

KCNQ Channels Enable Reliable Presynaptic Spiking and Synaptic Transmission at High Frequency

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The presynaptic action potential (AP) is required to drive calcium influx into nerve terminals, resulting in neurotransmitter release. Accordingly, the AP waveform is crucial in determining the timing and strength of synaptic transmission. The calyx of Held nerve terminals of rats of either sex showed minimum changes in AP waveform during high-frequency AP firing. We found that the stability of the calyceal AP waveform requires KCNQ (K_v7) K⁺ channel activation during high-frequency spiking activity. High-frequency presynaptic spikes gradually led to accumulation of KCNQ channels in open states which kept interspike membrane potential sufficiently negative to maintain Na⁺ channel availability. Blocking KCNQ channels during stimulus trains led to inactivation of presynaptic Na⁺, and to a lesser extent K_v1 channels, thereby reducing the AP amplitude and broadening AP duration. Moreover, blocking KCNQ channels disrupted the stable calcium influx and glutamate release required for reliable synaptic transmission at high frequency. Thus, while KCNQ channels are generally thought to prevent hyperactivity of neurons, we find that in axon terminals these channels function to facilitate reliable high-frequency synaptic signaling needed for sensory information processing.

Key words: action potential waveform; axon terminal; calyx of Held; channel inactivation; KCNQ channels; synaptic transmission

Significance Statement

The presynaptic spike results in calcium influx required for neurotransmitter release. For this reason, the spike waveform is crucial in determining the timing and strength of synaptic transmission. Auditory information is encoded by spikes phase locked to sound frequency at high rates. The calyx of Held nerve terminals in the auditory brainstem show minimum changes in spike waveform during high-frequency spike firing. We found that activation of KCNQ K⁺ channel builds up during high-frequency firing and its activation helps to maintain a stable spike waveform and reliable synaptic transmission. While KCNQ channels are generally thought to prevent hyperexcitability of neurons, we find that in axon terminals these channels function to facilitate high-frequency synaptic signaling during auditory information processing.

Introduction

At chemical synapses, the presynaptic action potential (AP) drives the activation of voltage-gated Ca²⁺ channels, and its repolarization enhances the driving force for Ca²⁺ influx into the terminal. Together, these two factors determine the intracellular Ca²⁺ levels needed for triggering vesicle fusion and neurotransmitter release (Borst and Sakmann, 1996; Sabatini and Regehr, 1999). Thus, the size and shape of the presynaptic AP is

a key determinant of the timing and strength of synaptic transmission (Sabatini and Regehr, 1997; Geiger and Jonas, 2000; Hoppe et al., 2014). Because of the differential expression of diverse voltage-dependent ion channels, the waveforms and firing patterns of action potentials vary considerably among various types of neurons in the mammalian brain (Bean, 2007). During repetitive firing of action potentials, voltage-gated Na⁺ (Na_v) and K⁺ (K_v) channels undergo cycles of activation and deactivation, inactivation, and repriming. Slow, cumulative inactivation of voltage-gated ion channels under repetitive high-frequency firing may affect the size and waveform of AP. For example, the AP waveforms of pituitary nerve terminals and hippocampal mossy fiber boutons are altered and the duration of action potentials is substantially increased during moderate- to high-frequency firing (Jackson et al., 1991; Geiger and Jonas, 2000).

The globular bushy cells of the cochlear nucleus faithfully relay auditory signals to the medial nucleus of the trapezoid body (MNTB) principal neurons through a giant glutamatergic

Received Feb. 16, 2020; revised Feb. 6, 2022; accepted Feb. 26, 2022.

Author contributions: Y.Z., L.O.T., and H.H. designed research; Y.Z., D.L., Y.D., and H.H. performed research; Y.Z., X.F., and H.H. analyzed data; Y.Z., L.O.T. and H.H. wrote the paper.

Financial support was provided by National Institutes of Health Grants DC-016324 and DC-012063 to H.H., and DC-004450 to L.O.T. We thank Dr. Laura Schrader for advice on data analysis and for critical reading of the manuscript.

The authors declare no competing financial interests.

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<https://doi.org/10.1523/JNEUROSCI.0363-20.2022>

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synapse, the calyx of Held (Mc Laughlin et al., 2008; Lorteije et al., 2009). Reliability and precision of synaptic transmission are required in the calyx–MNTB synapse to encode location of transient sounds in natural environments (Joris and Trussell, 2018). One factor that contributes to this precision is the remarkable stability of the presynaptic waveform during ongoing firing (Sierksma and Borst, 2017). Indeed, calyx of Held terminals show only minor AP waveform change at spike frequencies up to hundreds of hertz. However, the biophysical basis of this constancy is unclear. We explored the role of presynaptic KCNQ (K_v7) channels. KCNQ5 ($K_v7.5$) channels are expressed in the terminals of auditory neurons that fire AP up to hundreds of hertz (Caminos et al., 2007; Huang and Trussell, 2011). However, KCNQ channels are thought to temper spike activity in neurons, as naturally occurring mutations in KCNQ genes are associated with seizure, and the pharmacological blockade of KCNQ channels leads to neuronal hyperactivity (Biervert et al., 1998; Peters et al., 2005; Brown and Passmore, 2009; Qi et al., 2014), which would prevent spike firing at high frequency. Here, we found that KCNQ channels in calyx nerve terminals were cumulatively activated during high-frequency AP activity, thus minimizing inactivation of Na^+ and K_v1 channels, and maintaining a stable presynaptic AP waveform. Blocking KCNQ channels disrupted Ca^{2+} influx, reduced synaptic transmission, and disrupted high-fidelity synaptic signaling. These results illustrate how a slowly gating K^+ channel enables high-frequency firing needed for sensory coding.

Materials and Methods

Slice preparation. The handling and care of animals were approved by the Institutional Animal Care and Use Committee of Tulane University and in compliance with US Public Health Service guidelines. Brainstem slices containing the MNTB were prepared from P8–16 Wistar rats of either sex, as previously described (Huang and Trussell, 2014; Zhu et al., 2020). Briefly, 210 μm sections were cut in ice-cold, low- Ca^{2+} , low- Na^+ saline using a vibratome (model VT1200S, Leica), incubated at 32°C for 20–40 min in normal artificial CSF (aCSF) and thereafter stored at room temperature before experiments. The saline solution for slicing contained the following: 230 mM sucrose, 10 or 25 mM glucose, 2.5 mM KCl, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1.25 mM NaH_2PO_4 , 25 mM $NaHCO_3$, 0.4 mM ascorbic acid, 3 mM *myo*-inositol, and 2 mM Na-pyruvate, bubbled with 5% CO_2 /95% O_2 . The aCSF for incubation and recording contained the following: 125 mM NaCl, 10 or 25 mM glucose, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4 , 25 mM $NaHCO_3$, 0.4 mM ascorbic acid, 3 mM *myo*-inositol, and 2 mM Na-pyruvate, at pH 7.4 bubbled with 5% CO_2 /95% O_2 .

Whole-cell recordings. Brain slices were transferred to a recording chamber and were continually perfused with aCSF (2–3 ml/min) warmed to $\sim 32^\circ C$ by an inline heater (Warner Instruments). Neurons were viewed using a microscope (model BX51, Olympus) with a 40 $\times$ water-immersion objective and customized infrared Dodt gradient contrast optics. Whole-cell current-clamp and voltage-clamp recordings were made with a Multiclamp 700B amplifier and pClamp 10 software (Molecular Devices).

