and proximal dendrites of MSO neurons via a second patch pipette, the activation range of I_{h} was significantly shifted in the hyperpolarizing direction (−60.56 ± 1.96 mV to −71.25 ± 2.34 mV, ∆V_{i} = −10.69 ± 0.67 mV, P = 1.8 × 10^{-5}; n = 6; Fig. 6a,g,h). While instantaneous leak current was stable in the presence of 5-HT (change in instantaneous leak current (ΔInst. Leak) = −3.75 ± 11.93 pA, P = 0.77, n = 6), the maximum I_{h} was rapidly and significantly reduced (change in maximum I_{h} (ΔMax. I_{h}) = −11.93 ± 39.37 pA, P = 0.02; n = 6; Fig. 6b). Application of 5-HT also increased both the fast and slow activation time constants of I_{h} while increasing the relative proportion of total current made up by the slow component (Fig. 6i). The slow component likely reflected the presence of HCN4 subunits in MSO I_{h}.
amplitude and activation voltage of $I_h$ were eliminated in the presence of 10 µM WAY100135, a 5-HT1A antagonist ($\Delta V_{I_h} = -1.44 \pm 0.29$ mV, $P = 0.07$; $\Delta$Max. $I_h = -6.46 \pm 18.68$ pA, $P = 0.74$; $\Delta$Inst. Leak = 27.32 ± 16.66 pA, $P = 0.45$; $n = 7$; Fig. 6d–h). WAY100135 also strongly attenuated the 5-HT–mediated increase in both activation time constants (though $\tau_{\text{fast}}$ was still significantly increased versus control), and eliminated significant changes in the relative amplitudes of $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$ (Fig. 6i). The effects of 5-HT were reversible. In experiments with bath application of 20 µM 5-HT, the amplitude of $I_h$ was reduced and the $V_r$ was negatively shifted, as with local 5-HT applications, and these effects reversed within ~40 min (Supplementary Fig. 5). In addition to 5-HT1A receptors, 5-HT2 receptors have been reported to negatively shift the activation voltage of HCN channels. However, application of ketanserin (10–50 nM), a 5-HT2 antagonist, did not alter 5-HT induced changes in either maximal current amplitude or activation voltages in $I_h$ ($\Delta V_{I_h} = -11.00 \pm 2.08$, $P = 0.013$, $\Delta$Max. $I_h = -157.58 \pm 15.68$ pA, $P = 0.002$; $n = 4$).

To understand whether the modulation of HCN channels by 5-HT affects action potential initiation through a local action in the axon, we made whole-cell current-clamp recordings from MSO neurons and focally applied 5-HT (300 µM) to either the AIS or soma. Application of 5-HT to the AIS hyperpolarized the membrane potential ($\Delta V_{\text{rest}} = -1.29 \pm 0.04$ mV, $P = 0.001$; $n = 7$), decreased spike threshold (change in voltage threshold, $\Delta$Threshold = −2.29 ± 0.44 mV, $P = 0.002$; $n = 7$) and increased the maximum rate of rise of action potentials ($\Delta$Max. dV/dt = 121.21 ± 2.20 mV/ms, $P = 0.002$; $n = 7$) (Fig. 7a–d). However, these effects were non-significant when 5-HT was applied to the AIS during recordings in which HCN channels had been blocked by internal application of 20 µM ZD7288 through the recording pipette (change in resting membrane potential ($\Delta V_{\text{rest}}$), −0.03 ± 0.13 mV/ms, $P = 0.82$; $\Delta$Threshold, −0.08 ± 0.22 mV, $P = 0.78$; change in maximum rate of rise of membrane voltage ($\Delta$Max. dV/dt), −0.34 ± 3.3 mV/ms, $P = 0.92$; $n = 5$). Finally, focal application of 5-HT to the soma produced changes in the resting potential but no significant changes in spike threshold or maximum dV/dt, similarly to the focal blockade of HCN channels by ZD7288 (Fig. 7c,d; $\Delta V_{\text{rest}}$, −1.60 ± 0.20 mV, $P = 0.0004$; $\Delta$Threshold, −0.45 ± 0.23 mV, $P = 0.10$; $\Delta$Max. dV/dt, 3.60 ± 3.02 mV/ms, $P = 0.29$; $n = 6$).

