 Ionic Basis for Membrane Potential Resonance in Neurons of the Inferior Olive

Graphical Abstract

Inferior olivary neuron

Soma

Dendrite

HCN1 channel cluster

Cav3.1 channel cluster

Resonating conductance

Amplifying conductance

Membrane potential

Time

Electrical resonant property

Highlights

- Activation of Cav3.1 in resonance is membrane potential dependent
- Activation of HCN1 in resonance is dependent on frequency of input currents
- HCN1 and Cav3.1 puncta are clustered on dendrites of inferior olivary neurons
- HCN1 and Cav3.1 act as resonating and amplifying conductances, respectively

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In Brief

Matsumoto-Makidono et al. find that electrical resonance in inferior olivary neurons is mainly mediated by the activation of HCN1 and Cav3.1 channels, which act as frequency-dependent resonating conductance and depolarization-dependent amplifying conductance, respectively.

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Ionic Basis for Membrane Potential Resonance in Neurons of the Inferior Olive

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SUMMARY

Some neurons have the ability to enhance output voltage to input current with a preferred frequency, which is called resonance. Resonance is thought to be a basis for membrane potential oscillation. Although ion channels responsible for resonance have been reported, the precise mechanisms by which these channels work remain poorly understood. We have found that resonance is reduced but clearly present in the inferior olivary neurons of Cav3.1 T-type voltage-dependent Ca2+ channel knockout (KO) mice. The activation of Cav3.1 channels is strongly membrane potential dependent, but less frequency dependent. Residual resonance in Cav3.1 KO mice is abolished by a hyper-polarization-activated cyclic nucleotide-gated (HCN) channel blocker, ZD7288, and is partially suppressed by voltage-dependent K+ channel blockers. Resonance is inhibited by ZD7288 in wild-type mice and impaired in HCN1 KO mice, suggesting that the HCN1 channel is essential for resonance. The ZD7288-sensitive current is nearly sinusoidal and strongly frequency dependent. These results suggest that Cav3.1 and HCN1 channels act as amplifying and resonating conductances, respectively.

INTRODUCTION

Membrane potential oscillation is thought to play crucial roles in the generation of the neural rhythms that underlie various brain functions (Buzsaki, 2006; Hutcheon and Yarom, 2000; Llinás, 1988; Wang, 2010). It has been proposed that the ability to generate subthreshold membrane potential oscillations (STOs) is attributable to the electrical resonant properties of the neuronal membrane (Hutcheon and Yarom, 2000; Llinás, 1988). The magnitude of membrane voltage in response to sinusoidal current injection is strongly enhanced when the frequency of the injected current is close to a specific frequency (the resonant frequency) (Erchova et al., 2004; Hutcheon and Yarom, 2000; Lampl and Yarom, 1997; Pulil et al., 1986), which is the result of a frequency-dependent enhancement of membrane impedance. Enhanced impedance is caused by electrical resonance that occurs in circuits electrically equivalent to the parallel resonant circuit inherent to the plasma membrane (Erchova et al., 2004; Hutcheon and Yarom, 2000; Narayanan and Johnston, 2008; Pulil et al., 1986). The resistor and capacitor depend on the passive membrane property, and the phenomenological inductor is thought to be mediated by voltage-dependent ion channels.

There are several ion channel candidates for resonance, such as T-type voltage-dependent Ca2+ channels (T-type VDCCs), hyperpolarization-activated cyclic nucleotide-gated (HCN) channel blockers, ZD7288, and is partially suppressed by voltage-dependent K+ channel blockers. Resonance is inhibited by ZD7288 in wild-type mice and impaired in HCN1 KO mice, suggesting that the HCN1 channel is essential for resonance. The ZD7288-sensitive current is nearly sinusoidal and strongly frequency dependent. These results suggest that Cav3.1 and HCN1 channels act as amplifying and resonating conductances, respectively.

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Neurons in the inferior olive (IO) project climbing fibers to the cerebellar cortex, which forms strong excitatory synapses on Purkinje cells. IO neurons exhibit STOs ranging from 2 to 10 Hz (Benardo and Foster, 1986; Bleasel and Pettigrew, 1992; Devor and Yarom, 2002; Khosrohani et al., 2007; Llinás and Yarom, 1986). Previous analyses have demonstrated that T-type VDCCs crucial for olivo-cerebellar functions (Hildebrand et al., 2009; Ly ná s and Yarom, 1986; Placantonakis and Welsh, 2001) and can play a pivotal role in resonance and STOs in IO neurons. They are suppressed by T-type VDCC blockers (Benardo and Foster, 1986; Bleasel and Pettigrew, 1992; Devor and Yarom, 2002; Khosrohani et al., 2007; Llinás and Yarom, 1986, 1981). In some cases, Ca2+ spikes were associated with brief damped oscillation (Figure S1A). Depolarizing current injections elicited high threshold Ca2+ spikes (Figure S1B). The active and basic (Table S1) membrane properties of IO neurons were consistent with previous reports (Bal and McCormick, 1997; Best and Regehr, 2008; Choi et al., 2010).

