CaV3.1 is a tremor rhythm pacemaker in the inferior olive

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The rhythmic motor pathway activation by pacemaker neurons or circuits in the brain has been proposed as the mechanism for the timing of motor coordination, and the abnormal potentiation of this mechanism may lead to a pathological tremor. Here, we show that the potentiation of CaV3.1 T-type Ca2+ channels in the inferior olive contributes to the onset of the tremor in a pharmacological model of essential tremor. After administration of harmaline, 4- to 10-Hz synchronous neuronal activities arose from the IO and then propagated to cerebellar motor circuits in wild-type mice, but those rhythmic activities were absent in mice lacking CaV3.1 gene. Intracellular recordings in brain-stem slices revealed that the CaV3.1-deficient inferior olive neurons lacked the subthreshold oscillation of membrane potentials and failed to trigger 4- to 10-Hz rhythmic burst discharges in the presence of harmaline. In addition, the selective knockdown of CaV3.1 gene in the inferior olive by shRNA efficiently suppressed the harmaline-induced tremor in wild-type mice. A mathematical model constructed based on data obtained from patch-clamping experiments indicated that harmaline could efficiently potentiate CaV3.1 channels by changing voltage-dependent responsiveness in the hyperpolarizing direction. Thus, CaV3.1 is a molecular pacemaker substrate for intrinsic neuronal oscillations of inferior olive neurons, and the potentiation of this mechanism can be considered as a pathological cause of essential tremor.

Results

Mice Lacking CaV3.1 Are Selectively Resistant to the Harmaline-Induced Tremor. For insights into the role of CaV3.1 in motor functions, we first examined the motor-related capacity of CaV3.1+/− mice by using a number of behavioral experiments. In open field test, CaV3.1−/− mice showed a transient hyperactivity when first exposed to the test box, although they were eventually habituated as measured by normal baseline locomotor activities (Fig. 1A). However, the mice showed no other significant differences in overall motor capacity, including motor learning (Fig. 1B) and motor coordination of limbs during walking (Fig. 1 C and D), when compared with wild type. These findings suggest that CaV3.1−/− mice had no significant defects in motor learning and motor coordination, which has been associated with cerebellar motor functions and spinal reflexes (13). These findings suggested that the knockout of CaV3.1 gene causes no severe defects in physiological motor functions of mice.

Thus, we tried to examine the conditional phenotype induced by drugs that are known to disrupt motor coordination by inducing tremor, including oxotremorline, an agonist of muscarinic cholinergic receptors, for inducing 4- to 10-Hz resting tremor (14); physostigmine, an acetylcholinesterase inhibitor, for tremor with broad band frequencies (15); penitrem-A for 3- to 4-Hz action tremor (16); and harmaline for 5- to 14-Hz action tremor (14, 17, 18). Though CaV3.1−/− mice showed robust tremor activities when administered oxotremorine, physostigmine, or penitrem-A (Fig. 1E), these animals showed remarkably lower tremor activities than wild-type controls in response to harmaline (i.p. injection at 9 mg/kg; Movie S1), as measured by a decrease in the power (Fig. 1 F and G) and duration (Fig. 1H) of the tremor. Thus, this finding is consistent with the fact that those pharmacological tremor models are associated with different mechanisms (14–16) and suggests that CaV3.1 channels are indeed specifically associated with the harmaline-induced tremor.

www.pnas.org/cgi/doi/10.1073/pnas.1002995107

PNAS | June 8, 2010 | vol. 107 | no. 23 | 10731–10736
CaV3.1−/− Mice Lack 4- to 10-Hz Harmaline-Induced Oscillations Initiated from the Inferior Olive. The main neuronal target affected by the null mutation of CaV3.1 gene was determined by examining harmaline-induced oscillations in mice. Because harmaline is known to induce 4- to 10-Hz oscillation in olivocerebellar pathways (18, 19), we performed simultaneous recordings of local field potentials (LFP) and multiunit spikes in the IO and the DCN, which are the major input and output pathways, respectively, in cerebellar motor circuits (7, 8). After harmaline administration, 4- to 10-Hz rhythmic oscillations initially appeared in the IO before reaching the DCN (Fig. 2A and B and Fig. S1A; paired t test, P < 0.01). The delay between the IO and DCN oscillation was dose dependent, such that at 15 mg/kg, the mean delay was 71.82 s (n = 16), and at 9 mg/kg, the mean delay was 311.75 s (n = 5). Moreover, subtraction of spectrogram recorded from the IO with that of neighboring non-IO regions, which are at least 300 μm apart from the IO, showed that the 4- to 10-Hz oscillations originated directly from the IO neurons (Fig. 2E and Fig. S2). These findings support the idea that IO neurons are dominantly involved in the onset of the harmaline-induced oscillations in vivo (18).

