



# L-type $\text{Ca}^{2+}$ channels mediate regulation of glutamate release by subthreshold potential changes

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Subthreshold depolarization enhances neurotransmitter release evoked by action potentials and plays a key role in modulating synaptic transmission by combining analog and digital signals. This process is known to be  $\text{Ca}^{2+}$  dependent. However, the underlying mechanism of how small changes in basal  $\text{Ca}^{2+}$  caused by subthreshold depolarization can regulate transmitter release triggered by a large increase in local  $\text{Ca}^{2+}$  is not well understood. This study aimed to investigate the source and signaling mechanisms of  $\text{Ca}^{2+}$  that couple subthreshold depolarization with the enhancement of glutamate release in hippocampal cultures and CA3 pyramidal neurons. Subthreshold depolarization increased presynaptic  $\text{Ca}^{2+}$  levels, the frequency of spontaneous release, and the amplitude of evoked release, all of which were abolished by blocking L-type  $\text{Ca}^{2+}$  channels. A high concentration of intracellular  $\text{Ca}^{2+}$  buffer or blockade of calmodulin abolished depolarization-induced increases in transmitter release. Estimation of the readily releasable pool size using hypertonic sucrose showed depolarization-induced increases in readily releasable pool size, and this increase was abolished by the blockade of calmodulin. Our results provide mechanistic insights into the modulation of transmitter release by subthreshold potential change and highlight the role of L-type  $\text{Ca}^{2+}$  channels in coupling subthreshold depolarization to the activation of  $\text{Ca}^{2+}$ -dependent signaling molecules that regulate transmitter release.

$\text{Ca}^{2+}$  channels | exocytosis | calmodulin | readily releasable pool

Synaptic transmission, a core process of information flow mediated by neurotransmitter release from presynaptic terminals, is triggered by action potentials (APs). Although APs are generally considered all-or-none signals, they are modulated by subthreshold potential changes (1), which in turn affect synaptic strength (2, 3). In addition, subthreshold changes in somatic potentials can electrotonically spread to axon terminals and modulate spontaneous or asynchronous release (3). The involvement of subthreshold depolarization in the emergence of place field spiking (4) and the propensity to have place fields (5) has also been highlighted in recent studies. Considering that the resting membrane potential (RMP) is not fixed but fluctuates in the subthreshold range by the alteration of extracellular  $\text{K}^+$  concentrations, ion channel activities, and synaptic activities, the RMP is a key player in the analog–digital modulation of synaptic transmission and neural activities (6). However, the mechanisms underlying the modulation of synaptic transmission and neural activity by RMP changes are not well understood.

Neurotransmitter release at synapse is orchestrated by numerous molecular mechanisms that govern multiple steps of synaptic vesicle dynamics, including vesicle priming, fusion, and recycling (7). It is well known that the low-affinity  $\text{Ca}^{2+}$  sensor synaptotagmins transduce a large increase in presynaptic  $\text{Ca}^{2+}$  into the fusion pore opening of synaptic vesicles for transmitter release (8). Therefore, an AP-induced large increase in  $\text{Ca}^{2+}$  that persists for a short period is the initial step that triggers transmitter release. Interestingly, the slow dynamics of  $\text{Ca}^{2+}$  changes in a lower concentration range can regulate  $\text{Ca}^{2+}$ -triggered transmitter release by activating numerous  $\text{Ca}^{2+}$ -dependent signaling molecules. Calmodulin (CaM) plays a key role in vesicle priming and replenishment of the vesicle pool, thereby regulating short-term plasticity (9, 10). Diacylglycerol (DAG), a product of phospholipase C (PLC), increases transmitter release possibly by increasing fusion willingness (11, 12). Munc13 proteins (mammalian homologs of *Caenorhabditis elegans* UNC13), which are essential regulators of synaptic vesicle priming (13, 14), have binding sites for both CaM and DAG (9, 15, 16), suggesting a possibility that their function is regulated by these signaling molecules. The functional importance of  $\text{Ca}^{2+}$ -dependent signals in synaptic transmission has been mostly investigated to understand the short-term plasticity mechanism induced by high-frequency activity (17). It is of interest to determine whether these signaling cascades also contribute to basal synaptic transmission regulated by subthreshold potential changes.