To assess the electrical resonance in IO neurons, we utilized impedance amplitude profile (ZAP) stimulus (Lampl and Yarom, 1997; Puil et al., 1986) (see the Experimental Procedures). Because the voltage-dependent Na+ channels are not involved in resonance in IO neurons (Benardo and Foster, 1986; Llinás and Yarom, 1986), TTX was always added to the extracellular solution. As previously reported (Lampl and Yarom, 1997), the impedance-frequency (Z-F) profile is strongly dependent on membrane potential. Impedance was low and basically declined with input frequency at a membrane potential of ~40 mV (Figures 1A–1C). At ~60 mV, the magnitude of the voltage response was enhanced around 3 s (roughly corresponding to 3 Hz), and the Z-F profile exhibited a clear hump (Figure 1C). The majority of neurons (25/27; Figure S1G) exhibited a resonant frequency >2 Hz (Figure 1E). The resonant strength (see the Experimental Procedures) increased with hyperpolarization of the membrane potential, which peaked at ~75 mV, and then declined with further hyperpolarization (Figure 1D). The resonant frequency gradually increased with hyperpolarization (Figure 1E) (Hutcheon et al., 1996b). To examine possible developmental

**RESULTS**

**Electrical Resonance of IO Neurons**

We performed whole-cell recordings from neurons in the IO. In the current clamp mode, hyperpolarizing current injections elicited depolarizing sags as a result of hyperpolarization-activated potential (Figure S1A). Rebound low-threshold Ca2+ spikes followed the hyperpolarizing current offset (Figure S1A) (Choi et al., 2010; Llinás and Yarom, 1981). In some cases, Ca2+ spikes were associated with brief damped oscillation (Figure S1A). Depolarizing current injections elicited high threshold Ca2+ spikes (Figure S1B). The active and basic (Table S1) membrane properties of IO neurons were consistent with previous reports (Bal and McCormick, 1997; Best and Regehr, 2008; Choi et al., 2010).

However, the STO is small, but not completely ablated in mutant mice deficient in Cav3.1 (α1G) (Choi et al., 2010), the major alpha subunit of T-type VDCCs expressed in the IO (Talley et al., 1999). Moreover, several studies support that HCN channels are abundantly expressed (Monteggia et al., 2000; Notomi and Shige moto, 2004; Santoro et al., 2000) and participate in rhythmic firing of IO neurons (Bal and McCormick, 1997). It, therefore, remains undetermined whether the T-type VDCC is the major ion channel responsible for resonance in IO neurons.

In the present study, we have examined ion channels potentially involved in resonance of IO neurons and the behaviors of these channels. Results showed that Cav3.1 T-type VDCCs strongly amplified resonance in a membrane potential-dependent manner, although their activation was less dependent on the frequency of input current or potential. These data suggest that Cav3.1 T-type VDCCs act as an amplifying conductance. The resonating conductance was mainly mediated by HCN1 channels, and the HCN1-dependent conductance exhibited properties necessary for acting as a phenomenological inductor. Additionally, Kv channels were also involved in resonating conductance, although their contribution was restrictive. These results suggest that ion channels with distinct electrophysiological properties cooperatively contribute to resonance in IO neurons.

**Figure 1. Resonance in IO Neurons**

(A) Representative voltage waves in response to chirp current (50 pA) at membrane potentials of −40, −60, −75, and −90 mV. (B) Z-F profiles calculated from traces in (A). (C) Averaged Z-F profiles (n = 27 cells). The shaded area indicates the mean ± SEM. (D and E) Membrane potential dependency of resonant strength (D) and resonant frequency (E). The number of cells is 17 at −50, −65, −70, −80, and −85 mV and 27 at −40, −60, −75, and −90 mV. Averaged data are presented as the mean ± SEM. See also Figures S1, S4, and S6 and Table S1.
changes in the resonance of IO neurons, we compared Z-F profiles at P14–P15, P19–P20, and P30–P37. The membrane potential dependency of resonant strength and resonant frequency was not significantly different among these groups (Figure S1D), suggesting that the resonant properties were almost mature by P14–P15.