For presynaptic current-clamp and K^+ current recordings, the pipette solution contained the following: 110 mM K-gluconate, 20 mM KCl, 1 mM $MgCl_2$, 4 mM Mg-ATP, 0.3 mM Tris-GTP, 3 mM Na_2 -phosphocreatine, 10 mM Tris-phosphocreatine, 0.2 mM EGTA, and 10 mM HEPES (290 mOsm, pH 7.3 with KOH). To isolate KCNQ currents during high-frequency activity, tetrodotoxin (TTX; 0.5 μM), $CdCl_2$ (100 μM), CsCl (2 mM), 4-aminopyridine (4-AP; 2 mM), and tetraethylammonium chloride (TEA-Cl; 10 mM) were added to aCSF to block Na^+ , Ca^{2+} , HCN, K_v1 , and K_v3 channels, respectively. To isolate K_v1 currents, tetrodotoxin (0.5 μM), $CdCl_2$ (100 μM), CsCl (2 mM), XE991 (10 μM),

and TEA-Cl (1 mM) were added to aCSF to block Na^+ , Ca^{2+} , HCN, KCNQ, and K_v3 channels, respectively. To isolate K_v3 currents, tetrodotoxin (0.5 μM), $CdCl_2$ (100 μM), CsCl (2 mM), XE991 (10 μM), and margatoxin (10 nM) were added to aCSF to block Na^+ , Ca^{2+} , HCN, KCNQ, and K_v1 channels, respectively. For Na^+ and Ca^{2+} current recordings, the pipette solution included the following: 120 mM Cs-methanesulfonate, 10 mM CsCl, 10 mM TEA-Cl, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM EGTA, 0.4 mM Tris-GTP, 3 mM Mg-ATP, and 5 mM Na_2 -phosphocreatine (290 mOsm, pH 7.3 with CsOH). To isolate Na^+ currents, $CdCl_2$ (100 μM), CsCl (2 mM), TEA-Cl (10 mM), 4-aminopyridine (2 mM), and XE991 (10 μM) were added to aCSF to block Ca^{2+} , HCN, and K^+ channels, respectively. To isolate Ca^{2+} currents, tetrodotoxin (0.5 μM), CsCl (2 mM), TEA-Cl (10 mM), 4-aminopyridine (2 mM), and XE991 (10 μM) were added to block Na^+ , HCN, and K^+ channels, respectively. Equimolar NaCl was reduced in all aCSF solutions to keep the osmolarity constant. Leak subtraction was applied to the presynaptic voltage-clamp experiments. For EPSC recordings, the pipette solution contained 130 mM Cs-methanesulfonate, 10 mM CsCl, 10 mM HEPES, 5 mM EGTA, 0.3 mM Tris-GTP, 4 mM Mg-ATP, 5 mM Na_2 -phosphocreatine, and 2 mM QX-314 (290 mOsm, pH 7.3 with CsOH). Strychnine (1 μM), picrotoxin (50 μM), and (R)-CPP (5 μM) were added to the recording solution to block glycine, GABA, and NMDA receptor-mediated currents, respectively. For postsynaptic spike recordings, the pipette solution contained the following: 135 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 0.2 mM EGTA, 4 mM Mg-ATP, 0.3 mM Tris-GTP, and 7 mM Na_2 -phosphocreatine (290 mOsm, pH 7.3 with KOH).

Pipettes pulled from thick-walled borosilicate glass capillaries (WPI) had open tip resistances of 2–4 M Ω when filled with the above pipette solutions. Series resistances (4–15 M Ω) were compensated by 60–80% (bandwidth, 3 kHz). Presynaptic APs were evoked by afferent fiber stimulation using a bipolar stimulating electrode (0.2 ms, 4–8 V) placed close to the ventral midline of slices. Signals were filtered at 4–20 kHz and sampled at 10–50 kHz. Liquid junction potentials were measured and adjusted appropriately. Brain slices were not reused after drug application. No more than two recordings were obtained from each animal.

Drugs. Drugs were obtained from Alomone Labs (XE991) and Abcam (TTX), and all others from Sigma-Aldrich. Drugs were stored as aqueous stock solutions at $-20^\circ C$, and stock solutions were dissolved in aCSF to the final concentration immediately before experiments.

Analysis. Data were analyzed using Clampfit (Molecular Devices), Igor (WaveMetrics), Prism (GraphPad), and MATLAB (MathWorks). Statistical significance was established using one-way or two-way repeated-measures ANOVA followed by Dunnett's multiple-comparisons test or Sidak's multiple-comparisons test for mixed models, as well as paired *t* tests after passing Shapiro–Wilk normality test or nonparametric test if failed normality test, as indicated in the Results. Data are expressed as the mean $\pm$ SEM.

Results

KCNQ channels regulate the excitability of the calyx of Held terminal

Previous studies demonstrated KCNQ5 ($K_v7.5$) immunolabeling in excitatory synapses of auditory brainstem nuclei (Caminos et al., 2007). In the MNTB region, KCNQ5, but not KCNQ2–4, channels are expressed in the calyx of Held terminals, and not in the postsynaptic principal neurons (Huang and Trussell, 2011). The presynaptic KCNQ K^+ current, most probably mediated by KCNQ5 homomers, exhibits unusually negative activation voltage, and thus accounts for most resting outward ionic currents and regulates presynaptic resting properties (Huang and Trussell, 2011). KCNQ channels are well known to control neuronal excitability and prevent excessive neuronal firing in various preparations (Brown and Passmore, 2009). Here we studied the roles of presynaptic KCNQ channels in high-frequency synaptic signaling.

Using whole-cell recordings from the calyx of Held, we confirmed previous observations made in neuronal somata that

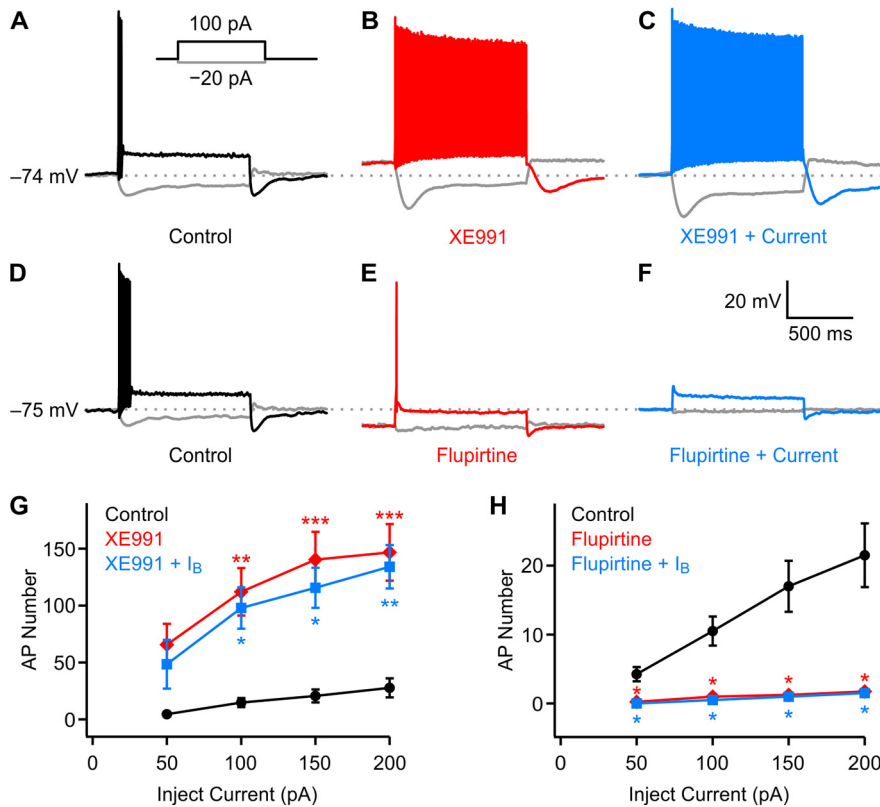


Figure 1. KCNQ channels suppress firing during prolonged current injection. **A, B**, APs elicited by 1 s current steps in control conditions (**A**) and after bath application of 10 μ M XE991 (**B**). **C**, The excitability change was not restored when bias currents were injected to correct for the depolarization of resting membrane potential. **D–F**, Similar to **A–C**, except bath application of 10 μ M flupirtine to open KCNQ channels. **G**, AP numbers elicited by 1 s depolarizing current steps to the values given on the x-axis before and after bath application of XE991, as well as XE991 in the presence of bias current (I_B). **H**, Similar to **G**, but with bath application of flupirtine. XE991 increased, while flupirtine decreased, the total number of APs elicited by the 1 s depolarizing current steps. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-way repeated-measures ANOVA followed by Dunnett's multiple-comparisons test. Error bars are the mean $\pm$ SEM.