To address whether 5-HT-induced modulation of spiking can be achieved by physiologically relevant concentrations of 5-HT, we electrically stimulated the plexus of serotonergic axons in and around the MSO during current-clamp recordings while blocking AMPA, NMDA, GABA$_A$, and glycine receptors with 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzofuran-4-ylquinoxaline-7-sulfonamide (NBQX) (10 µM), AP-5 (50 µM), gabazine (5 µM) and strychnine (1 µM) respectively. We injected simulated EPSCs through the somatic recording pipette every minute for 5 min and monitored both the resting potential and action potentials (Fig. 8a–d). Recordings were discontinued if the resting membrane potential was not maintained.

![Figure 7](image1.png)

**Figure 7** Spike threshold can be controlled by serotonergic modulation of HCN channels in the AIS. (a) Focal application of 300 µM 5-HT onto the AIS (red hyperpolarizes the resting membrane potential as well as action potential threshold, as compared to the artificial cerebrospinal fluid (ACSF) control (black traces). $V_{\text{rest}} = −58$ mV and −61 mV for control and 5-HT, respectively. (b) The shape and properties of spikes were not altered when 5-HT was applied to the AIS while recording with an intracellular pipette solution containing 20 µM ZD7288 (Int. ZD). $V_{\text{rest}} = −59$ mV for both control and 5-HT conditions. (c) Focal application of 300 µM 5-HT to the soma on the opposite side of the AIS hyperpolarized the resting potential but did not alter spike threshold or shape. $V_{\text{rest}} = −55$ mV and −57 mV for control and 5-HT, respectively. (d) Group data for focal 5-HT application to the AIS in control ACSF ($\Delta V_{\text{rest}}$, $P = 0.001$; $\Delta$Threshold, $P = 0.002$; $\Delta$Max. dV/dt, $P = 0.002$; $n = 7$), internal ZD7288 ($\Delta V_{\text{rest}}$, $P = 0.82$; $\Delta$Threshold, $P = 0.78$; $\Delta$Max. dV/dt, $P = 0.92$; $n = 5$), and somatic application ($\Delta V_{\text{rest}}$, $P = 0.0004$; $\Delta$Threshold, $P = 0.10$; $\Delta$Max. dV/dt, $P = 0.29$; $n = 6$). Paired two-tailed Student’s t-test. Error bars indicate s.e.m. ***$P < 0.001$; NS, non-significant.

![Figure 8](image2.png)