We also noticed that positive and negative voltage fluctuations were nearly symmetrical at a baseline of around –60 mV, although depolarizing deflections were clearly larger than hyperpolarizing ones around –75 mV (Figure 1A). These asymmetrical fluctuations were likely caused by regenerative potentials elicited at depolarizing phases. Interpretation of the calculated impedance in the linear RLC circuit requires an approximate linear relationship between input current and output voltage, but the generation of regenerative potentials may disturb the linear relationship (Hutcheon et al., 1996b; Ströhmann et al., 1994). Therefore, the magnitude of impedance from –65 mV to –85 mV might not be properly assessed as that in the linear RLC circuit. However, because the calculated value indicates the absolute ratio of output voltage relative to input current, these data indicate that output voltage significantly increased around a frequency of 3–5 Hz.

Cav3.1 T-type VDCCs Strongly Enhance Resonance

We first examined the contribution of VDCCs to resonance in IO neurons. First, we recorded resonance in a Ca2+-free external solution to suppress VDCC-mediated currents. The resonant strength in the Ca2+-free solution was significantly less than in the control solution (Figures 2A, 2B, and 2E). Moreover, positive and negative voltage fluctuations were almost symmetrical at all tested membrane potentials (Figure 2A), suggesting that the regenerative potentials were mediated by Ca2+ currents. This notion was confirmed by waveform analysis of Ca2+-dependent components (Figures S1E and S1F). These waveforms essentially comprised alternate depolarizing and hyperpolarizing potentials (Figures S1E and S1F). Positive and negative voltage fluctuations were almost symmetrical at –60 mV, but the amplitudes of depolarizing potentials were clearly larger than those of hyperpolarizing potentials at –75 mV (Figures S1E and S1F).

Next, we identified the VDCC subtype responsible for resonance in IO neurons. Bath application of Ni2+, a blocker of T-type and R-type VDCCs, strongly suppressed resonance to a level identical to the Ca2+-free experiment (Figure S2B). We further examined the contribution of T-type VDCCs using Cav3.1 knockout (Cav3.1 KO) mice (Petrenko et al., 2007) in which the generation of rebound Ca2+ spikes was abolished (Figure S2A) (Choi et al., 2010). Similar to the experiment with Ca2+-free external solution, voltage fluctuations were almost symmetrical at all tested membrane potentials in Cav3.1 KO mice (Figure 2C), indicating that the regenerative potentials were low-threshold Ca2+ spikes (Benardo and Foster, 1986). Importantly, the membrane potential dependence of resonant

Figure 2. Cav3.1 Acts as an Amplifying Conductance
(A) Representative voltage traces of a WT IO neuron in the Ca2+-free external solution at membrane potentials of –40, –60, –75, and –90 mV.
(B) Averaged Z-F profiles (n = 11 cells). Dotted lines represent the aforementioned WT data in control solution (Figure 1C). The shaded area indicates the mean ± SEM.
(C) Similar to (A), but voltage traces were recorded from an IO neuron in a Cav3.1 KO mouse.
(D) Averaged Z-F profiles of Cav3.1 KO mice (n = 19).
(E and F) Membrane potential dependency of resonant strength (E) and resonant frequency (F) in WT mice in Ca2+-free external solution (n = 11) and Cav3.1 KO mice (n = 19). Data for WT mice (dotted line) in (E) and (F) are the same as in Figures 1D and 1E, respectively. Averaged data are presented as the mean ± SEM. See also Figures S2, S4, and S5.
Conductance Mediated by the HCN1 Channels Is Essential for Resonance

Although resonant strength was significantly less, the Z-F profile still exhibited a clear hump in Cav3.1 KO mice (Figure 2D), and the resonant frequency was >2 Hz (Figure 2F). To corroborate the linearity between input and output of remnant resonance, we applied chirp currents with amplitudes of 20, 50, and 100 pA and compared the resultant Z-F profiles in Cav3.1 KO mice. Amplitudes of the resultant voltage responses were linearly increased with input currents (Figure 3I), but the Z-F profiles were not affected by this manipulation (Figure 3J). This result ensures a linear relationship between input current and voltage responses of residual resonance (Lampl and Yarom, 1997; Ströhmann et al., 1994).

The residual resonance in Cav3.1 KO mice was not mediated by high-voltage-activated Ca²⁺ channels or by the compensatory upregulation of other T-type VDCC subtypes, because the WT IO neurons exhibited similar resonance in the Ca²⁺-free solution (Figures 2E and 2F). Therefore, we pharmacologically examined other possible candidates. We focused on HCN channels, because they play a crucial role in resonance in some neurons (Hu et al., 2002; Sun et al., 2012; Ulrich, 2014; Zemankovics et al., 2010). In the presence of 20 μM ZD7288, a blocker of HCN channels, impedance declined with the frequency of input currents in Cav3.1 KO mice at all tested membrane potentials (Figures 3A, 3C, and 3G). The resonant frequency was <2 Hz (Figure 3H), indicating a disappearance of resonance. These results suggest that residual resonance in Cav3.1 KO mice is mediated by HCN channels.

