Brief Communications

Spontaneous Vesicle Release Is Not Tightly Coupled to Voltage-Gated Calcium Channel-Mediated Ca^{2+} Influx and Is Triggered by a Ca^{2+} Sensor Other Than Synaptotagmin-2 at the Juvenile Mice Calyx of Held Synapses

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It is well known that voltage-gated calcium channels (VGCCs)-mediated Ca^{2+} influx triggers evoked synaptic vesicle release. However, the mechanisms of Ca^{2+} regulation of spontaneous miniature vesicle release (mini) remain poorly understood. Here we show that blocking VGCCs at the juvenile mice (C57BL/6) calyx of Held synapse failed to cause an immediate change in minis. Instead, it resulted in a significant reduction (~40%) of mini frequency several minutes after the blockage. By recording VGCC activity and single vesicle fusion events directly at the presynaptic terminal, we found that minis did not couple to VGCC-mediated Ca^{2+} entry, arguing for a lack of direct correlation between mini and transient Ca^{2+} influx. Moreover, mini frequencies displayed a lower apparent Ca^{2+} cooperativity than those of evoked release. In agreement with this observation, abrogation of the Ca^{2+} sensor synaptotagmin-2 had no effect on apparent Ca^{2+} cooperativity of minis. Together, our study provides the first direct evidence that spontaneous minis are not mediated by transient Ca^{2+} signals through VGCCs and are triggered by a Ca^{2+}-sensing mechanism that is different from the evoked release at these microdomain VGCC–vesicle coupled synapses.

Key words: **Ca^{2+} sensor; spontaneous vesicle release; synaptic plasticity; VGCC**

Introduction

Three types of synaptic vesicle fusion have been identified so far, namely evoked synchronous release, evoked asynchronous release, and spontaneous release (Pang and Südhof, 2010). Evoked release of vesicles is the major form of synaptic transmission and is initiated by Ca^{2+} influx via voltage-gated calcium channels (VGCCs; Katz and Miledi, 1970; Borst et al., 1995). These readily releasable vesicles are coupled to VGCCs so that each vesicle can be driven by the opening of a single or multiple Ca^{2+} channels (Stanley, 1993; Wadel et al., 2007; Bucurenciu et al., 2010; Sheng et al., 2012; Vyleta and Jonas, 2014). Spontaneous minis are individual events functioning in synaptic maturation and synaptic plasticity (Axelsson and Thesleff, 1959; McKinney et al., 1999; Verhage et al., 2000; Tyler and Pozzo-Miller, 2003; Sutton et al., 2006; Lee et al., 2010), and are triggered by as yet unknown mechanisms. Despite a small fraction of Ca^{2+}-independent spontaneous miniature vesicle releases (minis; Yamasaki et al., 2006), the large fraction of minis appears to be Ca^{2+} dependent. Three factors involved in Ca^{2+}-dependent mini induction have been proposed: (1) resting Ca^{2+} concentrations; (2) stochastic Ca^{2+} channel opening; and (3) Ca^{2+} release from internal Ca^{2+} stores (Pang and Südhof, 2010). To a large extent, these mechanisms are related to VGCCs, because VGCC-mediated Ca^{2+} entry can increase intracellular Ca^{2+} concentrations, induce Ca^{2+} release from internal stores, or directly trigger vesicle release (Hubbard et al., 1968; Llano et al., 2000; Angleson and Betz, 2001). However, it has not been tested rigorously how exactly VGCCs are involved in triggering minis, and some earlier reports using different types of synapses remain under debate (Scanziani et al., 1992; Vyleta and Smith, 2011; Eggermann et al., 2012; Goswami et al., 2012; Vyleta and Jonas, 2014). Noticeably, there is no experimental evidence that has proven unequivocally that minis are directly and instantly triggered by VGCC-mediated Ca^{2+} entry.

It was shown previously that synchronous vesicle release is triggered by synaptotagmin (Syt)-1, Syt-2, and Syt-9 (defined as fast sensors; Geppert et al., 1994; Sun et al., 2007; Xu et al., 2007), whereas the Ca^{2+}-sensing mechanisms for asynchronous vesicle release are still under investigation. Syt-7 and Doc2 were proposed recently as the candidate Ca^{2+} sensors for asynchronous