inhibition of KCNQ channels results in hyperactivity, when assayed by delivering long depolarizing current pulses. Typically, calyces responded to long current pulses by generating only a few action potentials at the current onset, regardless of the length of the pulse (Fig. 1; Dodson et al., 2003; Ishikawa et al., 2003). Loss of KCNQ conductance had a striking effect on presynaptic excitability. Bath application of XE991 (10 μ M), a concentration that specifically blocks KCNQ current in the calyx of Held without affecting K^+ currents on postsynaptic MNTB neurons (Huang and Trussell, 2011), for 5–10 min caused calyces to fire continuously and at high rates for the duration of the 1-s current injections of 50–200 pA ($n = 6$; Fig. 1B). Conversely, the KCNQ channel activator flupirtine (10 μ M) decreased spike number during current injections ($n = 4$; Fig. 1E). Because of partial activation of KCNQ channels at rest (Huang and Trussell, 2011), the application of either drug caused a shift in resting membrane potential (see below). Therefore, after applying the drugs and recording responses to current pulses, we then injected positive or negative bias current to restore the membrane potential back to the control resting level and repeated the current pulse protocol. Similar results were obtained (Fig. 1C,F). Overall, the spike numbers were significantly increased in XE991 or XE991 plus bias current ($F_{(1,043,5,214)} = 31.03$, $p = 0.002$, $n = 6$; two-way repeated-measures ANOVA followed by Dunnett's multiple-comparisons test; Fig. 1G), while decreased in flupirtine or flupirtine plus bias current ($F_{(1,008,3,023)} = 25.65$, $p = 0.01$, $n = 4$; two-way repeated-

measures ANOVA followed by Dunnett's multiple-comparisons test; Fig. 1H). Together, these data show that the excitability of the terminal is dependent not only on the fast-gating channels that underlie the action potential, but also on slowly gating conductances like KCNQ.

KCNQ channels maintain AP waveform during high-frequency firing

We next asked how blocking KCNQ channels affected propagated, rather than locally triggered, action potentials. To do that, we first stimulated presynaptic axons and assessed the stability of the AP waveform when evoked at different rates. The AP waveforms were remarkably stable during prolonged repetitive stimulation (400 spikes) at high frequencies (Fig. 2). AP waveforms remained nearly unchanged during 10 and 33 Hz firing. Increasing the firing frequency to 100 Hz induced only a slight amplitude decrease and AP broadening in an activity-dependent manner. Comparing the 400th AP to the first AP, the amplitude decreased by $3.0 \pm 0.9\%$ and the half-width increased by $12.9 \pm 2.9\%$. When the stimulation frequency increased to 333 Hz, AP amplitude eventually decreased by $21.2 \pm 3.6\%$ and the half-width increased by $76.4 \pm 20.1\%$ ($n = 5$). Compared with the first AP, the amplitude of the last AP was significantly decreased while half-width was increased at 333 Hz, but not in other frequencies (amplitude: 10 Hz, $p = 0.99$; 33 Hz, $p = 0.48$; 100 Hz, $p = 0.10$; 333 Hz, $p < 0.001$; half-width: 10 Hz, $p > 0.99$; 33 Hz, $p > 0.99$; 100 Hz, $p = 0.46$; 333 Hz, $p < 0.001$; $n = 5$; two-way repeated-measures ANOVA followed by Sidak's multiple-comparisons test). Thus, the calyceal terminals have the capacity to maintain stable AP waveform during high-frequency firing.

We then investigated the underlying mechanisms that support reliable firing at the calyx terminal using the 333 Hz stimulation protocol. When measuring the interspike potential (ISP; i.e., the membrane potential between spikes; 0–0.2 ms before the next stimulation artifact), we noticed that 6 of 10 calyces exhibited a depolarization during repetitive firing during the first 20–50 ms of the stimulus protocol, and then gradually hyperpolarized (Fig. 3A,F, black trace). The average hyperpolarization of ISP was 3.2 ± 0.7 mV ($n = 6$). We hypothesized that a slow K^+ current, whose kinetics are similar to KCNQ channels (Huang and Trussell, 2011), may be activated during high-frequency spiking and is critical to maintaining the AP waveform. Indeed, in the presence of 10 μ M XE991, the delayed hyperpolarization of ISP was eliminated. Instead, the ISP continuously depolarized during high-frequency spiking (Fig. 3B,F). Accompanying the depolarization of ISP, the AP waveforms were greatly altered. When the first and last APs in the spike train were compared, it was apparent that XE991 had a significantly greater impact on the waveform of the last APs compared with the first APs. The

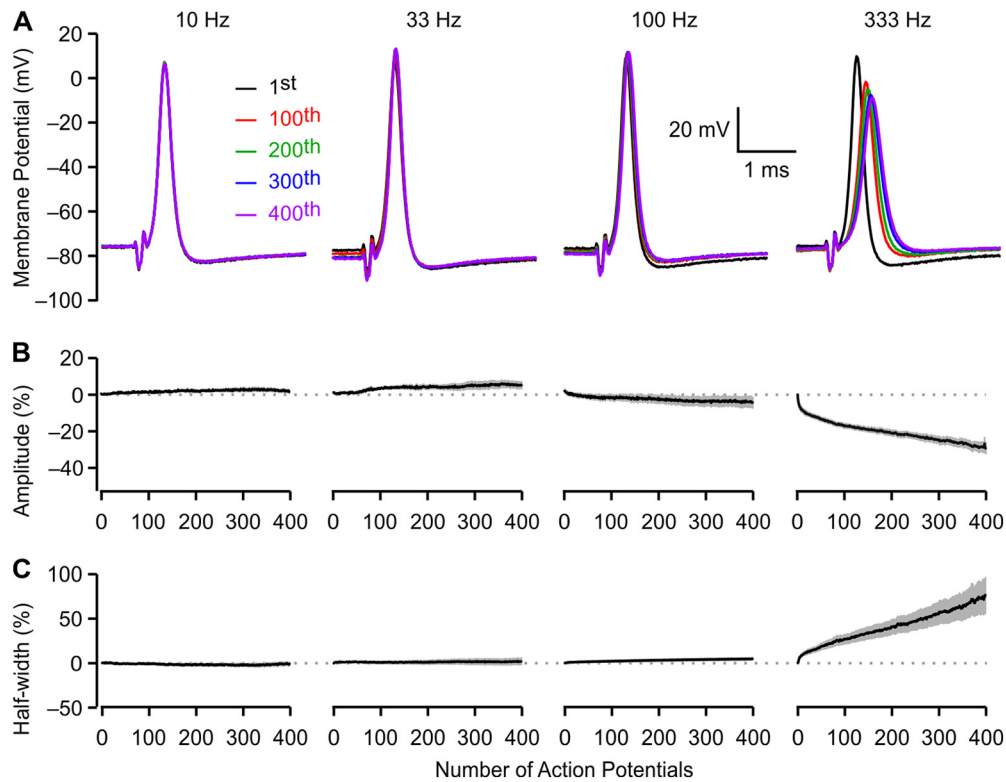


Figure 2. Stable AP waveform during high-frequency AP firing in the calyx of Held. **A**, Trains of APs evoked at the frequency of 10, 33, 100, and 333 Hz by afferent fiber stimulation. Every 100th AP is superimposed with the first AP. **B**, **C**, Plots of AP amplitude (**B**) and half-width (**C**) during spiking at each frequency. Data were normalized to the value of the first AP in each train. Shadings are the mean $\pm$ SEM.

amplitude was reduced from 69.6 ± 2.1 to 33.9 ± 4.5 mV, and the half-width was broadened from 0.46 ± 0.07 to 1.27 ± 0.09 ms after the application of XE991 (Fig. 3). KCNQ channels in the calyx of Held have an activation threshold more hyperpolarized than the resting membrane potential and thus contribute to the resting properties of the calyx of Held (Huang and Trussell, 2011). Blocking KCNQ channels with XE991 depolarized the membrane potential by 5.2 ± 1.3 mV, an effect that impacts the activation and inactivation of other voltage-gated channels. After applying XE991, we injected a hyperpolarizing bias current to restore the membrane potential and repeated the stimulating protocol. The waveform of the first APs was restored to the control level, while the late phase was not fully restored: the ISP continued depolarizing during the whole stimulation period (Fig. 3C,F) and the AP became shorter and broader. The AP amplitude was reduced from 91.3 ± 6.2 to 53.1 ± 4.6 mV, and the half-width was broadened from 0.33 ± 0.03 to 0.81 ± 0.09 ms (Fig. 3G,H; $n = 5$). Compared with control conditions (amplitude reduction from 92.2 ± 4.5 to 74.4 ± 4.2 mV, and half-width broadening from 0.30 ± 0.01 to 0.56 ± 0.05 ms), the AP amplitude reduction was significantly increased in XE991 or XE991 plus bias current ($F_{(1,980,7,920)} = 30.32$, $p < 0.001$, $n = 5$; one-way repeated-measures ANOVA followed by Dunnett's multiple-comparisons test: $p = 0.002$ for control vs XE991; control vs XE991 plus current, $p = 0.01$), and the AP half-width broadening was also increased in XE991 or XE991 plus bias current ($F_{(1,728,6,911)} = 16.75$, $p = 0.002$, $n = 5$; one-way repeated-measures ANOVA followed by Dunnett's multiple-comparisons test: control vs XE991, $p = 0.01$; control vs XE991 plus current, $p = 0.04$). Another KCNQ blocker, linopirdine, had a similar effect. Compared with the first AP, the amplitude of the last AP was reduced to $79.8 \pm 1.6\%$ for control, $50.4 \pm 6.5\%$ for linopirdine, and $72.9 \pm 2.2\%$ for linopirdine plus current, respectively ($F_{(1,076, 3,227)} = 29.56$, $p = 0.01$), and half-width