To address the precedence between ZD7288-sensitive and Cav3.1-dependent responses, we administered ZD7288 to WT mice. The generation of Ca²⁺ spikes was not blocked (discussed later), but the Z-F profile did not exhibit a clear hump in the presence of ZD7288 at all tested membrane potentials (Figures 3B and 3C), suggesting that HCN channel activation was essential for resonance in IO neurons (Figures 3G and 3H). Taken together, these results strongly suggest that the HCN channel acts as the major resonating conductance in IO neurons.

To identify the HCN channel subtype, we used mutant mice lacking HCN1, a HCN channel subtype strongly expressed in the IO (Monteggia et al., 2000; Notomi and Shigemoto, 2004; Santoro et al., 2000). In the HCN1 knockout (KO) mice, there was no depolarizing sag following the hyperpolarizing current injection (Figure S2C). Responses to chirp currents (Figure 3D) were identical to WT mice with ZD7288 (Figure 3B), and there was no clear hump in the Z-F profile (Figure 3F). In the Ca²⁺-free external solution, the generation of low-threshold Ca²⁺ spikes was eliminated (Figure 3E), and the Z-F profile (Figure 3F) was identical to that of Cav3.1 KO mice with ZD7288 (Figure 3C). These results strongly suggest that ZD7288-sensitive resonance is mediated by the HCN1 channel.

Activation of T-type VDCCs Is Dependent on the Depolarization of the Membrane Potential

Following administration of ZD7288 in WT mice, the frequency for Ca²⁺ spike activation shifted from around 4 Hz in the control extracellular solution (Figure 1A) to a lower frequency range (Figure 3B). If the membrane does not have phenomenological inducers, namely, the circuit is constructed only with resistors and capacitors, the amplitude of the voltage response is largest at the first positive peak and monotonically declines with input frequency (Hutcheon and Yarom, 2000) (Figures 3A and 3E).

Therefore, the shift in frequency for Ca²⁺ spike activation could be explained by a shift in frequency at which the membrane potential depolarization is larger (Hutcheon et al., 1996b; Ulrich, 2014). To test this possibility, we analyzed the threshold voltage of Ca²⁺-dependent responses (Figures 3K–3N). In the control solution, Ca²⁺ potentials were elicited at a membrane potential around ~70 mV, but were not elicited at the first or second peaks that were less than the threshold potential (Figures 3K and 3M). In the presence of ZD7288, Ca²⁺ spike activation shifted to the first peak (Figure 3L), but the thresholds remained around ~70 mV (Figures 3M and 3N). Importantly, hyperpolarizing potentials after Ca²⁺ spikes were smaller in the presence of ZD7288 (Figure 3L) or in HCN1 KO mice (Figure S2D), suggesting they are partially mediated by the closure of HCN1 channels (see Discussion). These results indicated that the activation of T-type VDCCs is strongly dependent on the depolarization of the membrane potential, but is much less dependent on the frequency of the input current.

Voltage-Clamp Analysis of ZD7288-Sensitive and Ca²⁺-Dependent Currents

To confirm the above-mentioned conclusions, ZD7288-sensitive and Ca²⁺-dependent currents were directly recorded under the voltage-clamp mode. We first theoretically verified the frequency dependence of the currents that flowed through electrical equivalent circuits shown in Figure 4A. When the input voltage was clamped in a sine wave pattern (Figure 4B), the current with a sinusoidal waveform mainly flowed through the inductor at a lower frequency range (Figures 4C–4F and 4I, blue lines and symbols). The magnitude of the current flowing through the inductor monotonically declined with increased frequency of input voltage (Figure 4I). Conversely, the sum of currents flowing through the capacitor and resistance monotonically increased (Figure 4H, purple symbols). As a result, the amplitude-frequency plots of total currents exhibited a bottom (Figure 4H, red symbols). In parallel, the phase of the current relative to the input voltage shifted from a lag mainly caused by the inductor to a lead as a result of the capacitor around the resonant frequency (Figure 4G, red symbols). We mainly focused on the following three current properties: (1) sinusoidal waveform, (2) phase shift from lag to lead, and (3) frequency dependence of current amplitude. First, we examined the properties of the ZD7288-sensitive current using Cav3.1 KO mice. As theoretically expected (Figure 4H, red symbols), the amplitudes of total currents in response to the sinusoidal voltage-clamp (Figure 5A) were smallest around the resonant frequency (Figures 5B–5E and 5G). In the presence of ZD7288, the amplitude of the total currents was monotonically increased (Figures 5B–5E and 5G), which represented the...
response in the RC circuit (Figure 4H, green). Moreover, the phases of the total currents changed from lag to lead in the control solution (Figure 5F, red), but they were always lead in the presence of ZD7288 (Figure 5F, green) (Narayanan and Johnston, 2008). The isolated ZD7288-sensitive currents exhibited nearly sinusoidal waveforms, and the peak amplitudes declined...
with increase in input frequency (Figures 5B–5E and 5H, blue). These results support the notion that the ZD7288-sensitive component exhibits properties required for a phenomenological inductor.