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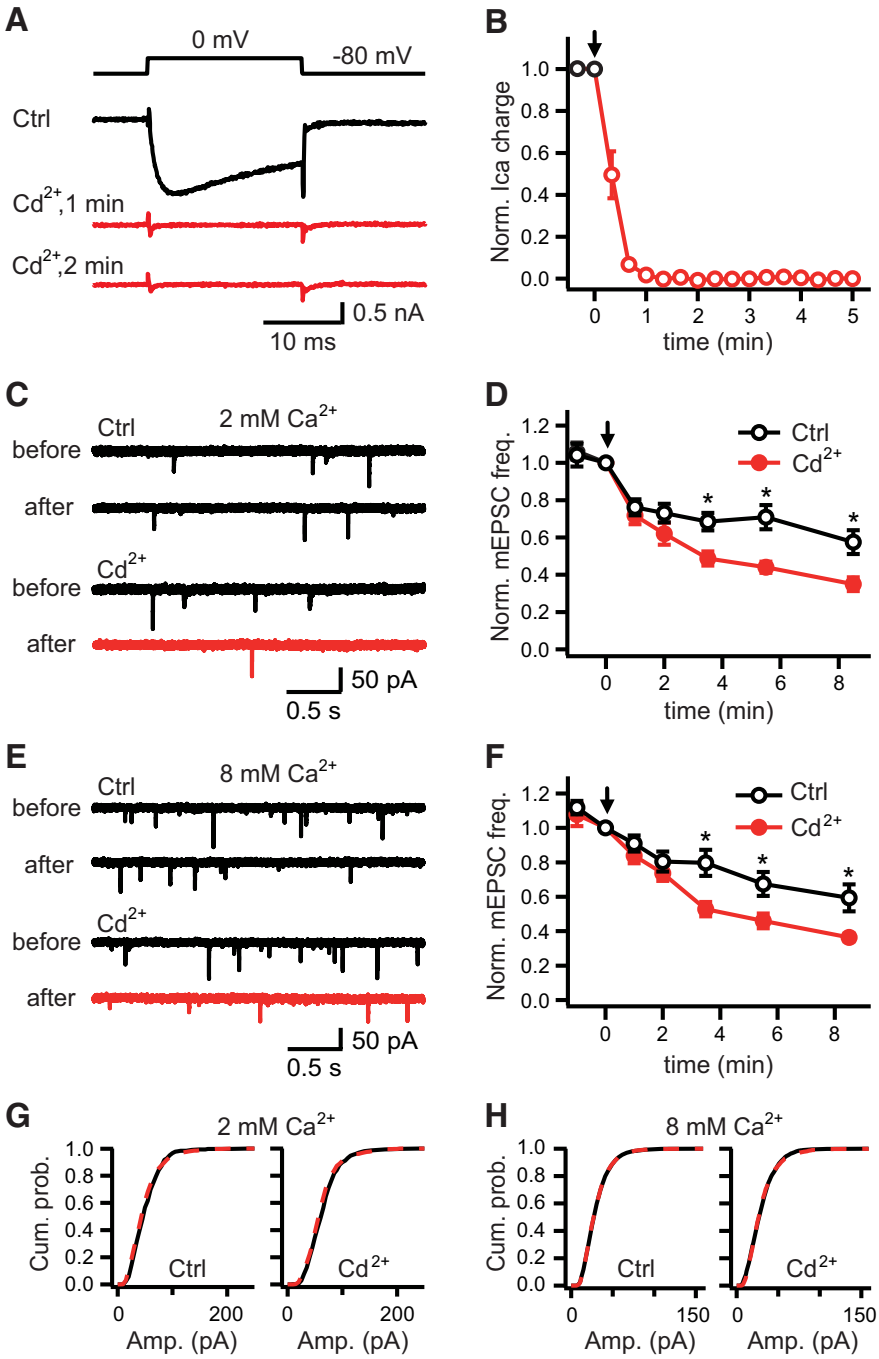

Figure 1. VGCC-mediated Ca^{2+} influx regulates spontaneous vesicle release. ***A***, Sampled presynaptic recording. Top, Depolarization at 20 ms from −80 to 0 mV; middle, Ca^{2+} current trace; bottom two panels, current traces (red) recorded after 1 and 2 min extracellular application of 0.2 mM Cd^{2+} contained bath solution. ***B***, Normalized electrical charge of Ca^{2+} influx versus time ($n = 5$). ***C***, Sampled mini traces before and after switching to normal (Ctrl, $n = 17$) and Cd^{2+} contained 2 mM $[Ca^{2+}]_e$ bath solution (Cd^{2+}, $n = 8$, red). ***D***, Average of normalized plot of mini frequency versus time at 2 mM $[Ca^{2+}]_e$. ***E***, Sampled mini traces before and after switching to normal (Ctrl, $n = 11$), and Cd^{2+} contained 8 mM $[Ca^{2+}]_e$ bath solution (Cd^{2+}, $n = 8$, red). ***F***, Average of normalized plot of mini frequency versus time at 8 mM $[Ca^{2+}]_e$. All the data in ***D*** and ***F*** were normalized to the recordings at the time of solution switching (as indicated by the arrowhead). ***G***, ***H***, Cumulative probability of mEPSC amplitude before (black solid) and after (red dash) switching to normal (left) or Cd^{2+} (right) contained bath solution, with $[Ca^{2+}]_e$ of 2 mM (***G***) and $[Ca^{2+}]_e$ of 8 mM (***H***), respectively. Error bars indicate ±SEM. *$p < 0.05$.