was increased by $77.4 \pm 9.3\%$ for control, $126.9 \pm 16.1\%$ for linopirdine, and $136.5 \pm 15.9\%$ for linopirdine plus current, respectively ($F_{(1,780,5,340)} = 42.38$, $p = 0.001$, $n = 4$; Fig. 3I–K). These results indicate that the accumulated activation of KCNQ channels during high-frequency AP firing produces a gradual hyperpolarization of ISP, which is essential in maintaining the AP waveform during high-frequency firing.

Activation of KCNQ current during high-frequency firing

We next used voltage clamp to monitor the buildup of KCNQ current during trains of spikes. The KCNQ current of the calyceal terminals is resistant to TEA and 4-AP (Huang and Trussell, 2011); thus, we were able to isolate the KCNQ current with these general K^+ channel blockers (see Materials and Methods). We measured the activation of KCNQ current during high-frequency AP firing in which a series of pseudo-APs (1 ms depolarizing voltage pulses from -80 to $+10$ mV) were used as voltage-clamp waveforms to trigger a gradual activation of presynaptic KCNQ current. In Figure 4A, this activation is seen as a gradual increase in the amplitude of current responses to voltage pulses: during each pulse, the driving force for K^+ current increases and decreases, but with successive pulses, more channels contribute to the current. At 333 Hz, we observed a cumulative KCNQ current activation of 213 ± 33 pA ($n = 5$; Fig. 4A). The activation of KCNQ current was fitted with a double exponential whose fast and slow components were 54.7 ± 28.2 and 387.7 ± 131.1 ms, respectively. The fast component was $31.2 \pm 5.1\%$, and the weighted time constant was 143.5 ± 37.2 ms ($n = 5$; Fig. 4B). Coapplication of XE991 (10 – 20 μ M) fully blocked this current ($n = 5$; Fig. 4C) and confirmed that the outward current is mediated by KCNQ channels. With 100 Hz AP train, the cumulative KCNQ current was 114 ± 50 pA ($n = 5$). The

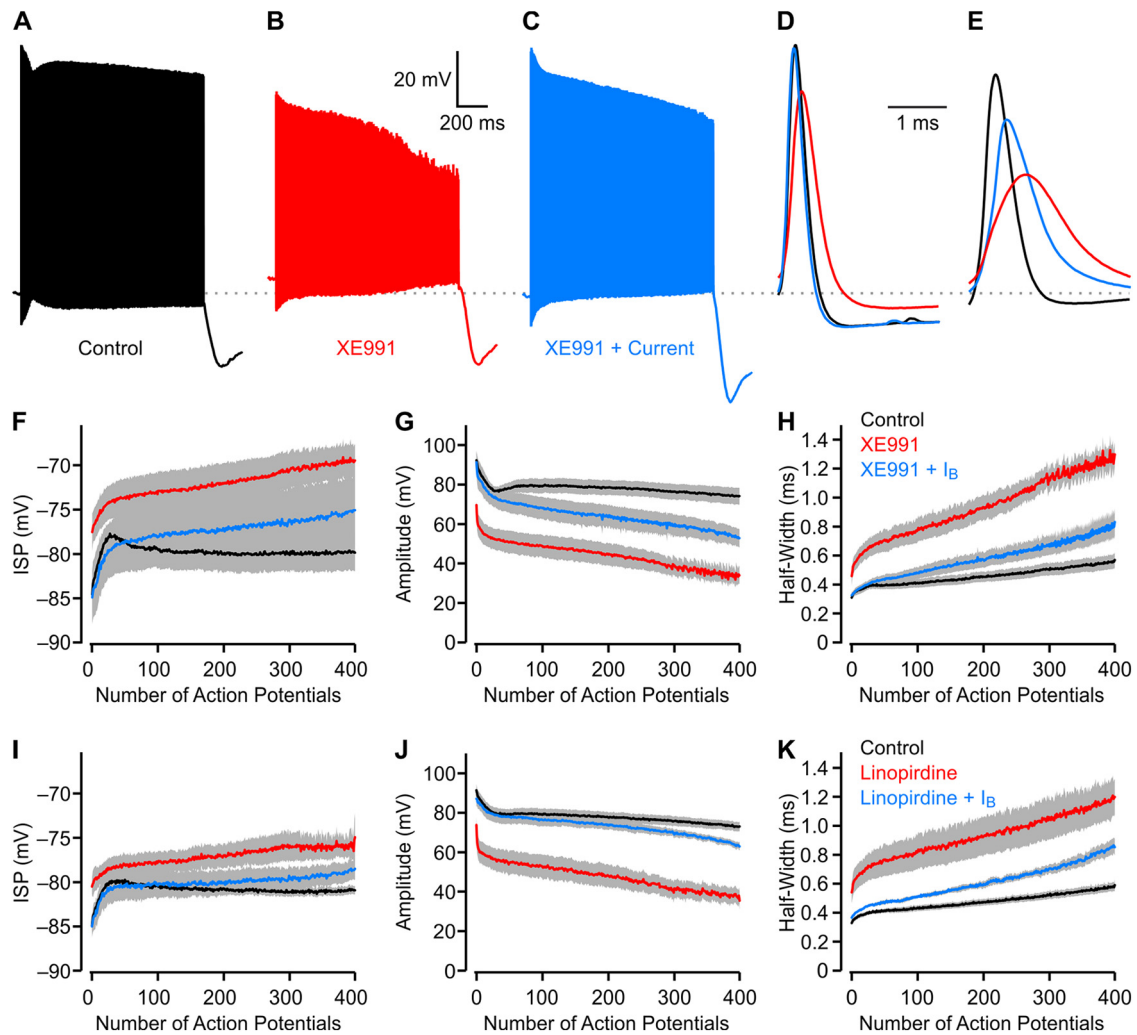


Figure 3. KCNQ channels contribute to reliable AP waveform during high-frequency firing. **A–C**, Trains of APs elicited at the frequency of 333 Hz (3 ms intervals) before (black, **A**) and after (red, **B**) bath application of 10 μ M XE991. **C**, Bias current was injected to restore the resting membrane potential to the value of the control AP train (blue). Stimulation artifacts were removed for clarity. **D**, **E**, The first (**D**) and 400th (**E**) APs were superimposed at an expanded timescale. **F–H**, Plots of ISP (**F**), AP amplitude (**G**), and half-width (**H**) of AP waveforms shown in **A–C**. **I–K**, similar to **F–H**, except bath application of 10 μ M linopirdine. Shadings are the mean $\pm$ SEM.

activation of KCNQ current was fitted with a double exponential whose fast and slow components were 313.6 ± 201.4 and 1655.6 ± 297.6 ms, respectively. The fast component was $44.1 \pm 10.7\%$, and the weighted time constant was 996.5 ± 265.8 ms ($n = 5$; Fig. 4D). The 10 Hz AP train, however, did not activate cumulative KCNQ current ($n = 5$; Fig. 4E), indicating that the activation of KCNQ channel during 1 ms depolarization is fully deactivated over the long interval of 99 ms. Therefore, the KCNQ current can be cumulatively activated during intense spiking activity (100 and 333 Hz), but not with mild spiking activity (10 Hz).