## Significance

Neuronal activities are encoded by action potentials, but subthreshold changes in resting membrane potentials also play important roles in regulating neuronal functions including synaptic transmission. It is, however, poorly understood how small changes in basal  $\text{Ca}^{2+}$  induced by subthreshold depolarization regulate transmitter release triggered by a large increase in local  $\text{Ca}^{2+}$  in presynaptic terminals. We demonstrate that L-type  $\text{Ca}^{2+}$  channels are the major source of presynaptic  $\text{Ca}^{2+}$  influx at the basal state and during subthreshold depolarization, resulting in the activation of signaling molecules such as calmodulin, which facilitate transmitter release by increasing both release probability and the readily releasable pool size. Our results provide mechanistic insight into how subthreshold potential changes contribute to regulating transmitter release.

The authors declare no competing interest.

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The role of presynaptic  $\text{Ca}^{2+}$  currents, including P/Q-, N-, and R-type  $\text{Ca}^{2+}$  currents mediated by  $\text{Ca}_v2.1$ ,  $\text{Ca}_v2.2$ , and  $\text{Ca}_v2.3$  channels, respectively, in  $\text{Ca}^{2+}$ -triggered transmitter release, is well established (18–20). However, it has been reported that L-type  $\text{Ca}^{2+}$  currents (LTCCs) do not participate in neurotransmitter release in most neurons (21, 22), except inner hair cells (23, 24) and bipolar cells in the retina (25, 26). However, increased GABA release by increasing L-type  $\text{Ca}^{2+}$  currents using Bay K 8644 was recently reported in cerebellar molecular layer interneurons (27), suggesting the contribution of LTCCs to synaptic transmission. Considering that substitution of the synaptic protein interaction site from  $\text{Ca}_v2.1$  channels in  $\text{Ca}_v1.2$  was sufficient to establish synaptic transmission initiated by LTCCs (28), the localization of  $\text{Ca}^{2+}$  channels at synaptic sites is important for their role in synaptic transmission. A recent study showed that chronic treatment with lipopolysaccharide increased  $\text{Ca}_v1.2$  channels at excitatory presynaptic terminals and their contribution to the increase in glutamate release (29), suggesting the possibility that  $\text{Ca}_v1.2$  localization and its contribution to synaptic transmission can be regulated. It would be intriguing to investigate whether LTCC-mediated  $\text{Ca}^{2+}$  influx plays a role in regulating neurotransmitter release under physiological conditions, such as subthreshold depolarization.

In the present study, we found that LTCCs played a key role in the enhancement of transmitter release by subthreshold depolarization. Furthermore, we demonstrated that the depolarization-induced increase in transmitter release is mediated by increases in readily releasable pool (RRP) size and release probability, which are attributable to CaM activation by LTCC-dependent elevation of presynaptic  $\text{Ca}^{2+}$  levels. Our results provide mechanistic insight into how RMP changes in the subthreshold range contribute to regulating transmitter release.