Next, we examined the Ca\textsuperscript{2+}-dependent current using WT mice. In contrast to the ZD7288-dependent current, the isolated Ca\textsuperscript{2+}-dependent current exhibited only inward components (Figures 6B–6E, blue) to the sinusoidal voltage-clamp (Figure 6A). Ca\textsuperscript{2+}-dependent currents were repeatedly elicited on every depolarizing phase of the clamping voltage when the depolarization overcame a threshold voltage (Figures 6F and 6G), suggesting strong membrane potential dependency. These results indicate that the properties of the Ca\textsuperscript{2+}-dependent component are not suitable for a phenomenological inductor.

Cav3.1 and HCN1 Clusters Do Not Overlap, but Are Closely Apposed, on Dendrites of IO Neurons

To better understand the morphological basis, we examined Cav3.1 and HCN1 distributions. We first examined mRNA expression by fluorescence in situ hybridization (FISH) and found that virtually all IO neurons co-expressed Cav3.1 and HCN1 (Figures 7A and 7B). Next, we virally labeled IO neurons with GFP (Figure S3D) and visualized Cav3.1 and HCN1 distributions using specific antibodies (Figures S3A–S3C). HCN1 (Milligan et al., 2006) and Cav3.1 expression was revealed as a punctate appearance on dendrites (Figures 7C–7E) and only occasionally overlapped (Figures 7D3 and 7E3). We further characterized subcellular distribution by utilizing pre-embedding immunogold electron microscopy. Cav3.1 (Figure S3E) and HCN1 (Figure S3F) immunoparticles were mainly associated with the plasma membrane, with higher densities on dendritic shafts and spines than on the soma (Figures S3G and S3H). We then performed SDS-digested freeze-replica immunogold labeling (SDS-FRL) to quantify the 2D distribution of Cav3.1 and HCN1 on the plasma membrane of dendrites. Cav3.1- or HCN1 immunogold particles frequently formed distinct clusters on the P-face of dendrites in WT mice (Figure 7F), and these clusters were not observed in the corresponding KO mice (Figures S3I and S3J). Measurements of nearest-neighbor distances between clusters revealed that most were located within 0.6 μm of each other (Figures 7G and 7H). These results morphologically confirm a close functional relationship between channels on IO dendrites.

The Contribution of Voltage-Dependent Potassium Channels to Resonance

We then examined the contributions of other factors potentially involved in STOs or resonance. Previous studies have shown that electrical coupling through gap junctions significantly influence STOs in IO neurons (Bazzigaluppi et al., 2012; De Zeeuw et al., 2003; Leznik and Llinás, 2005; Long et al., 2002; Placantonakis et al., 2006). Our results, however, showed that carbamoyl chloride (100 μM), a gap junction blocker, did not affect resonance in IO neurons (Figure S4). Next, we examined the possible contributions of K\textsuperscript{+} channels (Hu et al., 2002; Puil et al., 1988). Bath-applied UCL1684, a blocker of SK-type Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels, or intracellular loading of BAPTA did not block resonance in WT mice (Figure S5), suggesting that Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel activation plays a negligible role, if any, in resonance. Conversely, intracellular loading of tetraethylammonium (TEA), a broad-spectrum blocker of Kv channels (Hille, 2001), suppressed remnant resonance in Cav3.1 KO mice (Figures S6C and S6D). Kv channels strongly expressed in the IO include Kcnd2 (Kv4.2), Kcnd3 (Kv4.3), Kcnq3, and Kcnh7 (Kv11.3) (Allen Mouse Brain Atlas [available from http://mouse.brain-map.org]; Lein et al., 2007). However, the Kv4 blocker heteropodatoxin and the KCNQ blocker linopirdine did not alter resonance on the soma (Figures S3G and S3H).
but the Kv11 blocker E-4031 had an effect similar to that of TEA (Figures S6A–S6E). Although ZD7288 blocked resonance in all tested membrane potentials, TEA and E-4031 were less effective at hyperpolarized potentials (Figures S6A–S6D). Finally, we examined the components affected by these blockers. In the voltage-clamp mode, ZD7288 or E-4031 suppressed both inward and outward currents (Figure S6F). Suppression by E-4031 and ZD7288 was occluded (Figure S6G), suggesting that tight functional coupling between HCN and Kv channels plays a role in controlling resonance. Taken together, these results suggest that Kv channels modulate resonance around the resting membrane potential.