**Figure 8** Axonally stimulated release of 5-HT modulates the resting potential and spike threshold of MSO neurons. (a) Axonal stimulation (200 Hz, 50 pulses) of serotonergic fibers. Excitatory and inhibitory inputs are blocked (CTL) with 10 µM NBQX, 50 µM 6-nitro-1,2,3,4-tetrahydrobenzofuran-4-ylquinoxaline-7-sulfonamide (NBQX) (10 µM), AP-5 (50 µM), gabazine (5 µM) and strychnine (1 µM) respectively. Application of 5-HT to the AIS hyperpolarized the membrane potential ($\Delta V_{\text{rest}}$ = −1.29 ± 0.04 mV, $P = 0.001$; $n = 7$), decreased spike threshold (change in threshold, $\Delta$Threshold = −2.29 ± 0.44 mV, $P = 0.002$; $n = 7$) and increased the maximum rate of rise of action potentials ($\Delta$Max. dV/dt = 121.21 ± 2.20 mV/ms, $P = 0.002$; $n = 7$) (Fig. 7a–d). However, these effects were non-significant when 5-HT was applied to the AIS during recordings in which HCN channels had been blocked by internal application of 20 µM ZD7288 through the recording pipette (change in resting membrane potential ($\Delta V_{\text{rest}}$), −0.03 ± 0.13 mV/ms, $P = 0.82$; $\Delta$Threshold, −0.08 ± 0.22 mV, $P = 0.78$; change in maximum rate of rise of membrane voltage ($\Delta$Max. dV/dt), −0.34 ± 3.3 mV/ms, $P = 0.92$; $n = 5$). Finally, focal application of 5-HT to the soma produced changes in the resting potential but no significant changes in spike threshold or maximum dV/dt, similarly to the focal blockade of HCN channels by ZD7288 (Fig. 7c,d; $\Delta V_{\text{rest}}$, −1.60 ± 0.20 mV, $P = 0.0004$; $\Delta$Threshold, −0.45 ± 0.23 mV, $P = 0.10$; $\Delta$Max. dV/dt, 3.60 ± 3.02 mV/ms, $P = 0.29$; $n = 6$). To address whether 5-HT-induced modulation of spiking can be achieved by physiologically relevant concentrations of 5-HT, we electrically stimulated the plexus of serotonergic axons in and around the MSO during current-clamp recordings while blocking AMPA, NMDA, GABA$_A$, and glycine receptors with 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzofuran-4-ylquinoxaline-7-sulfonamide (NBQX) (10 µM), AP-5 (50 µM), gabazine (5 µM) and strychnine (1 µM) respectively. We injected simulated EPSCs through the somatic recording pipette every minute for 5 min and monitored both the resting potential and action potentials (Fig. 8a–d). Recordings were discontinued if the resting membrane potential was not maintained.
within ± 1 mV for 5 min. In these experiments, a train of 50 stimuli at 200 Hz triggered small membrane depolarizations (less than 1 mV) that rose and decayed over hundreds of milliseconds and lacked discrete synaptic events. These slow depolarizations were reduced on average by 85% in the presence of 100 μM granisetron, an antagonist of ionotropic 5-HT3 receptors (Fig. 8b; control: membrane voltage, 0.53 ± 0.05 mV; granisetron: membrane voltage, 0.06 ± 0.03 mV, P = 1.9 × 10−5; n = 6), suggesting that these events represent the paracrine release of 5-HT throughout the slice acting on ionotropic receptors. Correlated with this electrophysiological correlate of stimulated 5-HT release, we observed robust and more long-term hyperpolarization of both the resting membrane potential and spike threshold (Fig. 8c,d). Changes in resting potential and spike threshold lasted for the duration of recordings (tens of minutes) and were 68% and 95% blocked, respectively, in the presence of 0.1 μM WAY100135 (ΔVrest, −1.54 ± 0.31 mV, P = 0.0039; ΔThreshold: 0.19 ± 0.62 mV, P = 0.81; n = 8) or when recordings were made with 20 μM internal ZD7288 to block HCN channels (ΔVrest, −0.66 ± 0.45 mV, P = 0.40; ΔThreshold, 0.19 ± 0.49 mV, P = 0.56; n = 8). Together these results indicate that physiologically released 5-HT is capable of triggering robust changes in the resting potential and action potential threshold of MSO neurons.

**DISCUSSION**

In most neurons the AIS represents the final stage of synaptic integration, where the complex interplay between excitatory and inhibitory synaptic signals and voltage-gated ion channels determines whether the neuron generates an all-or-none action potential and successfully forwards its signal to its network targets. Action potential initiation sometimes has been regarded as static, exhibiting a discrete and constant threshold. Here we show that HCN channels are expressed in the AIS of auditory neurons in the sound localization circuitry and, by their proximity to the voltage-gated sodium and potassium channels mediating action potentials, regulate spike threshold through their influence on the local resting potential. We further show that modulation of Ih by HCN can modify spike threshold through activation of 5-HT receptors. Thus HCN channels in the AIS are in a position to adjust spike sensitivity in response to changes in motivation or state of attention.