release (Sun et al., 2007; Yao et al., 2011; Bacaj et al., 2013). In contrast, the mechanisms behind Ca^{2+} sensing during spontaneous vesicle release is far from elucidated (Kavalali et al., 2011). A number of previous studies suggested that a mini is simply an extension of evoked synchronous release and mediated by a fast sensor (Xu et al., 2009). However, other studies argued that proteins of the Doc2 family mediate Ca^{2+}-triggered minis (Groffen et al., 2010), which was challenged by subsequent studies (Pang et al., 2011; Bacaj et al., 2013).

Here, we used cell-attached patch recording and presynaptic/postsynaptic whole-cell recording at the calyx of Held synapses to address whether minis are directly triggered by Ca^{2+} influx through VGCCs. We further explored the mechanisms of Ca^{2+}-regulating spontaneous vesicle release by analyzing the Ca^{2+} dependence of minis at wild-type (WT) and Syt-2 knock-out (KO) synapses.

Materials and Methods

Preparation and solutions. Brain slices (200 μm) containing the medial nucleus of the trapezoid body were prepared in a transverse orientation from P7–P9 mice (C57BL/6 of either sex) using standard procedures as described previously (Sun and Wu, 2001; Sun et al., 2007). Breeding and genetic analysis of the mice lacking Syt-2 (Syt-2 KO, $Z2B^{-/-}$) were described in detail previously (Pang et al., 2006). All experiments involved presynaptic/postsynaptic whole-cell recordings with an intracellular pipette solution containing the following (in mM): 125 K-gluconate, 20 KCl, 4 MgATP, 10 Na-phosphocreatine, 0.3 GTP, 0.05 BAPTA/0.5 EGTA, and 10 HEPES, pH 7.2 adjusted with KOH, if not mentioned. All recordings were made at room temperature using a bath solution containing the following (in mM): 120–125 NaCl, 25 $NaHCO_3$ or 22.5 HEPES, 3 Myo-inositol, 2 Na-pyruvate, 2.5 KCl, 1.25 NaH_2PO_4, 0.4 ascorbic acid, 25 glucose, 0.1–8 $CaCl_2$, and 1–3 $MgCl_2$, pH 7.4 (when bubbled with 95% O_2 and 5% CO_2) if not mentioned. Pharmacologically isolated Ca^{2+} currents used bath solution containing the following (in mM): 105 NaCl, 20 tetraethylammonium (TEA)-Cl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 25 glucose, 0.4 ascorbic acid, 3 Myo-inositol, 2 Na-pyruvate, and 0.001 tetrodotoxin (TTX), pH 7.4. The presynaptic pipette solution contained the following (in mM): 125 Cs-gluconate, 20 KCl, 4 MgATP, 10 Na-phosphocreatine, 0.3 GTP, 0.05 BAPTA, and 10 HEPES, pH 7.2 adjusted with CsOH. Presynaptic cell-attached recording was made with the pipette solution containing the following (in mM): 122 NaCl, 3 Myo-inositol, 2 Na-pyruvate, 2.5 KCl, 0.4 ascorbic acid, 25 D-glucose, 20 HEPES, 2 $CaCl_2$, 0.001 TTX, 20 TEA-Cl, 0.01 CNQX, and 0.05 D-AP-5, pH 7.4. Changing Ca^{2+} concentrations was always balanced by adjusting the concentration of NaCl to keep the osmolarity.

Ca^{2+} current and mini recordings. Presynaptic and/or postsynaptic whole-cell recordings were obtained with a Multiclamp 700B amplifier (Molecular Devices). Presynaptic and postsynaptic currents were low-pass filtered at 10 and 4 kHz, respectively. At 8 mM $[Ca^{2+}]_e$, presynaptic and postsynaptic whole-cell recordings were obtained with an Axopatch 200B amplifier (Molecular Devices) and