In addition, the oscillation frequency was stabilized from the initial ~10-Hz frequency to ~5 Hz in both the IO and the DCN (Fig. 2C; one-way repeated ANOVA for time, IO, F(1, 15) = 10.063, *P < 0.001. (**, n = 7; *, n = 7). (B) Rotarod test. Motor coordination and learning were intact in CaV3.1−/− mice. Two-way repeated ANOVA for genotype and time, F(1, 238) = 0.0387, n.s. (***, n = 10; ***, n = 9). (C) Walking footprint pattern. d, diagonal; g, girdle; i, ipsilateral; LF, left front paw; RF, right front paw; LH, left hind paw; RH, right hind paw (e.g., gLFRF describes the relationship between the left front and right front paws). No significant difference was observed, χ2 test, P > 0.2 (***, n = 5; ***, n = 6). (E) Tremor induced by drugs: oxotremorine (Oxo) at 0.3 mg/kg; physostigmine (Phys) at 0.5 mg/kg; penitrem-A (PA) at 1.5 mg/kg; harmaline (HA) at 9 mg/kg. (F) Power spectral analysis of tremor induced by harmaline: color-coded power spectrum. Red-to-blue color means a variation in the intensity of tremors induced by drugs: oxotremorine (Oxo) at 0.3 mg/kg; physostigmine (Phys) at 0.5 mg/kg; penitrem-A (PA) at 1.5 mg/kg; harmaline (HA) at 9 mg/kg. (F) Power spectral analysis of tremor induced by harmaline: color-coded power spectrum. Red-to-blue color means a variation in the intensity of tremors induced by drugs: oxotremorine (Oxo) at 0.3 mg/kg; physostigmine (Phys) at 0.5 mg/kg; penitrem-A (PA) at 1.5 mg/kg; harmaline (HA) at 9 mg/kg. (F) Power spectral analysis of tremor induced by harmaline: color-coded power spectrum. Red-to-blue color means a variation in the intensity of tremors induced by drugs: oxotremorine (Oxo) at 0.3 mg/kg; physostigmine (Phys) at 0.5 mg/kg; penitrem-A (PA) at 1.5 mg/kg; harmaline (HA) at 9 mg/kg.
881.385, P < 0.001; in DCN, F(1, 15) = 424.059, P < 0.001). The dampening frequency reflects the increasing size of the neuronal population being synchronized, as described previously (20). Moreover, the latency of frequency stabilization was positively correlated with the onset delay of the DCN oscillations (Fig. 2D). Thus, the increase in synchronization among IO neurons seems to be a critical factor for the onset of tremor-related rhythms and the propagation of these rhythms to the DCN.

In contrast, in CaV3.1 /−/− mice, harmaline did not induce the 4- to 10-Hz bands in either the IO (Fig. 2E–H) or the DCN (Fig. S3). Though wild-type IO neurons generated highly synchronous rhythmic discharges that were phase locked to the 4- to 10-Hz bands in either the IO (Fig. 2E–H) or the DCN (Fig. S3). Consistently, the rhythmicity of spike activities before harmaline treatment, which reflects the presence of STO (21), was lower in the CaV3.1 /−/− than wild-type IO neuron (Fig. 3C). In addition, CaV3.1 /−/− IO neurons failed to trigger rhythmic spiking in the presence of harmaline (Fig. 3A). Harmaline induced the hyperpolarization of membrane potentials (Fig. 3 A and B), which is known to reverse the inactivation of T-type Ca2+ channels (1, 4, 22) in both genotypes without any quantitative differences (Fig. 3D). These findings reveal that CaV3.1 channels are involved in the generation of STO and STO-coupled rhythmic spiking of IO neuron in response to harmaline.

**CaV3.1 /−/− IO Neurons Lack a Capacity to Generate Rhythmic Action Potentials in Response to Harmaline-Induced Hyperpolarization.**

Next, we examined how the intrinsic properties of IO neurons were affected by the null mutation of CaV3.1. Intracellular recordings in brainstem slices indicated that in CaV3.1 /−/− IO neurons, typical protocol for activating T-type Ca2+ channel cannot induce low-threshold Ca2+ spike (Fig. 3B Left), a well-known physiological marker of T-type Ca2+ channel activity (1, 4, 5). In addition, we found a remarkable difference in the generation of the subthreshold oscillation (STO). Wild-type neurons exhibited STO at resting membrane potentials, the amplitude of which was increased in the presence of harmaline (Fig. 3A Upper). The augmented STO was eventually coupled with vigorous rhythmic action potentials at 4–10 Hz (Fig. 3A Upper), similar to the frequency range examined in vivo (Fig. 2 D and H). In contrast, the majority of CaV3.1 /−/− IO neurons showed no STO at resting membrane potential (Fig. 3A E; 0.138 ± 0.038 V2/Hz; n = 0.008 ± 0.001 V2/Hz, two-tailed t test, *P < 0.05).

Consistently, the rhythmicity of spike activities before harmaline treatment, which reflects the presence of STO (21), was lower in the CaV3.1 /−/− than wild-type IO neuron (Fig. 3C). In addition, CaV3.1 /−/− IO neurons failed to trigger rhythmic spiking in the presence of harmaline (Fig. 3A). Harmaline induced the hyperpolarization of membrane potentials (Fig. 3 A and B), which is known to reverse the inactivation of T-type Ca2+ channels (1, 4, 22) in both genotypes without any quantitative differences (Fig. 3D). These findings reveal that CaV3.1 channels are involved in the generation of STO and STO-coupled rhythmic spiking of IO neuron in response to harmaline.

**Knockdown of CaV3.1 Gene in the Inferior Olive Attenuates the Harmaline-Induced Tremor in C57BL/6J Mice.** Because the CaV3.1 gene is also expressed in cerebellar Purkinje cells and the DCN, which also show tremor-related rhythmicity (18, 19), we examined the role of CaV3.1 expression in the IO in the tremorogenesis. Local infusion of lentiviruses harboring CaV3.1-specific shRNA into the IO neurons (Fig. 4A and Fig. S5) efficiently attenuated harmaline-induced tremor (Fig. 4 B and D). Postmortem protein blotting confirmed the knockdown of CaV3.1 proteins in the IO (Fig. 4C). In addition, the local infusion of mibebradil, a nonspecific T-type Ca2+ channel blocker, to the IO also reduced the harmaline-induced tremors of wild-type animals.