DISCUSSION

Cav3.1 Acts as an Amplifying Conductance

In the present study, resonance in IO neurons was greatly suppressed, but not completely abolished, in WT IO neurons in Ca²⁺-free external solution (Figures 2E and 2F). This manipulation did not shift the resonant frequency (Figure 2F), but ZD7288 completely blocked resonance (Figures 3G and 3H), which suggests that T-type VDCCs strongly enhance resonance, but do not act as a resonating conductance. The Ca²⁺-dependent currents were almost inward and were only activated when the membrane potential exceeded the threshold (Figures 3K–3N and 6F–6G). The frequency dependence of the Ca²⁺-dependent component is not very strong, because ZD7288 shifts the generation of Ca²⁺ spikes to a lower frequency range. These properties are not suitable for resonating conductance, but rather enable Cav3.1 T-type VDCCs to act as an amplifying conductance. Cav3.1 might strongly enhance the small depolarizations induced by HCN1-dependent basal resonance.

Conversely, Cav3.1 might also influence HCN1 activation. Results showed that each Cav3.1-mediated Ca²⁺ spike was followed by a hyperpolarizing potential (Figures S1E and S1F). Because the hyperpolarizing potential was suppressed by ZD7288 (Figure 3L) and the amplitude was small in HCN1 KO mice (Figure S2D), it is likely to be mediated mainly by HCN1 channels. The duration of depolarization (119 ± 20 ms [n = 5, mean ± SEM] and hyperpolarizing (157 ± 20 ms) potentials of the Ca²⁺ spike was identical (p = 0.14, Mann-Whitney U test), and the total duration was 280 ms, which approximately corresponds to 4 Hz. Therefore, around the resonant frequency, the Ca²⁺ spike is in phase with the voltage oscillation and can most effectively amplify the voltage responses. Importantly, previous reports have suggested that HCN channels are partially activated at the resting membrane potential and strong depolarization facilitates their closure (Bal and McCormick, 1997). Partial activation of HCN1 at the resting membrane potential is also supported by the hyperpolarized membrane potential of HCN1 KO mice (Table S1). Large depolarization by Cav3.1 activation might facilitate HCN1 closure.

Our morphological analysis demonstrates that Cav3.1 and HCN1 are located on dendritic compartments and are close enough to electrically interact (Figure 7). This distribution might be a basis for tight functional coupling between Cav3.1 and HCN1. In some models of the IO neuron, T-type VDCCs are located on somatic compartments (De Gruijl et al., 2012; Schweighofer et al., 1999). However, the present study reveals that Cav3.1 is distributed on dendrites. Thus, the model of the IO neuron should be revised, taking into account the precise subcellular localization of Cav3.1 and HCN1.
HCN1 Is a Major Component for Frequency-Dependent Resonating Conductance

Although resonance in Cav3.1 KO mice was significantly less than in WT mice, it was clearly present and completely blocked by ZD7288 (Figures 3G and 3H). This indicates that the remnant resonance is attributable to HCN channels, and their activation can induce resonance without Cav3.1. Moreover, resonance in WT mice was blocked by ZD7288 (Figure 3B and 3C) and impaired in HCN1 KO mice (Figures 3D and 3F), suggesting that HCN1 is essential for resonance. The Z-F profiles of HCN1-dependent resonance were constant when the input current amplitudes were changed (Figures 3I and 3J), and this is consistent with a linear relationship between input current and output voltage. Moreover, it also suggests that HCN channel activation is strongly dependent on frequency rather than on the magnitude (at least within ±10 mV) of the membrane potential. Our voltage-clamp analysis revealed that the ZD7288-sensitive current exhibited frequency properties identical to that flowing in the inductor in the parallel resonant circuit (Figure 5). These lines of evidence indicate that HCN1 conductance displays properties that exhibit resonating conductance. Previous reports suggest that the HCN channel acts as a resonating conductance in several neuronal types (Marcelin et al., 2012; Nolan et al., 2007; Ulrich, 2002; Zemankovics et al., 2010). Moreover, theoretical analyses demonstrate that the HCN channel can reproduce resonance (Hutcheon et al., 1996a; Narayanan and Johnston, 2008). The HCN channel may be widely shared as a common resonating conductance in many brain regions. However, a previous study has concluded that HCN channels do not play a major role in resonance in IO neurons (Lampl and Yarom, 1997). This discrepancy might be due to the differences in animal species and/or the channel blockers used in the experiments.