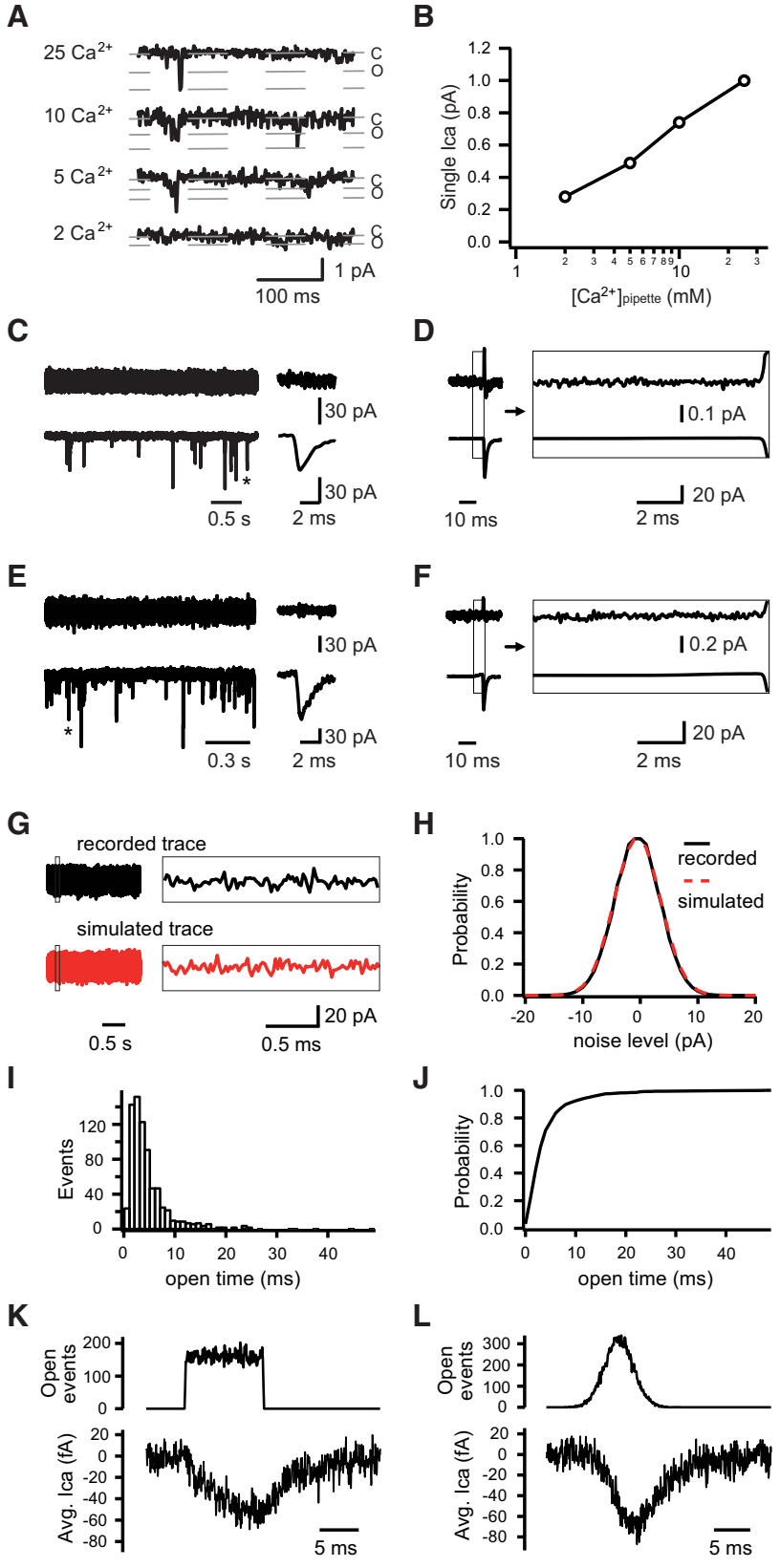

Figure 2. Ca^{2+} current and its temporal correlation with spontaneous minis. ***A***, Single calcium channel currents obtained by cell-attached patch recording at the release face, with the different $[Ca^{2+}]_{pipette}$ (holding potential of 0 mV). ***B***, Statistics of single Ca^{2+} channel current amplitude versus $[Ca^{2+}]_{pipette}$. ***C***, Left, Sampled presynaptic (at resting potential, −66 mV) and postsynaptic current recording (holding potential of −80 mV) at $[Ca^{2+}]_e$ of 2 mM. Right, The scaled single mini contained paired currents at the asterisk in the left. ***D***, Left, The averaged presynaptic current aligned at the onset (differential peak) of the minis (51,730 events); the inset shows the paired currents before the onset of the mini event. The presynaptic current was low-pass filtered at 5 kHz. ***E***, Same plot as in ***C*** but with $[Ca^{2+}]_e$ of 8 mM. ***F***, Same plot as in ***D*** but with $[Ca^{2+}]_e$ of 8 mM and 25,261 events averaging. ***G***, Top, Sample of a recorded presynaptic current trace. Bottom, Sample of a simulated trace with pseudo white noise; the current traces in the boxes of the left panels were displayed with greater details in the right panels. ***H***, The distribution of noise level of the recorded presynaptic currents (black solid) and the simulated pseudo white noise traces (red dash). ***I***, Histogram of opening lifetime of 751 single VGCC open events by cell-attached recording. ***J***, The cumulative probability distribution of single VGCCs opening lifetime. ***K***, ***L***, Top, Simulated uniform distribution (***K***) or Gaussian distribution (***L***) of single-channel opening time points (20,000 events for each distribution). Bottom, The simulated traces as average of 50,000 pseudo white noise traces including embedded 20,000 single-channel open events with corresponding distributions. Error bars indicate ±SEM.