The AIS of MSO neurons and the expression of HCN subunits

Action potential amplitude is unusually small in MSO principal neurons (5–20 mV), and yet immunolabeling for markers of the AIS and dendrites of MSO neurons22,23,24. HCN4 (but not HCN2) subunits are also expressed in the soma and dendrites of MSO neurons22,23,24. HCN4 (but not HCN2) subunits are active at rest is small relative to that of HCN channels22,31. The fraction of HCN channels in the AIS relative to the soma and dendrites. HCN channels expressed in the soma and dendrites are critical to synaptic integration: they shape temporal summation32–34 and contribute to resonance in firing activity in both cortical and hippocampal neurons35,36.

**The regulation of spike threshold by HCN channels**

HCN channels expressed in the soma and dendrites are critical to synaptic integration: they shape temporal summation32–34 and contribute to resonance in firing activity in both cortical and hippocampal neurons35,36. In auditory neurons concerned with preserving submillisecond timing information, including MSO principal neurons, HCN channels provide tonic membrane shunting, which enables fast-rising, precisely timed synaptic events22,31. By contrast, in the AIS, we found that HCN channels specifically reduced the probability of action potential firing (Fig. 4). Presumably this is mediated in part by an increase in sodium channel inactivation in the AIS, consistent with the increase in the rate of rise of the action potential during AIS-targeted HCN channel blockade. It is also possible that KCNA1 (Kv1) channels, which are enriched in the AIS of both auditory and non-auditory neurons8,38, may also activate through HCN channel activity, decreasing spike probability.

While hyperpolarization of the resting potential occurred with blockade or neuromodulation of HCN channels at both the soma and AIS, spike threshold reduction was far more sensitive to HCN channel manipulations in the AIS (Supplementary Fig. 3). Cable properties of the axon versus the soma and dendrites may at least partially account for this disparity. Even modest amplitudes of Ih may produce large local voltage changes in the relatively small-diameter (~0.5 μm) AIS, but the voltage at the soma would be expected to undergo strong attenuation from the unusually large capacitive load imposed by the soma and large caliber dendrites as well as shunting arising from their unusually high resting conductances22. The observations above may also reflect local differences in the density and/or properties of voltage-gated ion channels in the two compartments7,11,39.
Serotonergic modulation of HCN channels in the AIS

Serotonergic projections from the raphe nuclei ramify widely throughout the brain, and they include a dense projection to the MSO and other auditory brainstem nuclei. We found that 5-HT modulates HCN channels in MSO neurons via 5-HT1A receptors, which are coupled to the G proteins (Gq and Gi) that inhibit adenylate cyclase and decrease cAMP concentration.

Applications of 5-HT to whole MSO neurons decreased IBK, hyperpolarized the activation range by ~10 mV and slowed the activation and deactivation kinetics of the channels (Fig. 6), consistent with a decrease in adenylate cyclase activity and reduction in the allosteric modulation of IBK by cAMP.

Notably, activation of 5-HT1A receptors by 5-HT, released by stimulation of serotonergic fibers around the MSO, hyperpolarized the resting potential and spike threshold by modulating HCN channels (Fig. 8a–d), reflecting the local action of 5-HT on both the soma and dendrites, as well as the AIS (Fig. 7a–c).

In neurons from the spinal cord and prefrontal cortex, the action of 5-HT has been shown to decrease action potential firing through a modulatory decrease in voltage-gated Na+ current in the AIS. However, we observed no 5-HT-mediated changes in spike amplitude or shape in our experiments, consistent with effects on voltage-gated sodium channels. In MSO neurons, the release of 5-HT, as reported by voltage changes produced by coactivation of ionotropic 5-HT receptors, increased and decayed over hundreds of milliseconds to seconds, consistent with a paracrine release mechanism observed in spinal cord and other auditory brainstem neurons.

Functional implications

The 5-HT-induced decrease in resting HCN channel conductance in the AIS lowers spike threshold without strongly affecting the membrane potential of the soma and dendrites. AIS calcium channels have also been shown to affect both spike probability and pattern, and these influences too can be altered through modulation with and without coincidence detection. In neurons 44, 1069–1072 (2006).