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**Fig. 3.** Absence of STO and rhythmic burst discharges in CaV3.1 /−/− IO neurons. (A) In response to a continuous increase in harmaline concentration achieved by replacement of the bath medium with a 0.1 mg/mL harmaline solution, the amplitude of STO increased (Center, 5 min after) and then STO-coupled burst discharges appears (Right, 7 min after) in wild type, but those effects are not examined in CaV3.1 /−/− IO neurons. (B) Rebound LTS induced by hyperpolarizing current inputs is augmented in the presence of harmaline, whereas no rebound LTS was elicited in CaV3.1 /−/− IO neurons. (C) Autocorrelogram of action potentials of IO neurons. Note the lowered rhythmicity of action potentials in CaV3.1 /−/− IO neurons. (D) Harmaline-induced hyperpolarization is not different between genotypes (+/+, n = 5; /−/−, n = 4). (E) The power of STO is compared between genotypes (+/+, n = 6; /−/−, n = 6). HA, harmaline administration. Error bars indicate SEM.

**Fig. 4.** Effect of IO CaV3.1 knockdown on the harmaline-induced tremor. (A) Immunostaining of brain slices for EGFP and NeuN proteins, for detecting infected cell and the soma of neurons, respectively. Approximately 40–50% of IO neurons are infected with lentiviruses harboring either shRNA-CaV3.1 (sh3.1) or control (shC). DAO, dorsal accessory olivary nucleus; MAO, medial accessory olivary nucleus; PMn, paramedian reticular nucleus. (B) Tremor activity induced by harmaline administration is decreased by shRNA-CaV3.1. (C) Western blot analysis of CaV3.1 proteins isolated from infected IO regions by microdissection under microscope. (D) Duration of the harmaline-induced tremor. Two-tailed t test, *P = 0.05 (shC, n = 7; sh3.1, n = 12). (E) The effect of the mibebradil in the IO on the harmaline-induced tremor. Two-way repeated ANOVA for genotype and time, F(1, 42) = 34.674, *P < 0.05, saline, n = 5; mibebradil, n = 5. Error bars indicate SEM.
(Fig. 4E). These findings suggest that the CaV3.1 channels expressed in the IO were relevant for the tremorogenic action of harmaline.

**Harmaline Modifies the Voltage-Dependent Responsiveness of CaV3.1 Channels.** Given the findings described herein, we tried to address the effect of harmaline on the CaV3.1-dependent I_T. Voltage-clamp recording of IO neurons indicated that in a conventional protocol, which uses 20- to 40-mV hyperpolarization followed by depolarization for measuring the activation of I_T (4, 5), harmaline dose-dependently inhibited I_T in IO neurons (Fig. 5 A and C and Fig. S6). To avoid indirect effects that could result from the non-specific binding of harmaline on other ion channels (23) or an incomplete integrity of patch clamping due to well-developed electrical synapses of IO neurons (22), we measured I_T in HEK cells, which express CaV3.1 channels (HEK-CaV3.1). Consistently, HEK-CaV3.1 cells showed reduced I_T in the presence of harmaline, although the inhibitory effect was smaller when compared with that found in IO neurons (Fig. 5C). These findings suggest that harmaline directly modulates the activation of CaV3.1 channels.

Next, we examined the effect of harmaline on voltage dependence of CaV3.1 channels in HEK-CaV3.1 cells, because IO neurons have gap junctions that might cause CaV3.1-independent currents in IO neurons. In the plot of voltage dependence, both activation and inactivation curves were shifted toward negative potential in the presence of harmaline (Fig. 5D). The degree of shifting of both curves by harmaline were differential (Fig. 5E); In a range of 0.1 to 10 μM, the shifting of the activation curve (−mV) was linearly increased to increasing harmaline concentrations, but that of the inactivation curve was not significantly changed (Fig. 5E: one-way ANOVA, P > 0.05), suggesting that the activation effect of harmaline could be more dominant. Considering the voltage dependence of CaV3.1 channels, we quantified the effect of harmaline on membrane potentials (Fig. 5F). As previously described (9), harmaline dose-dependently hyperpolarizes the membrane potentials up to −60 to −70 mV, but more than 100 μM of harmaline was not effective at inducing more hyperpolarization (Fig. 5F).

Based on the complex action of harmaline on CaV3.1-dependent I_T, which seems to induce three different effects: inhibition of activation (Fig. 5 A–C), shifts of voltage dependence (Fig. 5 D and E), and induction of membrane potential hyperpolarization (Fig. 5F), we mathematically simulated the net influx of I_T in IO neuron in response to various types of variables, including the amplitude of hyperpolarizing prepulse, depolarizing pulse, and membrane hyperpolarization (Fig. S7). Results from this simulation showed that many types of inputs increased I_T, at around 10 μM, but that the inputs inhibited I_T at 1 μM or 100 μM (Fig. 5G). In addition, negative currents input causing less than 20 mV hyperpolarization usually increased I_T, but that with larger than 20 mV hyperpolarization led to the suppression of these currents in 10 μM harmaline (Fig. 5G).

Consistent with our findings, voltage clamping of HEK-CaV3.1 showed that, in the presence of 10 μM harmaline, 5- to 15-mV hyperpolarizing pre pulses increased I_T, but 20 mV led to the inhibition of I_T when compared with controls (Fig. 5H and Fig. S8). The hyperpolarization of more than 20 mV from the resting membrane potentials, however, may not be physiological because (i) almost all ions existing in naive neuron do not have reversal potential below −80 mV and (ii) many inward-rectifying potassium channels, which are also highly expressed in IO neurons (24), strongly depolarize membrane potential above −80 mV. Hence, these findings suggest that harmaline inhibits activation of CaV3.1 channels but can potentiate I_T through these channels in physiological conditions by modifying parameters related to the voltage-dependence responsiveness of these channels.

**Discussion**

Essential tremor is the most frequent movement disorder characterized by the onset of tremor during voluntary movement; however, as the name implies, its pathology and etiology remains unknown (17, 25). Harmaline, a derivative of β-carboline found in brewed coffee (26), cigarettes (27), and blood of patients with essential tremor (28), induces essential-like tremor in animals and human (25, 28). Because it causes 4- to 10-Hz tremor rhythms in the brain (14, 17, 25).