Activation of KCNQ limits cumulative inactivation of Na^+ and K_V1 channels

During repetitive firing, Na_V and K_V channels undergo cycles of activation and deactivation, inactivation, and repriming (recovery from inactivation). Repriming is strongly dependent on the spike afterpotential (Raman and Bean, 2001; Bean, 2007). Since activation of KCNQ channels keeps the ISP hyperpolarized, we hypothesized that the hyperpolarization induced by the opening of KCNQ channels during high-frequency firing helps Na_V and K_V channels to recover from inactivation. To test whether

KCNQ channels influence the recovery of voltage-gated ion channels from inactivation during high-frequency (400 APs at 333 Hz) firing, we recorded presynaptic Na_V and K_V currents before and after AP trains under voltage clamp in the presence of blockers for other voltage-gated channels (see Materials and Methods). The spike waveforms recorded in controls (Fig. 3A), with XE991 (Fig. 3B), and XE991 with tonic bias current (Fig. 3C) were used as voltage command templates. The 10 ms depolarizing square pulses from resting membrane potential to -10 mV were applied before and immediately after (3 ms after the onset of the last AP) the AP command train (Fig. 5A). The ratio of the peak inward current evoked by the second over the first square pulse represents the degree of inactivation developed during the train stimulation. The Na^+ current was reduced to $65.8 \pm 1.2\%$ after the control AP train template. When templates were used from spikes recorded in the presence of XE991 or XE991 plus bias current conditions, inactivation was more prominent. The peak inward Na^+ current decreased to $26.6 \pm 2.1\%$ and $25.8 \pm 2.1\%$, respectively ($F_{(1,730,12,11)} = 690.5$; $p < 0.001$, $n = 8$; one-way ANOVA followed by Dunnett's multiple-comparisons test; Fig. 5D), indicating that KCNQ channels serve to minimize the cumulative inactivation of Na^+ channels during repetitive firing.

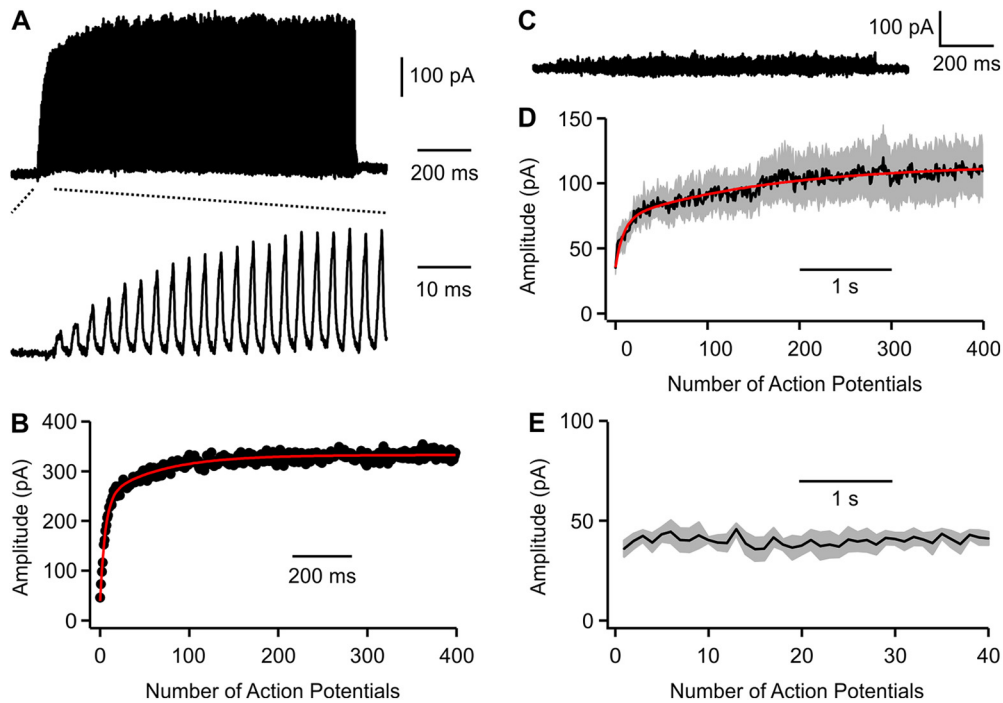


Figure 4. Activation of KCNQ current during high-frequency spike activity. **A**, KCNQ current in response to 1 ms depolarizations (−80 to +10 mV) at 333 Hz. The lower trace shows cumulative activation of KCNQ current at a faster timescale. **B**, The amplitudes of KCNQ current in **A** were plotted against the action potential number during the train. The trace was fitted by an exponential function (red) with fast and slow components. **C**, The outward currents were blocked by coapplication of 10 μ M XE-991. **D**, Similar to **B**, but the 1 ms depolarizations at 100 Hz. **E**, No cumulative KCNQ current was recorded in response to 1 ms depolarizations at 10 Hz. Recordings were made in the presence of TEA, 4-AP, TTX, Cd^{2+} , and Cs^+ to block voltage-gated K_v1 , K_v3 , Na^+ , Ca^{2+} , and HCN channels, respectively. Leak subtraction was applied to the traces. Shadings are the mean $\pm$ SEM.

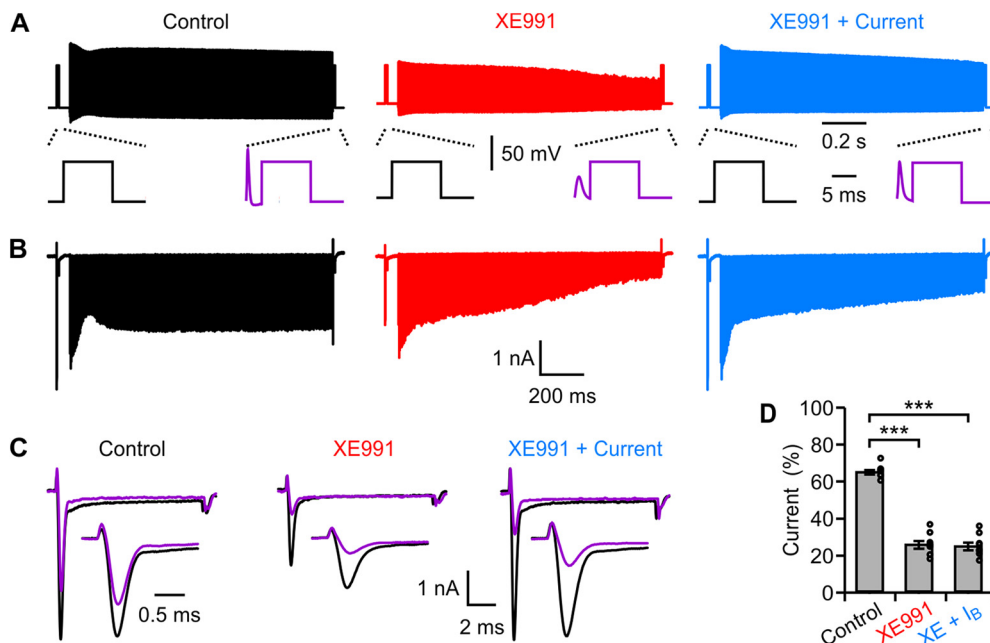


Figure 5. KCNQ activation relieves cumulative inactivation of Na^+ channels at high-frequency firing. **A**, Command templates were created by adding 10 ms depolarizing pulses before and immediately after AP trains obtained under the control, XE991, and XE991 plus current conditions shown in Figure 3A–C. **B**, Na^+ current (I_{Na}) evoked by the depolarizing pulses (10 ms depolarization to −10 mV) before (black) and after (purple) the AP train were superimposed at an expanded timescale. **C**, I_{Na} evoked by the depolarizing pulses (10 ms depolarization to −10 mV) before (black) and after (purple) the AP train were superimposed at an expanded timescale. **D**, Summary plot of the Na^+ current ratio, showing that blocking KCNQ with XE991 enhanced Na^+ channel inactivation. Recordings were made in the presence of TEA, 4-AP, XE991, Cd^{2+} , and Cs^+ to block voltage-gated K^+ , Ca^{2+} , and HCN channels, respectively. *** $p < 0.001$; one-way ANOVA followed by Dunnett's multiple-comparisons test. Error bars are the mean $\pm$ SEM.