Hu, W. et al. Distinct contributions of Na(v)1.6 and Na(v)1.2 in action potential initiation and backpropagation. Nat. Neurosci. 12, 996–1002 (2009).

Kole, M.H., Letzkus, J.J. & Stuart, G.J. Axon initial segment Kv1 channels control axonal action potential waveform and synaptic efficacy. Neuron 55, 633–647 (2007).

Grubb, M.S. & Burone, J. Activity-dependent relocation of the axon initial segment fine-tunes neuronal excitability. Nature 465, 1070–1074 (2010).

Kuba, H., Ochi, Y. & Ohmori, H. Presynaptic activity regulates Na(+)-channel distribution at the axon initial segment. Nature 465, 1075–1078 (2010).

Bender, K.J. & Trussell, L.O. Axon initial segment Ca(2+) channels influence action potential generation and timing. Neuron 61, 259–271 (2009).

Martineau, K. et al. Cholinergic afferent stimulation induces axonal plasticity in adult hippocampal granule cells. Neuron 85, 346–363 (2015).

Grotta, B. New roles for synaptic inhibition in sound localization. Nat. Rev. Neurosci. 4, 540–550 (2003).

Berghs, S. et al. betalF spectrin, a new spectrin localized at axon initial segments and nodes of Ranvier in the central and peripheral nervous system. J. Cell Biol. 151, 985–1002 (2000).

Van Wart, A., Trimmer, J.S. & Matthews, G. Polarized distribution of ion channels within microdomains of the axon initial segment. J. Comp. Neuro. 500, 339–352 (2007).

Mourot, A., Tochitsky, I. & Kramer, R.H. Light at the end of the channel: optical manipulation of intrinsic neuronal excitability with chemical photoswitches. Front. Mol. Neurosci. 6, 5 (2013).

Hurley, L.M. & Thompson, A.M. Serotonergic innervation of the auditory brainstem of the Mexican free-tailed bat, Tadarida brasiliensis. J. Comp. Neuro. 435, 78–88 (2001).

Thompson, A.M. & Hurley, L.M. Dense serotonergic innervation of principal nuclei of the superior olivary complex in mouse. Neurosci. Lett. 356, 179–182 (2004).

Lehnert, S. et al. Action potential generation in an anatomically constrained model of medial superior olive axons. J. Neurosci. 34, 5370–5384 (2014).

Scott, L.L., Mathews, P.J. & Golding, N.L. Perisomatic voltage-gated sodium channels actively maintain linear synaptic integration in principal neurons of the medial superior olive. J. Neurosci. 30, 2039–2050 (2010).

Khoura, S. et al. An essential role for modulation of hyperpolarization-activated current in the development of binaural temporal precision. J. Neurosci. 32, 2814–2823 (2012).

Koch, U., Braun, M., Kapfer, C. & Grotte, B. Distribution of HCN1 and HCN2 in rat auditory brainstem nuclei. Eur. J. Neurosci. 20, 79–91 (2004).

Kopp-Scheinfeld, C., Pigott, B.M. & Forsythe, I.D. Nitric oxide selectively suppresses Ih currents mediated by HCN1-containing channels. J. Physiol. (Lond.) 593, 1685–1700 (2015).

Santoro, B. Molecular and functional heterogeneity of hyperpolarization-activated pacemaker channels in the mouse CNS. J. Neurosci. 20, 5264–5275 (2000).

Wagner, B.J., DeGennaro, M., Santoro, B., Siegelbaum, S.A. & Tibbs, G.R. Molecular mechanism of cAMP modulation of HCN pacemaker channels. Nature 411, 805–810 (2001).