The present study shows that the tremorogenic action of harmaline depends on CaV3.1 channels (Fig. 1) expressed in IO neurons (Figs. 2–4) and reveals the mechanism of action of this drug (Fig. 5). Although harmaline inhibits the activation of CaV3.1 channels, it could potentiate these channels by modulating other parameters: (i) differential shifting of activation and inactivation voltage dependence, expanding the window of membrane potentials where CaV3.1 channels can be potentiated; (ii) hyperpolarization of membrane potentials, leading to the optimal range of membrane potentials to potentiate these channels. Furthermore, the positive effects of harmaline are concentration dependent, and are maximized at around 10 μM of harmaline (Fig. 5). Consistently, the 10-μM range is known as an effective dose to
induce tremor in cerebrospinal fluids of mice (29) and evokes tremor rhythms in IO neurons in vivo (Fig. 2). Thus, our findings suggest that potentiation or inhibition of CaV3.1 channels can be a unique measure for searching pathological causes of tremor or therapeutic mechanisms of antitremor drugs, which have been largely unknown.

The present experimental paradigm also afforded a possible explanation of how the potentiation of CaV3.1 channels contributes to tremor (Fig. 1) and tremor-related rhythms in IO neurons (Fig. 2). One classical hypothesis is that the STO of IO neurons can synchronize neuronal activities via electrical synapses (9, 30); however, a previous study showed that mice lacking connexin-36, a major component of the electrical synapses of IO neurons, exhibited robust tremor activities when administered harmaline (30). This suggests that the electrical synapse is not the only mechanism underlying network oscillation.

Our present findings indicate that CaV3.1−/− mice lack the STO in IO neurons (Fig. 3) and that they are impaired in the generation of tremor-related rhythms (Fig. 2) and behavioral tremor (Figs. 1 and 4). These findings support the relevance of STO in the synchronization of IO neurons (9, 30, 31). Harmaline increases the amplitude of STO, and this leads to rhythmic burst spikes (Fig. 3), which may efficiently activate neighboring neurons via electrical and chemical synapses (9, 30, 32), thereby increasing the number of neurons being synchronized (Fig. 2A–D). The synchronization of IO neurons may eventually lead to thalamic tremor-related oscillations (Fig. 2).

It has been reported that the IO and DCN neurons are synchronized when they are administered harmaline (19). Our finding shows, however, that the onset of IO oscillations is always preceded by that of DCN (Fig. 2A and Fig. S1L), and the propagation of tremor-related rhythms to the DCN is also modulated by the number of IO neurons being synchronized (Fig. 2D). Moreover, the onset of IO oscillations coincides with the time of tremor onset, and that of DCN oscillations is found in the period when the intensity of tremor is increasing (Fig. S1B). Hence, the synchronization of IO neurons seems to be critical for the onset of behavioral tremor, and the following synchronization of DCN neurons may contribute to the increase of tremor strength and affected body parts.

Although our findings show a critical role of CaV3.1 channels in the generation of IO oscillations, other mechanisms also play important supporting functions in these oscillatory behaviors (9, 33, 34); e.g., Ih currents were measured by depolarizing saccs in each genotype and it was found that CaV3.1−/− IO neurons have lower HCN channel activity (Fig. S9), which is consistent with previous findings that low-threshold calcium conductance leads to the persistent activation of Ih (35). Hence, this finding suggests that CaV3.1 channels can indirectly modulate HCN channels, which have been implicated in the oscillatory activities of IO neurons (33). It remains to be studied how other mechanisms can be associated with the potentiation of CaV3.1 channels.

Concerning the physiological significance of STO, it has been proposed that STO contributes to the timing of motor coordination (11), as IO neurons have been shown to relay peripheral sensory information back to the cerebellum (36) and function as a comparator in several motor-learning models (8, 37). However, we found no significant motor defects in CaV3.1−/− mice (Fig. 1), which have reduced capacity for generating intrinsic IO rhythms (Fig. 3). The relatively intact motor capacity of CaV3.1−/− mice can be due to physiological or developmental compensation, or that a more stringent test of motor timing may be required (31, 38). It is also plausible that STO is involved in higher motor functions that require faster processing of motor-related information (11), although this was not addressed in the present study. Detailed behavioral testing of mice with the CaV3.1 knocked down in adult IO neurons will provide clues concerning the function of STO.

The only notable difference found in CaV3.1−/− mice was that they showed a transient hyperactivity with normal habituation in open field test (Fig. L4). Considering that the increased activity in the early period of the test reflects an emotional response of mice to novel environmental stimuli (39), this phenotype can be explained, in part, by the enhanced vigilance of CaV3.1−/− mice reported in previous studies; they show longer waking episodes during sleep (40) and resistance to GABA-B agonists, which cause a loss of consciousness (2). In the thalamocortical pathways, T-type Ca2+ channels are involved in the generation of low-threshold burst spikes during sleep and drowsiness (41), which contribute to the sensory gating mechanism (42). Hence, it is plausible that the absence of thalamic burst spikes facilitates the thalamocortical relay of sensory information on novel stimuli in CaV3.1−/− mice.

In conclusion, the present study supports the view that tremor-related oscillations in the olivocerebellar pathways are a neural signature for essential tremor (9, 19). It also suggests that CaV3.1 channels play a critical role in the onset of tremor-related rhythms. It follows, then, that CaV3.1 can be directly linked with essential tremor. For example, the kinetic component of essential tremor (17, 25) may be explained by the activation of these channels in IO, by sensory inputs generated by movement, which were known to be conducted into IO (37). Further investigation for conditions to potentiate CaV3.1 channels may provide clues on the cause of essential tremor and on novel drug targets for the disease. Beyond the issue of essential tremor, the role of IO rhythmicity modulated by CaV3.1 channels in higher motor functions (11) remains to be elucidated.