The present analysis demonstrates that Kv channels influence resonating conductance, with the most likely candidate being Kv11 (Figure S6). However, although ZD7288 completely blocked resonance at all tested membrane potentials, TEA or E-4031 blocking was weaker with hyperpolarization of the membrane potential (Figures S6C and S6D), suggesting that Kv channel contribution is restrictive. The question remains as to how HCN and Kv channels are involved in resonance around a resting potential. Because ZD7288 or E-4031 alter inward and outward currents (Figure S6F), and the blocking effect of E-4031 was not additive to ZD7288 (Figure S6G), the functional interplay between HCN and Kv channels should be so tight that blockade of either channel alone could disrupt resonance in IO neurons. Membrane potential-dependent alteration of ion channels mediating resonance has been reported in several neuronal types (Hu et al., 2002; Xue et al., 2012). Because the resting membrane potential of IO neurons is near the activation limit of HCN channels, support by Kv channels might be required to ensure sufficient opening of the HCN channels.

Ionic Mechanisms Underlying Subthreshold Oscillations

Hutcheon and Yarom proposed that a stable STO requires interplay between resonating and amplifying conductances (Hutcheon and Yarom, 2000). Therefore, HCN1 and Cav3.1 channels might also participate in the generation of STOs in IO neurons.
In IO neurons, STOs are dependent on VDCCs (Benardo and Foster, 1986; Choi et al., 2010; Lampl and Yarom, 1997; Llinás and Yarom, 1986; Placantonakis and Welsh, 2001) and rhythmic firing is affected by HCN channels (Bal and McCormick, 1997). In addition, STOs also show a similar waveform transition to that observed in resonance when membrane potential is hyperpolarized. STO waveforms are nearly sinusoidal at resting potentials, but depolarizing phases become larger at more hyperpolarized membrane potentials (Benardo and Foster, 1986; Bleasel and Pettigrew, 1992; Lampl and Yarom, 1997). These lines of evidence suggest that resonance and STO in IO neurons share common ionic mechanisms.

However, there are also several differences between resonance and STO. Previous studies show that IO neurons generate STOs with a wide frequency range from 2 to 10 Hz (Devor and Yarom, 2002; Placantonakis et al., 2000). However, resonant frequency has a narrower normal distribution that peaks around 3 Hz (Figure S1 G) and does not cover the higher frequency range (8–10 Hz). Moreover, some IO neurons exhibit multi-peaks in the power spectra (Bleasel and Pettigrew, 1992; Lampl and Yarom, 1997), but the Z-F profile has a single peak, suggesting that neuronal membranes do not have the intrinsic resonant property to respond to two or more frequencies at once. These results suggest that STO frequency is more variable than what was theoretically predicted from resonant frequency and that factors other than resonance might contribute to STOs in neurons. Electrical coupling through gap junctions appears to be an additional factor that enables neurons to generate STOs with a wide frequency range. Although we have shown that resonance in IO neurons is not dependent on gap junctions (Figure S4), previous reports have suggested that gap junction modulation alters membrane potential dependency of STO frequency (De Zeeuw et al., 2003; Leznik and Llinás, 2005; Long et al., 2002; Placantonakis et al., 2006) and the bursting pattern of IO neurons (Bazzigaluppi et al., 2012). Further studies are required to identify additional factors and to elucidate the mechanisms of STOs.

**EXPERIMENTAL PROCEDURES**

Detailed experimental procedures are described in the Supplemental Experimental Procedures.

**Animals**

All animal experiments were performed in accordance with guidelines from the experimental animal ethics committees of Hiroshima University, Hokkaido University, Fukushima Medical University, the University of Tokyo, and Niigata University. C57BL/6 mice were used in most experiments. Details for the generation of Cav3.1 KO mice (RBRC01465, Riken BRC) (Petrenko et al., 2007) and HCN1 KO mice (#016566, Jackson Laboratory) (Nolan et al., 2003) have been previously described.