Ishii, T.M., Takano, M., Xie, L.-H., Noma, A. & Ohmori, H. Molecular characterization of the hyperpolarization-activated cation channel in rabbit heart sinoatrial node. J. Biol. Chem. 274, 12835–12839 (1999).
28. Bucchi, A., Tognati, A., Milanesi, R., Baruscotti, M. & DiFrancesco, D. Properties of ivabradine-induced block of HCN1 and HCN4 pacemaker channels. J. Physiol. (Lond.) 572, 335–346 (2006).
29. Kramer, R.H., Mourot, A. & Adesnik, H. Optogenetic pharmacology for control of native neuronal signaling proteins. Nat. Neurosci. 16, 816–823 (2013).
30. Mathews, P.J., Jercog, P.E., Rinzel, J., Scott, L.L. & Golding, N.L. Control of submillisecond synaptic timing in binaural coincidence detectors by Kv1 channels. Nat. Neurosci. 13, 601–609 (2010).
31. Baumann, V.J., Lehnert, S., Leibold, C. & Koch, U. Tonotopic organization of the hyperpolarization-activated current (Ih) in the mammalian medial superior olive. Front. Neural Circuits 7, 117 (2013).
32. Harnett, M.T., Magee, J.C. & Williams, S.R. Distribution and function of HCN channels in the apical dendritic tuft of neocortical pyramidal neurons. J. Neurosci. 35, 1024–1037 (2015).
33. Magee, J.C. Dendritic Ih normalizes temporal summation in hippocampal CA1 neurons. Nat. Neurosci. 2, 848 (1999).
34. Poolor, N.P., Migliore, M. & Johnston, D. Pharmacological upregulation of Ih-channels reduces the excitability of pyramidal neuron dendrites. Nat. Neurosci. 5, 767–774 (2002).
35. McCormick, D.A. & Page, G.C. Properties of a hyperpolarization-activated cation current and its role in rhythmic oscillation in thalamic relay neurones. J. Physiol. (Lond.) 431, 291–318 (1990).
36. Narayan, R. & Johnston, D. Long-term potentiation in rat hippocampal neurons is accompanied by spatially widespread changes in intrinsic oscillatory dynamics and excitability. Neuron 56, 1061–1075 (2007).
37. Golding, N.L., Robertson, D. & Oertel, D. Recordings from slices indicate that octopus cells of the cochlear nucleus detect coincident firing of auditory nerve fibers with temporal precision. J. Neurosci. 15, 3138–3153 (1995).
38. Dodson, P.D., Barker, M.C. & Forsythe, I.D. Two heteromeric Kv1 potassium channels differentially regulate action potential firing. J. Neurosci. 22, 6953–6961 (2002).
39. Colbert, C.M. & Pan, E. Ion channel properties underlying axonal action potential initiation in pyramidal neurons. Nat. Neurosci. 5, 533–538 (2002).
40. Steinbusch, H.W. Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. Neuroscience 6, 557–618 (1981).
41. Polter, A.M. & Li, X. 5-HT1A receptor-regulated signal transduction pathways in brain. Cell. Signal. 22, 1406–1412 (2010).
42. Wang, J., Chen, S., Nolan, M.F. & Siegelbaum, S.A. Activity-dependent regulation of HCN pacemaker channels by cyclic AMP: signaling through dynamic allosteric coupling. Neuron 36, 451–461 (2002).
43. Coté, F., Exley, R., Cragg, S.J. & Perrier, J.F. Serotonin spillover onto the axon initial segment of motoneurons induces central fatigue by inhibiting action potential initiation. Proc. Natl. Acad. Sci. USA 110, 4774–4779 (2013).
44. Yin, L. et al. Selective modulation of axonal sodium channel subtypes by 5-HT1A receptor in cortical pyramidal neuron. Cereb. Cortex http://dx.doi.org/10.1093/cercor/bht245 (2015).
45. Tang, Z.Q. & Trussell, L.O. Serotonergic regulation of excitability of principal cells of the dorsal cochlear nucleus. J. Neurosci. 35, 4540–4551 (2015).
46. Sturgill, J.F. & Isaacson, J.S. Somatostatin cells regulate sensory response fidelity via subtractive inhibition in olfactory cortex. Nat. Neurosci. 18, 531–535 (2015).
47. Wilson, N.R., Runyan, C.A., Wang, F.L. & Sur, M. Division and subtraction by distinct cortical inhibitory networks in vivo. Nature 488, 343–348 (2012).
48. Yamada, R., Koba, H., Ishii, T.M. & Ohmon, H. Hyperpolarization-activated cyclic nucleotide-gated cation channels regulate auditory coincidence detection in nucleus laminaris of the chick. J. Neurosci. 25, 8867–8877 (2005).
49. Franken, T.P., Roberts, M.T., Wei, L., Golding, N.L. & Joris, P.X. In vivo coincidence detection in mammalian sound localization generates phase delays. Nat. Neurosci. 18, 444–452 (2015).
50. Roberts, M.T., Seemann, S.C. & Golding, N.L. A mechanistic understanding of the role of feedforward inhibition in the mammalian sound localization circuitry. Neuron 78, 923–935 (2013).
ONLINE METHODS