Similar experiments were used to test the inactivation of K_v1 and K_v3 channels, which are the predominant K^+ channel subtypes in the calyx of Held (Dodson et al., 2002; Ishikawa et al., 2003; Dodson and Forsythe, 2004; Song et al., 2005; Yang et al.,

2014). K_v1 currents (measured in the presence of 1 mM TEA) were reduced by repetitive spike activity to $64.1 \pm 3.6\%$ under control spike train, and further reduced to $60.5 \pm 2.6\%$ and $54.9 \pm 2.7\%$ under XE991 and XE991 plus bias current

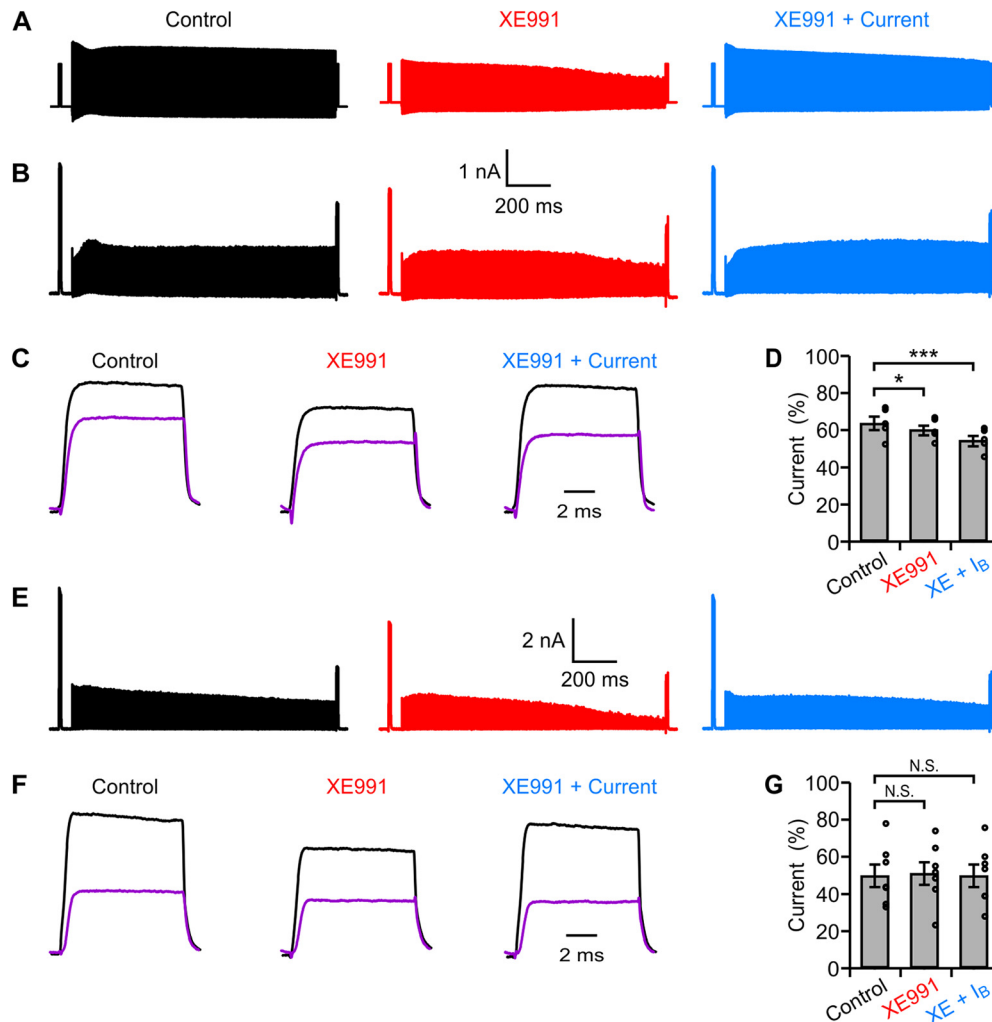


Figure 6. KCNQ activation relieves cumulative inactivation of K_v1 , but not of K_v3 . **A**, Recordings were similar to Figure 5 while K^+ currents were recorded. **B**, Representative traces of K_v1 current evoked by the command templates. Recordings were made in the presence of TTX, Cd^{2+} , Cs^+ , XE991, and TEA to block Na^+ , Ca^{2+} , HCN, KCNQ, and K_v3 channels, respectively. **C**, K_v1 current evoked by the first (black) and second (purple) depolarizing pulses (10 ms depolarization to -10 mV) were superimposed at an expanded timescale. **D**, Summary data of the remaining K_v1 currents for the control, XE991, and XE991 plus current command templates. **E–G**, Similar to **B–D**, except K_v3 currents were recorded in the presence of TTX, Cd^{2+} , Cs^+ , XE991, and margatoxin to block Na^+ , Ca^{2+} , HCN, KCNQ, and K_v1 channels, respectively. N.S., $p > 0.05$, $*p < 0.05$, $***p < 0.001$; one-way ANOVA followed by Dunnett's multiple-comparisons test. Error bars are the mean $\pm$ SEM.

conditions, respectively (Fig. 6A–D), indicating a slightly larger inactivation after blocking KCNQ channels ($F_{(1.475,5.898)} = 45.00$, $p < 0.001$, $n = 5$; one-way ANOVA followed by Dunnett's multiple-comparisons test; Fig. 6D). K_v3 currents were recorded in the presence of the K_v1 channel blocker margatoxin (10 nM). In control conditions, K_v3 currents were reduced to $50.1 \pm 6.1\%$, likely because of the use-dependent inactivation (Marom and Levitan, 1994), which was not different from recordings in the presence of XE991 ($51.4 \pm 6.1\%$) or XE991 plus current conditions ($50.2 \pm 6.1\%$; Fig. 6E–G; $F_{(1.677,10.06)} = 0.1784$, $p = 0.80$, $n = 7$; one-way ANOVA followed by Dunnett's multiple-comparisons test; Fig. 6G). Therefore, the gradual hyperpolarization of the membrane during repetitive firing primarily serves to minimize the inactivation of Na^+ channels.

KCNQ channels enable reliable calcium influx during high-frequency firing

Presynaptic AP directs Ca^{2+} influx required for vesicle fusion and neurotransmitter release (Katz and Miledi, 1970; Borst and Sakmann, 1996). We therefore tested how changes in AP waveform during high-frequency firing affect Ca^{2+} currents. AP

trains recorded during current clamp (Fig. 3A–C) were used as command templates to evoke Ca^{2+} current (I_{Ca}) under voltage-clamp recordings (Fig. 7A,B). In control conditions, the I_{Ca} amplitudes were relatively stable, reducing slightly from 2.00 ± 0.14 nA at the first AP to 1.75 ± 0.07 nA after the 400th APs. However, I_{Ca} amplitudes were reduced even more, from 1.17 ± 0.09 to 0.43 ± 0.06 nA in XE991, and from 1.87 ± 0.10 to 1.43 ± 0.15 nA for the conditions of XE991 plus current (Fig. 7E). Compared with the first AP, the I_{Ca} amplitude reduction of last AP was significantly greater in XE991 or XE991 plus bias current than in the control ($F_{(1.446,7.230)} = 85.70$, $p < 0.001$, $n = 6$; one-way repeated-measures ANOVA followed by Dunnett's multiple-comparisons test: control vs XE991, $p < 0.001$; control vs XE991 plus current, $p = 0.06$).

Since the I_{Ca} duration was altered (Fig. 7C,D), we also calculated the AP-induced Ca^{2+} charge. The charge was slightly increased from 0.51 ± 0.05 to 0.67 ± 0.06 pC in control conditions, mainly because of AP broadening. In XE991, the AP Ca^{2+} charge was initially 0.45 ± 0.05 pC, briefly increased during the train, and then declined to 0.37 ± 0.06 pC. In the XE991 plus bias current, the Ca^{2+} charge was gradually increased from

0.50 ± 0.06 to 0.86 ± 0.08 pC ($p < 0.0001$). Together, these results indicate that KCNQ channels during high-frequency firing have profound effects on regulating the I_{Ca} and that the KCNQ-enabled stable AP waveform is crucial for stably evoking Ca^{2+} influx. Compared with the first AP, the reduction of Ca^{2+} charge of the last AP was also significantly greater in XE991 or XE991 plus bias current than in control ($F_{(1.196,5.979)} = 67.44$, $p < 0.001$, $n = 6$; one-way repeated-measures ANOVA followed by Dunnett's multiple-comparisons test: control vs XE991, $p = 0.002$; control vs XE991 plus current, $p < 0.001$).