**Electrophysiology**

Coronal brain slices including the IO were prepared from C57BL/6, Cav3.1 KO, and HCN1 KO mice at P14–P37. The mice were anaesthetized with CO2 gas and subsequently decapitated. A tissue block that included the medulla was sliced in the sucrose-based cutting solution. Whole-cell recordings were made from neurons in the principal and the dorsal accessory nuclei of the IO using an upright microscope equipped with an IR-CCD camera system. The properties of resonance were not significantly different between the IO subnuclei (Figure S1C). NBQX (10 μM), (R)-CPP (5 μM), bicuculline methchloride

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**Figure 7. Cav3.1 and HCN1 Distribution in the IO**

(A and B) Double-labeling FISH for Cav3.1 (A1) and HCN1 (A2) mRNA expression in the IO. At a higher magnification, Cav3.1 (green) and HCN1 (red) mRNA are co-expressed in IO neurons (B).

(C–E) Triple immunofluorescence showing Cav3.1 and HCN1 localization on dendrites of GFP-labeled neurons. Squared areas in (C) are shown in (D) and (E). Arrows and arrowheads indicate Cav3.1- and HCN1-positive puncta, respectively.

(F) SDS-FRL confirms Cav3.1 and HCN1 localization on the dendritic plasma membrane. Immunogold clusters for Cav3.1 and HCN1 are pseudo colored in green and red, respectively.

(G and H) Summary histograms show the nearest-neighbor distance measured from Cav3.1- to HCN1-positive clusters (G) and vice versa (H) on IO dendrites. A total of 58 Cav3.1-positive and 87 HCN1-positive clusters containing two or more immunogold particles were analyzed from ten dendrites (average of 2 μm) in two mice. Scale bars, 100 μm (A), 20 μm (B), 10 μm (C), 2 μm (D and E), and 500 nm (F).

See also Figure S3.
Impedance Measurement

Impedance was measured when neurons did not show the STO as previously described (Hutcheon and Yarom, 2000; Lampl and Yarom, 1997; Pull et al., 1986). A sinusoidal current with a constant amplitude (50 pA), but linearly altered frequency at a range of 0 to 40 Hz in 40 s (a chirp current), was applied 1986). A sinusoidal current with a constant amplitude (50 pA), but linearly described (Hutcheon and Yarom, 2000; Lampl and Yarom, 1997; Puil et al., 2009), goat anti-GFP (Takasaki et al., 2010), rabbit anti-HCN1 against amino acid residues 888–910 of mouse HCN1 (NP_034538), and commercial rabbit anti-HCN1 (APC-056, Alomone).

Fluorescent In Situ Hybridization

Under deep diethyl ether anesthesia, mice were decapitated and their brains were immediately frozen in powdered dry ice. Frozen sections were cut on a cryostat. Non-isotopic in situ hybridization was employed with fluorescein- and digoxigenin (DIG)-labeled cRNA probes for Cav3.1 and HCN1 mRNAs.

Immunofluorescence

Under deep pentobarbital anesthesia, mice were fixed and coronal sections were prepared. Sections were incubated overnight with a mixture of primary antibodies, followed by incubation with fluorescent secondary antibodies for 2 hr. Images were taken with a laser-scanning microscope.

Pre-embedding Immunogold Electron Microscopy

Under deep pentobarbital anesthesia, mice were fixed and coronal sections were prepared. Sections were incubated overnight with primary antibodies, followed by secondary antibodies linked to 1.4-nm gold particles for 4 hr. Immunogolds were intensified with a silver enhancement kit. Sections were treated with osmium tetroxide, stained with uranyl acetate, and, finally, embedded in Epon 812. Photographs were taken with an electron microscope.

SDS-Digested Freeze-Fracture Replica Labeling

Under deep pentobarbital anesthesia, mice were fixed and coronal sections were prepared. Sections were immersed in graded glycerol and then rapidly frozen. Frozen samples were fractured into two parts at –140 °C and replicated by carbon deposition, platinum, and carbon. Tissue debris was dissolved, and the replicas were washed in graded polyethylene glycol in washing buffer. The replicas were then incubated with anti-HCN1 antibody, followed by incubation with gold-particle-conjugated secondary antibody. Cav3.1 was similarly immunolabeled using anti-Cav3.1 antibody. Photographs were taken with an electron microscope.

Statistical Analysis

Averaged data are presented as mean ± SEM. Statistical significance was assessed using the Mann-Whitney U test. Differences between groups were assessed by one-way ANOVA and judged to be significant at p < 0.05. When the difference was judged significant, data were processed using the Holm-Sidak test as a post hoc test. One and two asterisks represent p < 0.05 and p < 0.01, respectively. In morphological analysis, the differences between groups were assessed using the Steel-Dwass test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.053.

AUTHOR CONTRIBUTIONS


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