Animal use. All procedures were conducted in accordance with The University of Texas at Austin Institutional Animal Care and Use Committee, following guidelines of the National Institutes of Health.

Immunostaining. Gerbil brains (21 d postnatal) were acutely dissected and drop-fixed in ice cold 4% paraformaldehyde, pH 7.2, for 1 h. We then transferred the brains to 20% sucrose in 0.1 M phosphate buffer (PB) overnight, and then to 30% sucrose in 0.1 M PB until sectioning. We cut 40 µm thick coronal sections of brainstem on a cryostat and mounted them on coverslips. Sections were then immunostained as described using mouse antibodies against HCN1 (clone N70/28, NeuroMab), PanNav52, or Caspr (clone K65/35, NeuroMab), and rabbit antibodies against HIF spectrin or Nav1.6 (ref. 54). AIS lengths were measured using Zeiss Zen software (Zeiss, Thornwood, NY) or Image J (NIH). For AIS lengths, the start and end of the AIS was defined as the point at which the immunoreactivity fell below 10% of the maximum fluorescence intensity along the AIS. Statistics were performed using GraphPad Prism.

Brainstem slice preparation. Mongolian gerbils (Meriones unguiculatus) of both sexes were obtained from Charles River Laboratories or bred at the Animal Resource Center of the University of Texas at Austin. Litters were group housed and kept on a 12-h light/dark cycle. Gerbils (P18–P23) were anesthetized with isoflurane, decapitated, and the brain rapidly removed in artificial cerebrospinal fluid (ACSF) at 32 °C. ACSF was bubbled with 95% O2 and 5% CO2 and contained using Zeiss Zen software (Zeiss, Thornwood, NY) or Image J (NIH). Pipette solutions were identical to those in current-clamp recordings. Experiments were conducted at room temperature in ACSF. To reduce the effects of whole-cell dialysis on the activation voltage of HCN channels we used patch-pipettes with open tip resistances of between 4 and 5 MΩ (as in ref. 22) and the time of data collection was limited to 15 min. Series resistance was compensated by at least 95% and pipettes were coated with Sylgard to reduce capacitance. For pharmacologically isolating Ih, the following were added to the external ACSF: 1 mM 3,4-diaminopyridine (3,4-DAP), 10 mM TEA-Cl, 0.2 mM 4-aminoypyridine, 0.2 mM BaCl2, 0.001 mM TTX, 0.05 mM NiCl2, 0.2 mM CoCl2, 0.01 mM NBQX, 0.05 mM p-AP5, and 0.001 mM strychnine. DENAQ exhibits the cis (unblocked) conformation in visible light (450–550 nm) and relaxes rapidly to the trans (blocked) conformation in the dark17,57. Thus, in DENAQ experiments cells were maintained in the dark in the blocked state and then Ih was unblocked by scanning regions of interest with the 488 nm laser. Ih in ROIs was revealed by subtracting Ih records in 488 nm illumination from those recorded in the dark. All reported membrane potentials recorded with K-glucuronate are corrected for a liquid junction potential of 10 mV.