an EPC-10 amplifier (HEKA), respectively. Presynaptic and postsynaptic currents were low-pass filtered at 10 and 5 kHz, respectively. All recordings were digitized at 50 kHz, if not mentioned. The pipette resistances were 4–6 MΩ for presynapses and 3–5 MΩ for postsynapses. Ca^{2+} current and single Ca^{2+} channel current recordings were obtained with an EPC-10 amplifier and were low-pass filtered online at 5 kHz. Single Ca^{2+} channel currents were digitized at 20 kHz and were filtered offline at 300 Hz to display.

Capacitance measurement. Cell-attached capacitance measurements were performed with a lock-in amplifier SR850 (Stanford Research Systems) that was coupled with a modified EPC-8 patch-clamp amplifier (HEKA), the gain was set to 20/50 mV/pA, the filter was set to 30 kHz, and capacitance (C-slow) and conductance (G-series) were set to 0.2 pF and 0.2 μS, respectively, using the pipette solution with 10 mM Ca^{2+}. A sine wave with root mean square (rms) amplitude of 150 mV at 20 kHz was superimposed on a command potential of 0 mV. The real and imaginary outputs of the lock-in amplifier were filtered with time constant 1 ms, 24 dB. Pipettes were fire polished with a microforge and were coated with Sylgard or wax to reduce the capacitance noise.

Data analysis. An IgorPro program (WaveMetrics) was used to offline detect minis (amplitudes > 5 pA) and capacitance jumps. Mini frequency was analyzed by each minute recording. Statistical significance was determined using Student's *t* test. *p* values < 0.05 were considered to be significant. Data values were reported as mean ± SEM.

Simulation. The process that a single Ca^{2+} channel opening drives vesicle fusion was simulated by a self-developed Igor program. The time points of single Ca^{2+} channel opening were set randomly by uniform or Gaussian distribution within a 10 ms time window and preceded the mini event. The size of unitary single-channel current and opening lifetime probability are the same as those obtained from cell-attached recording. The simulated single-channel current was embedded into the pseudo white noise traces with an SD similar to that of

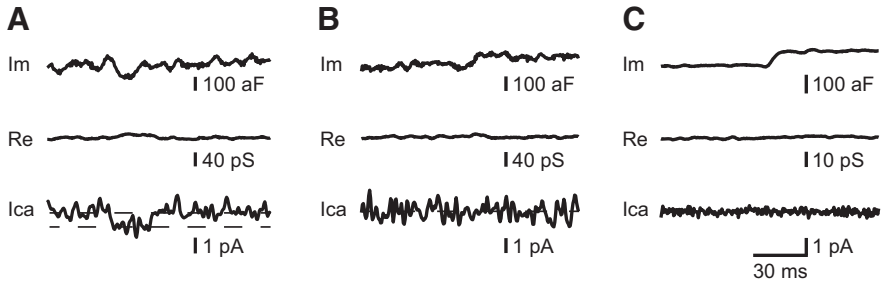

Figure 3. Ca^{2+} current and its temporal correlation with capacitance up-step in cell-attached recording at presynaptic release site. ***A***, Sampled presynaptic imaginary (Im) and real (Re) part of lock-in signals, as well as I_{Ca} trace by cell-attached patch recording at the release face. The inward unitary current indicates single VGCC activity. ***B***, Sampled presynaptic imaginary (Im) and real (Re) part of lock-in signals, as well as I_{Ca} trace by cell-attached patch recording at the release face. The up-step of Im corresponds to a single vesicle fusion event-related membrane capacitance change. ***C***, The averaged Im, Re, and I_{Ca} traces aligned by the onset of capacitance from 52 spontaneous events. The timescale calibration is applicable to all the plots.

the recorded presynaptic currents. A total of 50,000 of these simulated traces were generated, and 40% of these traces were defined as containing single-channel events.