Methods

Drug-Induced Tremor. A mouse was placed in the test cage for 7 min, and one of the following tremorogenic drugs was injected i.p.: oxotremorine sesquifumarate (0.3 mg/kg), pentetrem-A (1.5 mg/kg), or harmaline hydrochloride dihydrate (9 or 15 mg/kg). Tremor activity was measured by using DC accelerometer (DC Response Accelerometer, model 3711D1FA3G; Piezotronics) or ICP accelerometer (Integrated Circuit Piezoelectric Accelerometer, model 352C66; Piezotronics) attached to the bottom of the test cage, which was hung in the air by two elastic wires (Fig. S10 and Movie S1).

Electrophysiological Recording in Vivo. Male C57BL/6J mice (10–28 wk old) were fixed on a stereotaxic device under urethane anesthesia (1.65 g/kg). Body temperature of mice was monitored and maintained by a temperature controller (Homeothermic Blanket System; Harvard Apparatus). Quartz-coated tetrodes (0.5–2.25 MΩ; Thomas Recording) were placed either at the sublamina of the inferior olive [laminar I (DL) and MO; distance from bregma (in mm), −7.4, ±0.5, 4.5–5.3: anterior-posterior (AP), mediolateral (ML), dorsal-ventral (DV) axes] or at the medial cerebellar nuclei (−6.4 ± 0.8, 2.0 ± 2.6: AP, ML, DV), and harmaline (9 or 15 mg/kg) was injected i.p. To localize the site of the recordings, the tips of the tetrodes were briefly dipped in fluorescein dye solution (DII, 50 mg/mL; Sigma) before tissue penetration, and electrode tracks in the brainstem slices were visualized under a confocal microscope using a rhodamine filter, as previously described (43). Signals were amplified using an AC amplifier (PDMGA; Thomas Recording) and then sampled at 30 kHz (DT3010; Neuralynx) and filtered at either 480–6,000 Hz (for the measurement of multunit activity) or 1.52–50 Hz (for the measurement of LFP).

Whole-Cell Patch-Clamp Recording. In HEK cells. Ca3.1-Kir2.1 transfected HEK cell line (44) were used in demonstrating harmaline effect to Ih. Borosilicate glass electrodes with a resistance of 3–4 MΩ were pulled and filled with internal solution containing (in mM): 130 LiCl, 4 MgCl2, 10 Hepes, 5 TEA-Cl, 10 EGTA, 4 Mg-ATP, and 0.3 Na2-GTP. Ih was recorded in external solution containing (in mM) 100 NaCl, 25 TEA-Cl, 3 KCl, 20 Hepes, 2 MgCl2, and 5 BaCl2, For holding potential, −60 mV was used. In IO neurons. Preparing IO slices for patch-clamp recording were performed similarly to a previous study (30). Ca3.1 wild-type and knockout mice (postnatal day 12–15) were intracardially perfused with an ice-cold solution containing (in mM): 130 GCI, 4 MgCl2, 10 Hepes, 5 TEA-Cl, 10 EGTA, 4 Mg-ATP, and 0.3 Na2-GTP. Ih was recorded in external solution containing (in mM) 100 NaCl, 25 TEA-Cl, 3 KCl, 20 Hepes, 2 MgCl2, and 5 BaCl2, For holding potential, −60 mV was used.
were transferred to a submersion-type recording chamber held at 32 °C. Solution used for slice incubation contained 126 NaCl, 3 KCl, 1.25 NaH2PO4, 2 MgSO4, 26 NaHCO3, 10 dextrose, and 2 CaCl2 with 95% O2/5% CO2; the recording solution was the same, except for reduced divalent ion levels (1 MgSO4 and 1 CaCl2) and a slight increase in potassium (3.5 KCl). Recording pipettes (4–7 MΩ) were filled with (in mM) 115 CsOH, 115 D-glucocic acid, 10 Hepes, 0.5 EGTA, 4 Mg-ATP, 0.3 GTP-Tris, 7 PC-Tris, 2 NaCl, 20 TEA-CI, and 2 MgCl2. After rupturing, IO neurons were maintained at ~60 mV, and recording solution was substituted with Ca2+-free current recording solution, which is composed of (in mM) 66 NaCl, 3.5 KCl, 1.25 NaH2PO4, 1.2 MgSO4, 26 NaHCO3, 10 dextrose, 2.5 CaCl2, 60 TEA-CI, 5 4-aminopyridine, 3 CsCl, 0.0125 bicuculline methiodide, and 0.001 tetrodotoxin. Because of high Cm value of IO neurons (80–130 pF) and presence of gap junctions, which cause incomplete voltage clamping, we also recorded in CaV3.1 wild-type and mutant IO neurons (80–190 pF). Signals were amplified by Multiclamp 700B or Axopatch 200B (Axon Instrument) and digitized by Digidata 1322A (Axon Instruments). Cells were visualized by Ikon camera (Andor).

Computational Modeling of \( I_f \). \( I_f \) has been known to be activated only after the deactivation, a process of recovery from inactivation by hyperpolarizing prepulse (4, 5). Thus, the amplitude of \( I_f \) by Ca3.1 can be determined by the following function,

\[
T_{control} = f(r_m - d - a) * f(r_m + b),
\]

where \( r_m \) is the IO neuron resting membrane potential, \( a \) and \( b \) are the degree of hyperpolarizing/depolarizing voltage step sequence (Fig. 5F Left), \( f(\cdot) \) is the ratio of recovered channels from inactivation (Fig. 5FA), and \( f(\cdot) \) is the current amplitude resulting from depolarizing pulse normalized with the maximal current before harmaline treatment (Fig. 5B).