Synaptic stimulation. We activated synaptic inputs to MSO cells by delivering brief (0.1 ms) electrical pulses to the slice through patch pipettes (tip diameter: 10 µm). Stimulation electrodes were placed either medial or lateral to the MSO, activating either contralateral or ipsilateral excitatory inputs from the cochlear nucleus, respectively. The probability of action potentials driven by synaptic excitation was not affected by illumination of AAQ in photoswitch experiments when light-induced changes in postsynaptic potential were offset with direct current, precluding significant presynaptic effects of AAQ (Supplementary Fig. 2).

Focal application of ZD7288 or 5-HT. The puffing solution contained 10 mM glucose, 125 mM NaCl, 2.5 mM KCl, 3 mM HEPES, and 1% fast green for monitoring the spread of drugs. ZD7288 (50 µM) or 5-HT (300 µM) was included in the puffing solution. Initially we visualized the AIS of MSO neurons through a confocal microscope before gently applying either ZD7288 or 5-HT to the AIS.

Data analysis and statistics. All data analyses were performed using custom routines implemented in Igor Pro (WaveMetrics, Lake Oswego, OR). For voltage-clamp recording, MSO neurons were held at −60 mV, and then depolarized to −30 mV for 1 s to deactivates Ih. Subsequently, Ih was activated with voltage steps from −30 to −110 mV in 10 mV increments and followed by a 1 s step to −100 mV to elicit tail currents. Peak tail currents were averaged from the 10 ms immediately following the capacitive transient and normalized to minimum and maximum tail currents obtained from −110 mV and −30 mV prepulses, respectively. We quantified the activation range of HCN channels by plotting peak tail currents to maximum and minimum Ih and fitting this relationship to a Boltzmann equation of the form

$$V = \frac{V_0 + (V_1 - V_0) + \frac{1}{1 + \exp((V_1 - V_0)/k)}}{1 + \exp((V_1 - V_0)/k)}$$

where V is the membrane voltage, V1 is the half-maximal activation voltage, and k is the slope factor. Time constants of activation of Ih were measured using bi-exponential fits of tail currents in response to voltage steps from −30 mV to −110 mV. In all experiments, values are presented as mean ± s.e.m., and statistical significance was assessed using a...
two-tailed Student’s \( t \) test or a two-way ANOVA for repeated measures at a significance level of 0.05 unless otherwise indicated. Data distribution was assumed to be normal but this was not formally tested. No statistical methods were used to predetermine sample sizes but they were consistent with other publications in the field. Numbers of replications (\( n \)) are equal to the number of animals used unless indicated otherwise.

A Supplementary Methods Checklist is available.

51. Chang, K.J. et al. Glial ankyrins facilitate paranodal axoglial junction assembly. Nat. Neurosci. 17, 1673–1681 (2014).
52. Rasband, M.N. et al. Dependence of nodal sodium channel clustering on paranodal axoglial contact in the developing CNS. J. Neurosci. 19, 7516–7528 (1999).
53. Yang, Y., Lacas-Gervais, S., Morest, D.K., Solimena, M. & Rasband, M.N. BetaIV spectrins are essential for membrane stability and the molecular organization of nodes of Ranvier. J. Neurosci. 24, 7230–7240 (2004).
54. Schafer, D.P., Custer, A.W., Shraga, P. & Rasband, M.N. Early events in node of Ranvier formation during myelination and remyelination in the PNS. Neuron Glia Biol. 2, 69–79 (2006).
55. Scott, L.L., Mathews, P.J. & Golding, N.L. Posthearing developmental refinement of temporal processing in principal neurons of the medial superior olive. J. Neurosci. 25, 7887–7895 (2005).
56. Banghart, M.R. et al. Photochromic blockers of voltage-gated potassium channels. Angew. Chem. Int. Edn. Engl. 48, 9097–9101 (2009).
57. Tochitsky, I. et al. Restoring visual function to blind mice with a photoswitch that exploits electrophysiological remodeling of retinal ganglion cells. Neuron 81, 800–813 (2014).