Results

To determine whether Ca^{2+} influx through VGCCs is required to trigger minis, we tested the effects of a nonselective inorganic VGCC blocker $CdCl_2$ (0.2 mM) on the minis. Direct recording of Ca^{2+} influx at the presynaptic calyx terminal revealed a substantially inhibited Ca^{2+} current by Cd^{2+} (Fig. 1*A*,*B*). We then evaluated the effect of Cd^{2+} on minis (recorded as mEPSCs). We normalized mini frequencies to those of minis released during 1 min before perfusion of Cd^{2+} and found an ~40% reduction in the mini frequency after the 8.5 min incubation (Fig. 1*D*). Notably, the Ca^{2+} influx was diminished within 1 min of Cd^{2+} application, whereas the significant suppression of minis could be detected at least 2 min later (Fig. 1*B*,*D*). The elevated extracellular Ca^{2+} to 8 mM significantly facilitated mini frequency (Fig. 1*E*). However, the similar reduction (~40%) in the mini frequency after the 8.5 min incubation of $CdCl_2$ was found as $[Ca^{2+}]_e$ of 2 mM (Fig. 1*F*). In addition, we analyzed the mEPSC amplitudes before and after addition of Cd^{2+} at $[Ca^{2+}]_e$ of either 2 or 8 mM and found that Cd^{2+} application does not change the distribution of mEPSC size (Fig. 1*G*,*H*). Therefore, it is rather unlikely that the Cd^{2+}-caused reduction of mini frequency is attributable to the rundown in mEPSC amplitude. Thus, our results demonstrate a significant and $[Ca^{2+}]_e$-independent time lapse between the VGCCs blockage and the resulting reduction in mini frequency.

To study the activity of single VGCCs, we used cell-attached patch recording at resting status (pipette holding potential, 0 mV) in combination with different $[Ca^{2+}]_{pipette}$. The amplitude of single Ca^{2+} channel current was measured as 0.28 ± 0.01 pA at 2 mM $[Ca^{2+}]_{pipette}$, and there was a positive correlation between this unitary current size and $[Ca^{2+}]_{pipette}$ (Fig. 2*A*,*B*). Because P/Q-, N-, or R-type channels were reported to have similar conductance at the calyceal terminal (Sheng et al., 2012), we did not categorize them further. If a mini is directly caused by VGCC-mediated Ca^{2+} entry, then the Ca^{2+} current signal should occur before the mini event. To test this hypothesis, we performed paired whole-cell patch-clamp recording on the calyceal terminal (at resting potential) and the postsynaptic principle cell at 2 mM $[Ca^{2+}]_e$. In these experiments, the peak-to-peak noise in the presynaptic current recording was ~20 pA, which was 100 times larger than single Ca^{2+} channel current amplitude (Fig. 2*C*, top). To improve the signal-to-noise ratio, we averaged the presynaptic current traces corresponding to 51,730 mini events from 46 synapses and obtained the peak-to-peak noise at ~0.07 pA (Fig. 2*D*, top; rms, ~0.05 pA). Surprisingly, with the averaging, a lack of the inward current signal was observed, indicating that there was no significant temporal correlation between VGCC-mediated Ca^{2+} influx and spontaneous vesicle release. This phenomenon was confirmed by the fact that we failed to detect the temporal correlation, even when $[Ca^{2+}]_e$ was increased to 8 mM and the amplitude of single Ca^{2+} channel current was as high as ~0.6 pA (Fig. 2*F*). To validate that our method can indeed discriminate Ca^{2+} channel signals from the background noise in a reproducible manner, we performed a simulation in which a vesicle fusion is driven by single-channel opening within a 10 ms time window preceding the mini event. The simulated single-channel current was embedded into the pseudo white noise traces on the basis that these traces had a deviation similar to that of the recorded presynaptic currents (Fig. 2*G*,*H*). We set the size of single-channel current as 0.28 pA, and the channel opening lifetimes had the same probability as those obtained from cell-attached recording (Fig. 2*I*,*J*), whereby the time points of channel opening were set randomly by uniform or Gaussian distribution (Fig. 2*K*,*L*, top panels). We generated 50,000 simulated traces and defined 40% of them as containing single-channel events. After the averaging of these traces, one single-channel signal could be observed clearly (Fig. 2*K*,*L*, bottom panels).

To further rule out any mutual interference of electrical signaling between the presynaptic and postsynaptic measurement, we simultaneously assayed the presynaptic current and vesicle fusion (upward capacitance step) using cell-attached patch recording at 10 mM $[Ca^{2+}]_{pipette}$. Under this condition, the amplitude of single-channel Ca^{2+} current should become detectable (Figs. 2*B*, 3*A*; He et al., 2006). Thus, we think that our system is competent for detecting a single-channel current signal. Consistently, no presynaptic signal was detected that correlated to the capacitance jump (Fig. 3*B*). Fifty-two capacitance jump events were aligned, and the presynaptic current traces were averaged, resulting in a reduction of the peak-to-peak noise to ~0.58 pA (rms, ~0.41 pA; Fig. 3*C*). Even after this procedure, no presynaptic signal was detected. In summary, our data indicated that spontaneous vesicle release is not coupled, in any direct manner, to the Ca^{2+} influx through VGCCs.