Because harmaline shifts both inactivation and activation curve and induces membrane potential hyperpolarization, amplitude of net \( I_f \) after harmaline treatment can be described with a slightly different equation:

\[
T_{harmaline} = g_i(r_m - d - a) * g_o(r_m + d + b),
\]

where \( d \) is the level of preconditioned membrane potential hyperpolarization (Fig. 5F) and \( g_i \) and \( g_o \) are the shifted inactivation and activation curve, respectively, at certain harmaline concentration. For calculating \( g(\cdot) \), the ratio of channels recovered from inactivation after harmaline is calculated by normalizing inactivation curve with the percentage of current remaining at a given harmaline concentration (Fig. 5C).

ACKNOWLEDGMENTS. D. K. thanks Walton Jones for critical comments on our manuscript. Y.G. thanks Boeun Yoon for helping with the voltage-clamp experiment. We thank Dr. Dong-jin Kim for approving the use of HEK-CaV3.1 cell line. This work was supported by the Biotechnology Development Program of the Ministry of Education, Science and Technology Grants R01-2006-000-10799-0, M10708000035-08R4800-34510, and 2009083354 (to D.K.) and by National Institutes of Health Grant NS13742 (to R.R.L.).

Supporting Information

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SI Methods

Animal Preparation. Mice heterozygous for the Ca\textsubscript{v}3.1 gene (Ca\textsubscript{v}3.1\textsuperscript{+/−} animals) were generated by using embryonic stem cells with 129/Sv genetic background (1). To make C57BL/6J congenic strain, F1 heterozygous mice obtained from mating chimera and C57BL/6J females were backcrossed with C57BL/6J strains for \( \approx 25 \) generations (N25). Ca\textsubscript{v}3.1\textsuperscript{−/−} mice and wild-type littersmates obtained from mating between C57BL/6J (N25) Ca\textsubscript{v}3.1\textsuperscript{+/−} mice were used for this study. The genotypes were determined by PCR analysis, as described previously (1). Animal care and all animal procedures used in this study were carried out following the directives of the Animal Care and Use Committee of KAIST and New York University Medical School. Mice were maintained with free access to food and water under a 12:12-h light/dark cycle, with the light cycle beginning at 7:00 AM.

Physiological Behavior Tests. Open field test. Mice were placed in the center of a 40 x 40 x 40 cm cage and recorded for 60 min in the dark using an infrared camera. Videos were analyzed to quantify the total distance covered by each mouse using EthoVision software (Noldus Information Technology).

Rotarod test. Mice were tested on a 3-cm (diameter) rotarod and 5% CO\textsubscript{2} to (one-way 0.3), power densities of a 0.6 Hz range fit \( > 40 \times \% \) mice. Walking patterns were analyzed using the Cat-40 cm cage and recorded for 60 min in the dark using an infrared camera. Videos were analyzed to quantify the total distance covered by each mouse using EthoVision software (Noldus Information Technology).

Intracellular Recordings in IO Neurons in Vitro. Parasagittal slices were obtained from brains of mice (postnatal day 15–19) as previously described (6). Animals were deeply anesthetized with pentobarbital (Nembutal, 120 mg/kg, or ethylene, 1 mL per mouse) and decapitated after loss of limb-withdrawal reflex. The brainstem was isolated and placed in chilled high-sucrose artificial cerebrospinal fluid (ACSF) containing (in mM) 248 sucrose, 1.25 NaHPO\textsubscript{4}, 5 KCl, 2 MgSO\textsubscript{4}, 0.5 CaCl\textsubscript{2}, and 10 glucose, aerated with 95% O\textsubscript{2} and 5% CO\textsubscript{2} to a final pH of 7.4. Parasagittal slices (350 \( \mu \)m in thickness) were sectioned using a vibratome (VT1000S; Leica Microsystems). Slices were transferred to a holding chamber containing a continuously oxygenated combination of 50% high sucrose (see above) and 50% normal ACSF [in mM, 124 NaCl, 5 KCl, 1.25 NaHPO\textsubscript{4}, 26 NaHCO\textsubscript{3}, 2 MgSO\textsubscript{4}, 0.5 CaCl\textsubscript{2}, and 10 glucose (pH 7.4)]. Slices were incubated at 32 °C for at least 1 h before use. Intracellular recordings were obtained from principal olivary (PO) and MAO neurons using glass micropipettes filled with 3 M KOAc (60–100 MΩ). Recording solution was same with normal ACSF, except increased CaCl\textsubscript{2} concentration to 2.4. Electrodes were introduced into the slice using a Narashige manipulator. We used only healthy cells with a membrane potential below \(-50\) mV. Signals were amplified using the Axoclamp 2A amplifier (Axon Instruments) or the IR183 amplifier (Neurodata Instrument) and acquired at 10 kHz using a digital oscilloscope (Nicolet 4094; Nicolet Instrument) or Digidata 1322A (Axon Instrument) for offline computer analysis. Data were analyzed using Igor-based software (Wave Metrics), Clampfit 9.2 (Axon Instrument), or NeuroExplorer (Nex Technologies).

For the quantification of the amplitude of subthreshold oscillations in wild type, the power densities in the range of 0.3 Hz above and below the peak frequency were averaged. Because the absence of the oscillation with significant power in Ca\textsubscript{v}3.1\textsuperscript{−/−} (one-way ANOVA for frequency, \( P > 0.3 \)), power densities of a 0.6 Hz range in 6–10 Hz was randomly selected and compared with wild type.