KCNQ channels enable reliable synaptic transmission at high frequency

As blocking KCNQ channels affects the AP-evoked Ca^{2+} influx, we tested the role of KCNQ channels in neurotransmitter release and synaptic transmission. Postsynaptic cells were voltage clamped, and presynaptic axons were stimulated with an extracellular electrode to evoke 400 EPSCs at 3 ms intervals. Despite synaptic depression, EPSCs were evoked in response to each presynaptic AP (Fig. 8A). The application of $10 \mu\text{M}$ XE991 increased the EPSC amplitudes at the beginning of the train because of the previously described role of KCNQ channels on presynaptic resting membrane potential (Huang and Trussell, 2011). However, as the AP train proceeded, the EPSCs were reduced in amplitude by 23% (Fig. 8B; mean amplitude of the 301st to the 400th EPSC reduced from 266 ± 34 to 202 ± 46 pA; $p = 0.04$, $n = 5$; paired t test).

High-frequency signals are faithfully transmitted from globular bushy cells to the postsynaptic MNTB neurons through the calyx terminal, and each presynaptic spike triggers an AP at its postsynaptic MNTB neuron, with few failures (Mc Laughlin et al., 2008; Lorteije et al., 2009). However, the loss of KCNQ activity distorted the presynaptic spike and Ca^{2+} influx, and reduced the EPSC. Therefore, we next tested whether KCNQ contributes to faithful one-to-one synaptic signaling. Postsynaptic cells were current clamped to record the AP firing in response to the presynaptic stimulation. At 333 Hz, each presynaptic stimulation evoked an AP in MNTB neurons under control condition (Fig. 8C,E). Bath application with XE991 did not affect the reliability of synaptic transmission at the beginning of the train, but eventually disrupted one-to-one fidelity and caused failures in postsynaptic spikes (Fig. 8D,E). The failure rate increased after the 251st stimulation ($F_{(1,6)} = 17.9$, $p = 0.006$, $n = 7$; two-way repeated-measures ANOVA followed by Sidak's multiple-comparisons test; Fig. 8E). A previous study showed that $20 \mu\text{M}$ XE991 did not affect K^+ currents on postsynaptic MNTB neurons (Huang and Trussell, 2011). We further tested the effects of XE991 on postsynaptic resting membrane potential and firing properties. As shown in Figure 8F–H, $10 \mu\text{M}$ XE991 did not affect resting membrane potential, action potential amplitude, or half-width ($n = 5$).

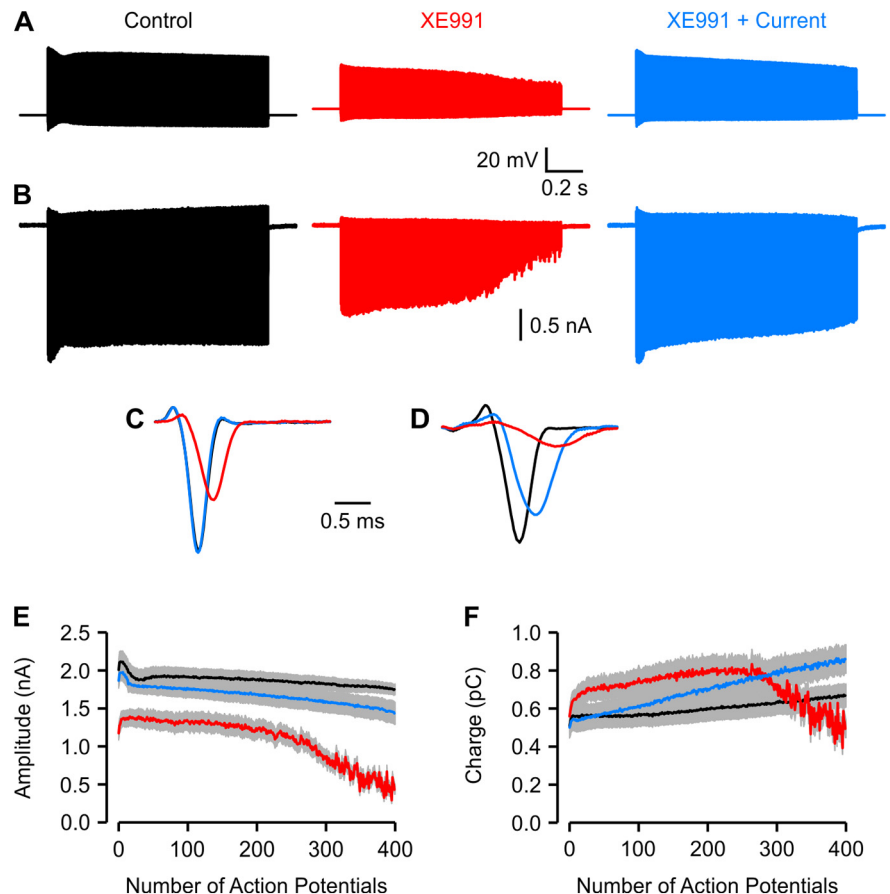


Figure 7. KCNQ activation maintains reliable Ca^{2+} influx during high-frequency firing. **A**, Naive control, XE991, and XE991 plus current AP trains were used as voltage-clamp command templates. **B**, Representative traces of I_{Ca} evoked by three command templates with the same calyx. **C**, **D**, First (**C**) and last (**D**) I_{Ca} in each recording are superimposed at an expanded timescale. **E**, **F**, Amplitudes (**E**) and charges (**F**) of I_{Ca} were measured and plotted against the number of the APs within the three command templates. Recordings were made in the presence of TEA, 4-AP, XE991, TTX, and Cs^+ to block voltage-gated K^+ , Na^+ , and HCN channels, respectively. Shadings are the mean $\pm$ SEM.

Together, these results indicate that the activation of presynaptic KCNQ current during high-frequency firing aids in reliable neurotransmitter release and synaptic signaling across the synapse.

Discussion

Calyx of Held terminals exhibit minimum changes in AP waveform during high-frequency spiking. Spike amplitude and width degraded only when spike number and frequency reached extreme values. Here, we showed that KCNQ channels, formed by KCNQ5 homomers (Huang and Trussell, 2011), are cumulatively activated during high-frequency AP firing, and this activation keeps the presynaptic membrane potential between spikes hyperpolarized, which minimizes ongoing inactivation of Na^+ and K_v1 channels. The resulting consistency in the AP waveform ensures reliable calcium influx and glutamate release.

KCNQ channels and reliable AP waveform

While the somata of globular bushy cell and postsynaptic MNTB neurons may exhibit significant AP depression during brief high-frequency firing (Smith and Rhode, 1987; Lorteije et al., 2009; Zhang and Huang, 2017; Li et al., 2020), the AP waveform of the calyceal terminals was remarkably stable during high-frequency firing. In postnatal day 12 (P12) to P14 rat brain slices, reliable synaptic transmission was generally possible at