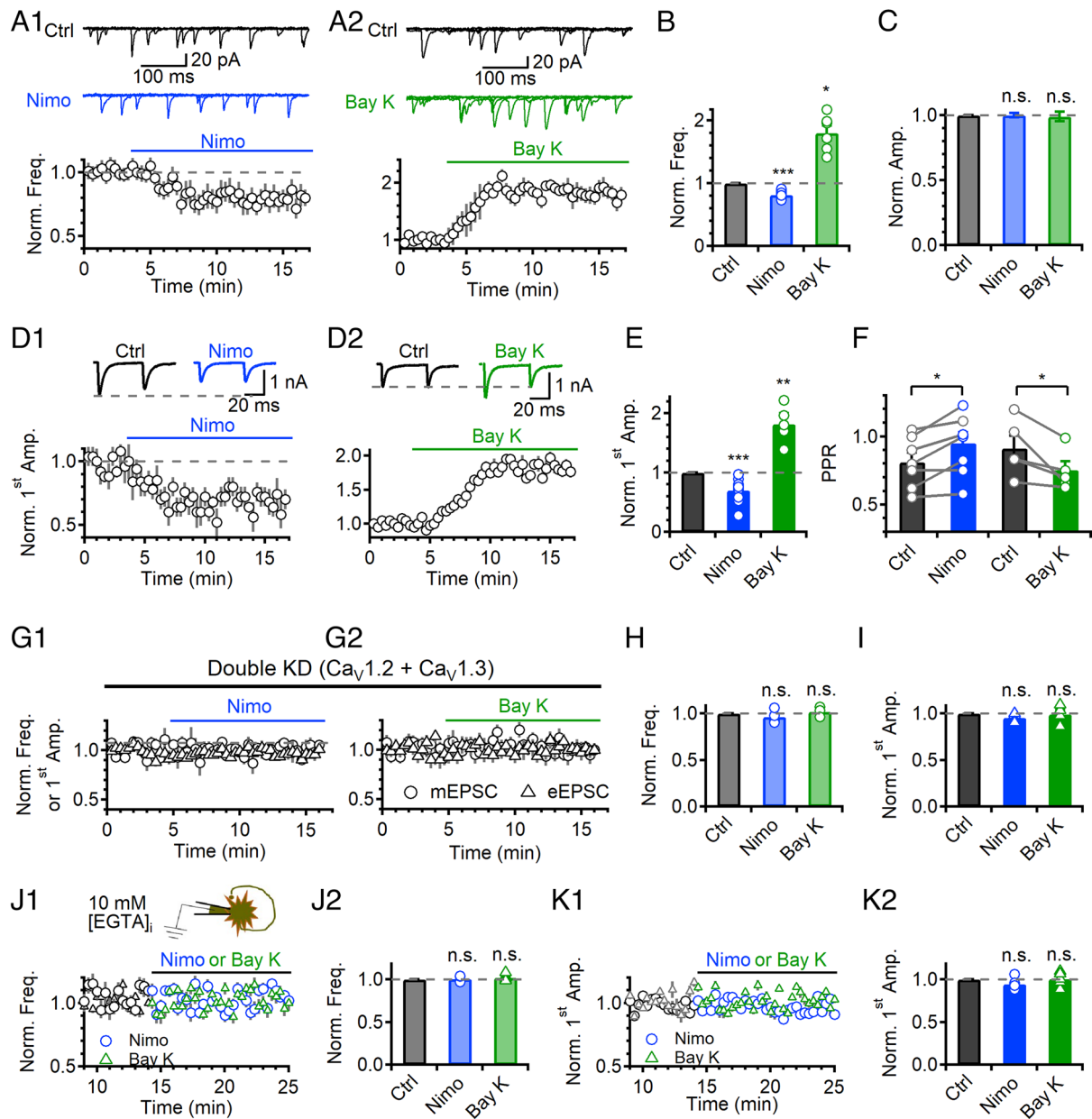
## Results

**LTCCs Regulate Both Spontaneous and Evoked Glutamate Release.** To test the contribution of L-type calcium channels (LTCCs) at presynaptic terminals to neurotransmitter release, we used autaptic cultured hippocampal neurons that enable manipulation of the presynaptic compartment environment. In the control condition, whole-cell voltage-clamp recordings were performed in aCSF containing 2 mM  $\text{Ca}^{2+}$  and 2.5 mM  $\text{K}^+$  with a pipette solution containing 0.1 mM ethylene glycol bis ( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). Excitatory neurons were distinguished from inhibitory neurons by fast decay kinetics of synaptic currents (ranged from 3.4 to 13.8 ms with the mean value of  $6.9 \pm 0.2$  ms, *SI Appendix, Fig. S1*,  $N = 94$ ) as previously described (30). Miniature excitatory postsynaptic currents (mEPSCs) were recorded at the holding potential (HP) of  $-70$  mV, while evoked excitatory postsynaptic currents (eEPSCs) were recorded by applying 2-ms depolarizing step pulses to 0 mV from the HP every 20 s. The mEPSC frequency and eEPSC amplitude measured under the experimental conditions were normalized to the control level. To assess the role of LTCCs in glutamate release, we examined the effects of drugs that inhibit or activate LTCCs on the mEPSC frequency or eEPSC amplitude. Nimodipine (Nimo, 10  $\mu\text{M}$ ), a blocker of LTCCs, significantly decreased mEPSC frequency (Fig. 1 *A1* and *B*, blue,  $0.81 \pm 0.01$ ,  $N = 17$ , normalized to the control value) without affecting mEPSC amplitudes (Fig. 1 *C*), whereas LTCC activator (10  $\mu\text{M}$  Bay K 8644, Bay K) induced a significant increase in mEPSC frequency (Fig. 1 *A2* and *B*, green,  $1.79 \pm 0.11$ ,  $N = 7$ , normalized to control). Nimo and Bay K also altered the eEPSC amplitude in the same direction as the mEPSC frequency (Fig. 1