Because spontaneous releasable vesicles do not tightly couple to VGCCs, it is difficult for Syt-2 to function as a Ca^{2+} sensor in minis attributable to its low Ca^{2+} affinity (Fedchyshyn and Wang, 2005; Kaeser and Regehr, 2014). To test whether Syt-2 is a Ca^{2+} sensor for spontaneous minis, we compared the Ca^{2+} dependence of minis between WT and Syt-2 KO synapses. The mini frequency was measured at $[Ca^{2+}]_e$ of 0.1, 1, 2, and 5 mM (Fig. 4*A*). Surprisingly, our results showed clearly that there is no difference in the $[Ca^{2+}]_e$ dependence of mini frequency between WT and KO (apparent cooperativity of 0.43 in WT and 0.47 in KO; Fig. 4*B*). Furthermore, the apparent Ca^{2+} cooperativity for minis was significantly lower than that observed for evoked vesicle release (Borst and Sakmann, 1996). These results suggest that, within the physiological $[Ca^{2+}]_e$ range, minis are essentially triggered by a sensor other than Syt-2.

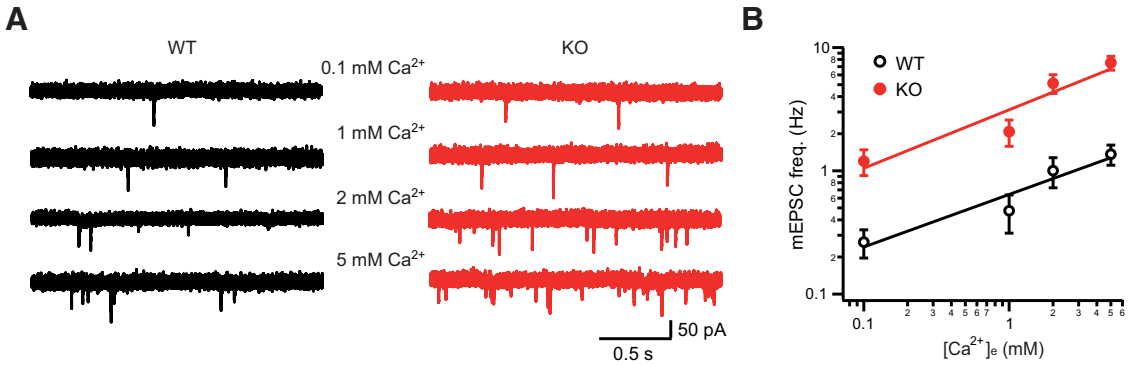

Figure 4. $[Ca^{2+}]_e$ dependence of mini frequency in WT and Syt-2 KO synapses. ***A***, Sampled mini traces recorded at different $[Ca^{2+}]_e$ in WT and KO synapses. ***B***, $[Ca^{2+}]_e$ dependence of mini frequency obtained from WT (black) and KO (red) synapses. Data were scaled in logarithm coordinates and linearly regressed with a cooperativity of 0.43 for WT (black) and 0.47 for KO (red). Error bars indicate ±SEM.

Discussion

The present work demonstrated that at least 40% of minis can be attributed to VGCCs at the calyx of Held synapse, because application of 0.2 mM $CdCl_2$ decreased the mini frequency by ~40% (Fig. 1). The remaining 60% of minis were possibly caused by Ca^{2+}-independent mechanisms (Yamasaki et al., 2006), resting intracellular Ca^{2+} concentration (Lou et al., 2005; Sun et al., 2007), Ca^{2+} release from internal Ca^{2+} stores (Friel and Chiel, 2008), or Ca^{2+}-sensing G-proteins (Vyleta and Smith, 2011). Our results for VGCC-dependent minis are consistent with most of the previous reports showing that blocking of VGCCs reduces mini frequency by ~50% (Goswami et al., 2012; Williams et al., 2012). However, a number of previous reports demonstrated that the addition of Cd^{2+} failed to alter spontaneous minis, as shown for CA3 pyramidal neurons (Scanziani et al., 1992) and excitatory cultured cortical synapses (Vyleta and Smith, 2011). We attribute the inconsistency in those previous reports to the specificity of synapses studied, because the latter synapses either display looser VGCC–vesicle association and/or have stronger intracellular Ca^{2+} buffers than most other synapses (Eggermann et al., 2012; Vyleta and Jonas, 2014). Alternatively, extracellular activation of presynaptic G-protein-coupled receptors in some synapses regulates spontaneous vesicle fusion more dominantly (Vyleta and Smith, 2011).