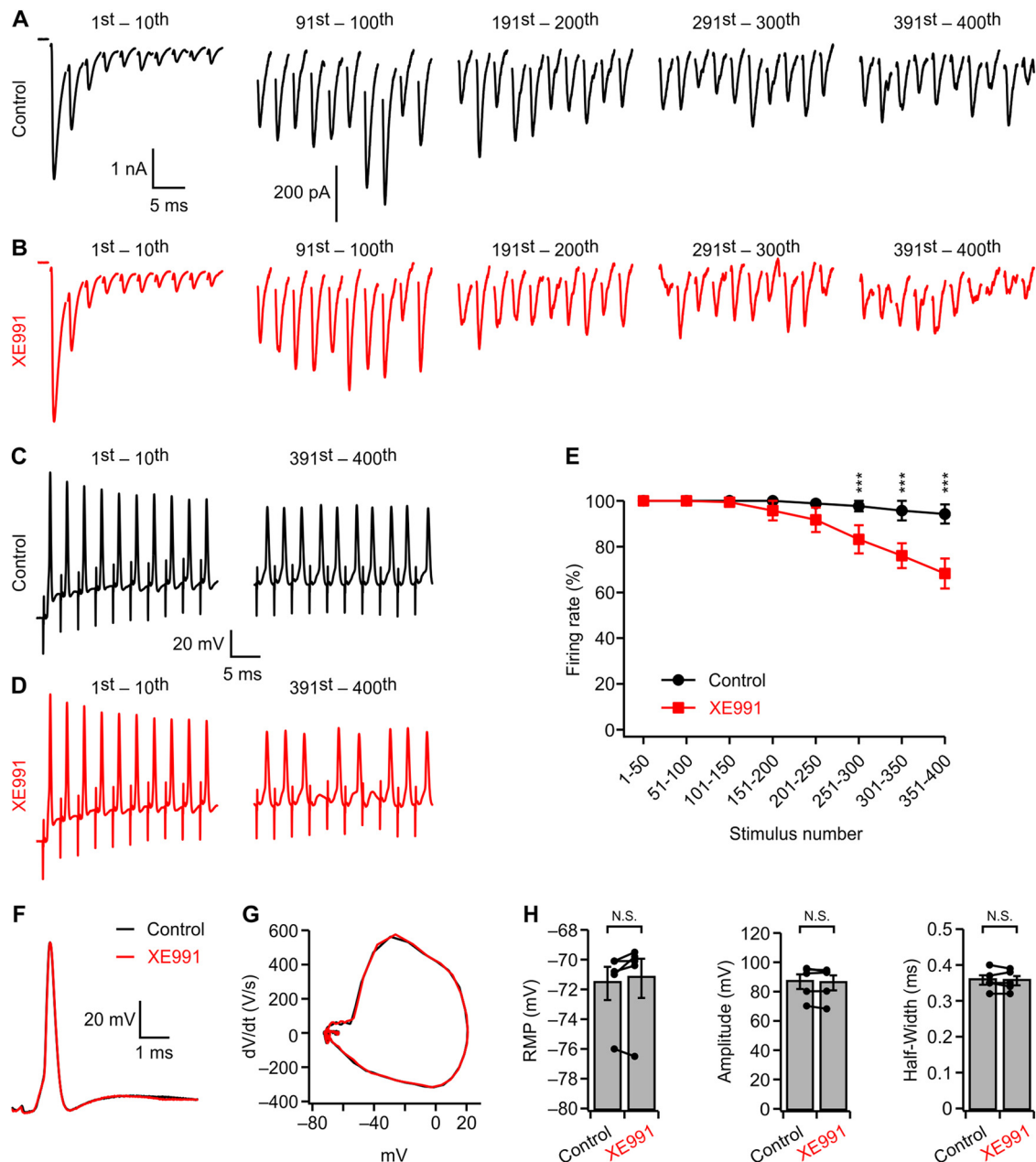


Figure 8. Blocking of KCNQ channels reduces the reliability of synaptic transmission at high frequency. **A**, EPSCs at different time points evoked at 333 Hz. **B**, The same EPSC recordings in the presence of 10 μ M XE991. **C**, **D**, Representative trace showed the same range of number of APs before (black) and after (red) bath application of XE991. **E**, Statistical results showing that XE991 decreased the reliability of synaptic transmission at the later phase of high-frequency trains. *** $p < 0.001$; two-way ANOVA followed by Sidak's multiple-comparisons test. **F**, **G**, Representative postsynaptic AP waveform (**F**) and its phase plane (**G**) obtained before (black) and after (red) bath application of XE991. **H**, XE991 did not significantly affect resting membrane potential (RMP), AP amplitude, or half-width. N.S., $p > 0.05$, nonparametric test for RMP, and paired t test for amplitude and half-width. Error bars are the mean $\pm$ SEM.

frequencies up to 600 Hz for 50 stimuli (Taschenberger and von Gersdorff, 2000). *In vivo* recordings from mice showed that APs showed little or no (<4%) depression when instantaneous firing frequencies were >200 Hz (Sierksma and Borst, 2017). Here we showed AP amplitude decrease during 100 Hz firing for 4 s was 3%, while 333 Hz firing decreased the AP amplitude by 20% (Fig. 2), indicating very stable AP waveforms during prolonged high-frequency firing. The following two factors appeared to be crucially important for the lack of a change in the shape of the AP: fast recovery of Na^+ channels from inactivation (Leão et al., 2005; Huang and Trussell, 2008); and relatively stable, negative membrane potential following the AP (Sierksma and Borst, 2017). The recovery of Na^+ from inactivation is sharply

dependent on the membrane potential, as a hyperpolarizing shift of 20 mV doubled the speed of recovery (Leão et al., 2005). Because of its slow activation and deactivation kinetics (in tens of milliseconds; Huang and Trussell, 2011), the amplitude of KCNQ current gradually increases during repetitive high-frequency AP firing (Fig. 4). This accumulation of KCNQ current hyperpolarized the afterpotential and sped the recovery of the Na^+ channel from inactivation, whereas blocking KCNQ channels elevated the membrane potential between action potentials and led to a distortion of the presynaptic Ca^{2+} current. Therefore, the activation of KCNQ channels during high-frequency activity enables the AP firing with stable waveform and high-fidelity synaptic signaling. In conditions where extracellular

$[K^+]_o$ is elevated to 11.5 mM, XE991 reduced and retigabine increased EPSPs in hippocampal and striatum neurons (Vervaeke et al., 2006), suggesting that KCNQ channels are more critical for synaptic transmission in pathologic high $[K^+]_o$ conditions. Our results showed that the inhibition of KCNQ lowered postsynaptic firing rate during high-frequency signaling, suggesting complexity in using KCNQ channel openers to treat hyperexcitability conditions.

KCNQ channels and control of firing properties

KCNQ channels are a subfamily of voltage-gated K^+ channels that are widely expressed in the CNS. Their slow activation and lack of inactivation make them suitable for a variety of roles associated with controlling excitability in the brain. In particular, extensive studies reported that KCNQ channels function to prevent excessive activity in various neurons. KCNQ mutation or pharmacological block of KCNQ channels elevated neuronal excitability (Soh et al., 2014; Martinello et al., 2019). *In vivo* studies showed that the injection of XE991 leads to hyperactivity (Schwarz et al., 2006) and that mutations in KCNQ channels are associated with epilepsy (Biervert et al., 1998). Consistent with these observations, we found that KCNQ blockers caused calyces to fire continuously and at high rates in response to prolong current injection.

However, such analyses do not address the role of K^+ channels in maintaining the membrane in a spike-ready state, in particular with a membrane potential appropriate for optimal repriming of Na^+ channels. Loss of Na^+ channel availability is particularly acute during high-frequency AP activity, and thus a mechanism is needed to ensure stable responses across different activity rates. KCNQ channels are well suited to this role. At rest, a small proportion of KCNQ channels are activated (Wladyka and Kunze, 2006; Brown and Passmore, 2009; Huang and Trussell, 2011; Shah et al., 2013; Battefeld et al., 2014; Hu and Bean, 2018), contributing to establishing the resting potential and the baseline responsiveness of soma, axon, and synapse. The resting membrane potential also determines the availability of channels that inactivated strongly, such as Na^+ and A-type K^+ channels, and thus indirectly affect AP waveform (Huang and Trussell, 2011; Battefeld et al., 2014). We show here that blocking KCNQ depolarizes the resting membrane potential of the calyx of Held terminals and increase the neurotransmitter release, consistent with previous results (Huang and Trussell, 2011). Moreover, we show that during prolonged repetitive activity, gradual depolarization of the membrane reduces the availability of Na^+ channels, and to a lesser extent K_v1 channels. This loss of channel function broadens and attenuates the spike. At the nerve terminal, these changes are particularly problematic because of their effect on Ca^{2+} flux and Ca^{2+} -dependent exocytosis. Indeed, blocking KCNQ channels reduces the neurotransmitter release and attenuates EPSCs, thus lowering the reliability of synaptic transmission during prolonged high-frequency firing.

Although KCNQ channels activate slowly on depolarization (Biervert et al., 1998; Peretz et al., 2010; Huang and Trussell, 2011), fast spikes will contribute slightly but incrementally to a gradual enhancement of the KCNQ current and will help to maintain the membrane potential between spikes at an optimal level. This effect will be of particular importance in axons and terminals of sensory pathways, where driven spike rates can reach hundreds of hertz in response to strong stimuli. Thus, in addition to their role in preventing hyperexcitability, KCNQ channels may function to enable sensory coding across the full dynamic range of the system.

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