Whole-Cell Patch-Clamp Recording. Amplitudes of \( I_f \) were measured by using the level of fully inactivated states of each \( I_f \) as a baseline. Degrees of curve shift were calculated in each cell, respectively. Clampfit 9.2 (Axon Instruments) was used for measuring \( I_f \), and SigmaPlot 9.0 was used for data fitting. In the evaluation of \( I_f \) modeling, HEK-Ca\textsubscript{v}3.1 experiments were performed as in Fig. S8.
Contributions of each of the three actions of harmine on \(I_T\) were calculated as follows: Comparison of \(I_T\) induced by same voltage steps before and after harmaline treatment were used to calculate potentiation by harmaline in the absence of the hyperpolarization effect. By subtracting the percent inhibition at given harmaline concentration (Fig. 5C), the contributions of curve shift on \(I_T\) potentiation was investigated.

**Delivery of Drugs into the Inferior Olive.** Ten- to 20-wk-old male C57BL/6j mice were anesthetized by i.p. injection of tribromoethanol (Avertin, 20 mg/mL). After the development of surgical anesthesia, the scalp was incised and a small trepanation was made above the location of the IO (−7.4 mm, +0.15 mm: AP, ML). A cannula (Brain Infusion Kit 1; Alzet) connected to an osmotic pump (model 1002, 0.25 μL/h; Alzet) filled with mibe-fradil dihydrochloride (20 mM; M5441; Sigma) in physiological saline was stereotactically positioned just above the MAO and the DAO. A fluorescent dye (DiI) was coated on the outer surface of the cannula, and the infusion positions were confirmed by postmortem histological examination. A tremor test was implemented 2 d after cannulae implantation.

**Immunohistochemistry.** Some mice were used for EGF immunohistochemistry, for finely visualizing lentiviral-infected neurons in IO. Immediately after the tremor test, mice were anesthetized with tribromoethanol (Avertin, 20 mg/mL) and perfused transcardially with heparin solution (10 units/mL) followed by 4% formaldehyde dissolved in PBS. Brains were removed and postfixed for overnight at 4°C. Fifty-micrometer sections were prepared with a vibratome (VT1000S; Leica Microsystems). Sections were permeabilized in 0.5% Triton X-100 in PBS, and incubated with EGF and NeuN primary antibodies, followed by Cy3-, or FITC-conjugated secondary antibodies. A polyclonal antibody to enhanced green fluorescent protein (EGFP; 1432) in guinea pig was generated using H6-EGFP (amino acids 1–240). The NeuN antibody was purchased from Chemicon International.

**In Vivo Transduction of Lentivirus.** The shRNA-expressing lentiviral vector to target the Cav3.3.1 was constructed by inserting a synthetic double-stranded oligonucleotide (5′–CGGAAATTCGG-GA AGATCGTAGATAGACAAAttagaagatTTCTATCTCAGATTTTTTATATCTAAGACA−3′) into the EcoRI–XbaI restriction enzyme sites of the shLentisyn3.4G lentiviral vector (Macrogen LentiVector Institute, Seoul, Korea). Construct integrity was verified by sequencing. The shLentisyn3.4G lentiviral vector was designed to express shRNAs from the U6 promoter and to express EGFP from the synapse promoter. The target sequences did not overlap with any mRNAs from the database of the National Center for Biotechnology Information other than of Cav3.1. A scrambled version of the Cav3.1 shRNA oligonucleotide (5′–CGGAAATTCGGGGTA AGTGAACTGA-CAAGAAttagaagatTTCTATCTCAGATTTTTTATATCTAAGACA−3′) was also inserted into the shLentisyn3.4G vector as described previously, and used as a control. This sequence did not have any homology to known mammalian genes. The recombinant lentiviral vectors were produced and concentrated commercially (Macrogen LentiVector Institute). We routinely use lentivirus titers of \(\geq 1 \times 10^9\) transduction units (TU)/μL. One-microliter solution containing viruses carrying the Cav3.1 shRNA or the scrambled control were injected into the IO using Nanoilf 33 G blunt needle and a Nanoilf syringe (World Precision Instruments) coupled to a micro syringe pump (Eicom). The detailed surgical procedures were the same as described. A tremor test was performed 10 d after viral transduction. Only the results from mice with infected inferior olive neurons were gathered and analyzed.

**Analysis of Cav3.1 Knockdown Animals.** Immediately after the tremor test, mice were anesthetized by i.p. injection of tribromoethanol (Avertin, 20 mg/mL). The brainstem and cerebellum were isolated, and coronal brainstem slices (200 μm in thickness) were cut in 4°C high-sucrose ACSF solution. Viral infection of IO neurons was confirmed by the presence of EGFP expression, which was visualized using a confocal microscope (Zeiss). Tissue slices were not allowed to dry before being frozen at −70°C. IO tissue containing infected IO neurons was microdissected with stainless-steel punch under microscopic control. Punched samples were lysed by pipetting in 2x SDS sample buffer [121.7 mM Tris (pH 6.8), 3.4% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue], denatured for 30 min at room temperature, and spun down. Equal amounts of protein (20 μg as estimated using the BCA reagent; Pierce) were loaded onto individual lanes of a 7.5% polyacrylamide gel. Extracts of wild-type or Cav3.1−/− mouse brains were used as controls for the confirmation of Cav3.1 band. Samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane using a PowerPac Basic (Bio-Rad). Cav3.1 protein was detected using an anti-Cav3.1 primary antibody (1:500; Alomone Laboratories), followed by detection with an HRP-conjugated anti-rabbit IgG (1:20,000; GE Healthcare) secondary antibody. Detection of α-tubulin, which was used as a protein loading control, was performed using an anti-α-tubulin primary antibody (1:1,000; Sigma), followed by detection with an HRP-conjugated anti-mouse IgG (1:10,000; GE Healthcare) secondary antibody. Immunoreactivity was detected using the ECL detection reagent (GE Healthcare) visualized on Fuji Medical X-ray films. The quantification of protein expression levels was performed by measuring the intensity of bands using Multi Gauge 3.0 software (Fuji Photo Film Co., Ltd.).