Here, we established three pieces of evidence supporting the notion that minis are not immediately triggered by Ca^{2+} entry. First, there is a time lapse between VGCC blockage and the resulting reduction in mini frequency after the addition of $CdCl_2$ (Fig. 1*D*,*F*). Second, no presynaptic current was detected corresponding to mini in averaging >25,000 events (Fig. 2*D*,*F*). Third, no temporal coupling between Ca^{2+} influx current with respect to mini-related capacitance jumps was observed at $[Ca^{2+}]_{pipette}$ above 8 mM (Fig. 3*B*,*C*). The lack in temporal correlation between Ca^{2+} influx and spontaneous minis suggests strongly that Ca^{2+}-dependent minis are determined by resting $[Ca^{2+}]_i$ (Fredj and Burrone, 2009), and the Ca^{2+}-sensing mechanism is different from that for synchronous release (Kavalali et al., 2011; Ramirez and Kavalali, 2011). Our argument is supported by previous studies that reported for juvenile mice calyx type of synapses that VGCCs and vesicles are coupled in microdomains rather than nanodomains (Fedchyshyn and Wang, 2005; Eggermann et al., 2012) .

As a Ca^{2+} sensor for synchronous release, Syt-2 has a low Ca^{2+} affinity with cooperativity of 4–5 (Borst and Sakmann, 1996; Schneggenburger and Neher, 2000; Sun et al., 2007; Kaeser and Regehr, 2014). However, the observed apparent Ca^{2+} cooperativity of minis is lower than that for synchronous release (Fig. 4*B*; Borst and Sakmann, 1996). It was argued that Syt-2 might have an allosteric modification that reduces the cooperativity at low Ca^{2+} concentrations (Lou et al., 2005). However, the apparent Ca^{2+} dependence of minis at Syt-2 KO synapses displayed a similar apparent Ca^{2+} cooperativity, indicating that the absence of Syt-2 does not affect the Ca^{2+} cooperativity. Thus, we concluded that spontaneous vesicle release is not mediated by a fast Ca^{2+} sensor at the juvenile mice calyx of Held synapses, which is mechanistically distinct from evoked release (Kavalali et al., 2011; Ramirez and Kavalali, 2011). It was suggested previously that spontaneous vesicle release is triggered by the Ca^{2+} sensors for asynchronous release (Sun et al., 2007). Doc2 protein was proposed to be such a Ca^{2+} sensor, because Doc2 deficiency significantly reduced both spontaneous and asynchronous release without altering synchronous release (Groffen et al., 2010; Yao et al., 2011). However, this notion was challenged by the result that Doc2 modulates spontaneous vesicle release by a Ca^{2+}-independent mechanism (Pang et al., 2011). It will be of great interest to determine the Ca^{2+} sensor(s) that has lower Ca^{2+} cooperativity and is essential for spontaneous vesicle release and to reveal the mechanisms that mediate spontaneous vesicle release.

Previously, it was reported that the coincidental opening of either multiple VGCCs or uncorrelated stochastic VGCC opening can trigger spontaneous vesicle release and that the fast sensors (Syt-1, Syt-2, or Syt-9) can function as Ca^{2+} sensors for spontaneous release (Xu et al., 2009). These tightly VGCC-coupled vesicles should be, in principle, releasable under action potential and belong to the readily releasable vesicle pool. It was suggested that these evocable spontaneous minis functionally modulate subthreshold cellular membrane potential in these synapses (Ermolyuk et al., 2013). However, these results could only be obtained so far in the synapses with nanodomain VGCC–vesicle coupling, such as GABAergic synapse onto cultured neocortical neurons (Williams et al., 2012) or glutamatergic synapse onto small hippocampal synapses (Ermolyuk et al., 2013). Our data from the juvenile mice calyx of Held synapse provide the first set of experimental evidence that spontaneous vesicle release can be triggered indirectly by the Ca^{2+} entry through VGCCs and be mediated via a different Ca^{2+}-sensing mechanism than evoked release. This view is likely applicable to most synapses with a microdomain coupling between VGCCs and vesicles (Ohana and Sakmann, 1998; Meinrenken et al., 2002; Eggermann et al., 2012). Possibly, this view can be partly applied to the synapses characterized by tight VGCC-vesicle coupling. For instance, in GABAergic synapses of cultured cortical neurons, 50% of vesicle release is EGTA sensitive

(Maximov and Südhof, 2005). In this regard, they can, in part, be described as loosely coupled microdomain synapses.

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