The relative lentiviral infections in IO and non-IO neurons were measured by GFP fluorescent imaging (LSM510; Zeiss) with Imaged software (http://rsbweb.nih.gov/ij/), and the percentage of IO infection was compared between groups.

**Statistical Analyses.** All data were analyzed by t test and ANOVAs using the SigmaStat 3.1 software package, followed by analysis using a post hoc Holm–Sidak test.
Fig. S1. Comparison of the onset times between neuronal oscillations and behavioral tremor. (A) The onset of IO (blue) and contralateral DCN oscillation (red) after 15 mg/kg harmaline i.p. injection is marked with vertical bars. Note that the onset of IO oscillation is always preceded by that of DCN in every set of the experiment. (B) Temporal relationship between neural oscillations and behavioral tremor after 9 mg/kg harmaline. Note that the onset of IO oscillations is found around the onset of behavioral tremor, and DCN oscillations are initiated during the period when tremor activities are intensified.

Fig. S2. Rhythmic oscillations of IO induced by harmaline are absent in Ca\textsubscript{V}3.1\textsuperscript{−/−} mice. (A Left) Electrode tracks are marked by red fluorescence. (Right) Diagram for the location of the electrode tip inside (●) and outside (▴) of the medial or dorsal accessory olivary nuclei (MAO or DAO). (B) Representative LFP spectrograms from IO (●) or non-IO (▴) region. Subtracted result (● − ▴) is presented in Fig. 2E. (C) Raw LFP traces of IO and non-IO region at 14 min after harmaline injection. Two traces of Ca\textsubscript{V}3.1\textsuperscript{−/−} look very similar, suggesting that no region-specific oscillations were induced by harmaline from Ca\textsubscript{V}3.1\textsuperscript{−/−} IO neurons.

Fig. S3. Spectrograms of LFPs simultaneously recorded in IO and DCN neurons after harmaline injection at 9 mg/kg. No harmaline-induced oscillations are detected in either IO or DCN neurons of Ca\textsubscript{V}3.1\textsuperscript{−/−} animals. Color bar indicates the magnitude of oscillation, ranging from −60 to 20 dB. Fundamental oscillations are marked by asterisks, and other oscillations with higher frequencies are their harmonics. ▴, the time at which the frequency of the oscillation was stabilized.
**Fig. S4.** The firing rate of IO neurons is increased after harmaline injection (9 mg/kg) in wild type (●), but that of Ca$_{3.1}^{-/-}$ neurons is significantly decreased after harmaline administration (○). Two-way repeated ANOVA for genotype and time, $F(1, 77) = 11.944, *p < 0.05$ ($n = 9$ and $6$, for wild type and mutant). Arbitrary unit means the fold changes of firing rate relative to that before harmaline treatment. Error bars indicate SEM.

**Fig. S5.** Lentiviral infection in the IO and the non-IO regions. The intensity of green fluorescence induced by lentiviral infection of EGFP proteins is measured. The percent intensity of fluorescence on each area is plotted. No significant difference between control (shC) and Ca$_{3.1}$-shRNA (sh3.1) group was found. Two-tailed $t$ test, $P > 0.3$. PMn, paramedian reticular nucleus; Gi, gigantocellular reticular nucleus; Py, pyramidal tract.

**Fig. S6.** $I_T$ recorded in IO neurons. (A) Absence of $I_T$ in Ca$_{3.1}^{-/-}$ IO in voltage-clamping experiments. (B) Quantification of peak amplitude of $I_T$ between genotypes. Two-tailed $t$ test, $*p < 0.05$. (+/+; $n = 3$; −/−, $n = 3$).
Fig. S7. Mathematical simulation of the net influx of \( I_T \) in IO neurons. (A) The effect of harmaline on deinactivation of Ca\(_{\text{v}}\)3.1 channels in HEK-Ca\(_{\text{v}}\)3.1 measured by preceding hyperpolarizing voltage steps with a depolarizing step to \(-30\) mV for inducing \( I_T \). (B) The effect of harmaline on the activation of Ca\(_{\text{v}}\)3.1 channels. As described in Fig. 5C, harmaline inhibits the activation of \( I_T \) dose-dependently. (C) Simulation of net \( I_T \) induced by harmaline. The net \( I_T \) induced by an input consist of hyperpolarizing (\( \Delta a \)) and depolarizing pulses (\( \Delta b \)) at given harmaline concentration can be simulated by multiplication of two values obtained from curves of A and B.

Fig. S8. Confirmation of results expected in the mathematical model for \( I_T \). (A) Simulation of the net \( I_T \) in the presence of 10 \( \mu \)M harmaline in response to inputs that consist of hyperpolarizing (\( \Delta a \)) and depolarizing (\( \Delta b \)) pulses. (B) Two combination of inputs marked on the simulation (■, ●) are used for experimental confirmation in HEK-Ca\(_{\text{v}}\)3.1 cells. Because hyperpolarization is unable to be considered in voltage clamping, \(-10\) mV is added to the hyperpolarizing prepulse as simulated in the mathematical model (red lines). Note that the \(-40\) mV hyperpolarization with 10 \( \mu \)M induces less \( I_T \) than \(-30\) mV hyperpolarization without harmaline (Right), whereas more \( I_T \) is induced when less than 20 mV hyperpolarization is used (Left).
Movie S1. Resistance of Ca$_{\text{v}3.1}^{-/-}$ mice to the harmaline-induced tremor. Whereas a wild-type mouse shows a robust tremor after harmaline injection, a Ca$_{\text{v}3.1}^{-/-}$ mouse does not show any tremor activities.