*D* and *E*). Nimo and Bay K decreased and increased the eEPSC amplitude, respectively. In addition, the paired-pulse ratio (PPR) was increased by Nimo and decreased by Bay K application (Fig. 1*F*), suggesting that presynaptic mechanisms are involved in the LTCCs' effects on eEPSCs. To further confirm that the effects of Nimo and Bay K were specifically mediated by LTCCs, we prepared  $\text{Ca}_v1.2$  and  $\text{Ca}_v1.3$  double knockdown autapses (*SI Appendix, Materials and Methods*), since LTCCs mainly comprise  $\text{Ca}_v1.2$  and  $\text{Ca}_v1.3$  subunits in the hippocampus (31), and tested the effects of Nimo and Bay K. Neither the eEPSC amplitude nor the mEPSC frequency was affected by Nimo or Bay K (Fig. 1 *G–I* and *SI Appendix, Fig. S2*). These results showed that LTCCs contribute to evoked and spontaneous glutamate release at the physiological RMP.

In addition, we interestingly found that the effects of Nimo and Bay K on the mEPSC frequency (Fig. 1*J* and *SI Appendix, Fig. S3B*) and eEPSC amplitude (Fig. 1*K* and *SI Appendix, Fig. S3C*) were completely abolished by 10 mM EGTA in the pipette solution, which inhibited global  $\text{Ca}^{2+}$  changes without affecting local  $\text{Ca}^{2+}$  increases near  $\text{Ca}^{2+}$  channels upon  $\text{Ca}^{2+}$  channel opening (32). The effect of 10 mM EGTA on the LTCC contribution differed from its effect on P/Q-, N-, and R-type contributions. Intracellular 10 mM EGTA did not affect the effects of P/Q-, N-, or R-type blockers (0.1  $\mu\text{M}$   $\omega$ -agatoxin-IVA, 0.1  $\mu\text{M}$   $\omega$ -conotoxin GVIA, and 100  $\mu\text{M}$   $\text{NiCl}_2$ , respectively) on mEPSCs (*SI Appendix, Fig. S4*), which is consistent with the notion that the contribution of P/Q-, N-, and R-type  $\text{Ca}^{2+}$  channels is mediated by local  $\text{Ca}^{2+}$  increases (30). There was no additive effect of Bay K on the mEPSC frequency and eEPSC amplitude when all P/Q-, N-, and R-type VGCCs were blocked by the mixture of 0.1  $\mu\text{M}$  Aga, 0.1  $\mu\text{M}$  Cono, and 100  $\mu\text{M}$   $\text{NiCl}_2$  (3-mix, *SI Appendix, Fig. S5*), implying that  $\text{Ca}^{2+}$  influx via LTCCs does not directly trigger exocytosis by increasing local  $\text{Ca}^{2+}$  near primed vesicles with nanodomain or microdomain coupling but indirectly augments vesicle release triggered by P/Q-, N-, and R-type VGCCs.

**$V_m$ -Dependent Regulation of Spontaneous Release Is Mediated by LTCC-Dependent Changes in Basal  $\text{Ca}^{2+}$ .** Several studies have reported the enhancement of neurotransmitter release by subthreshold depolarization (2, 3, 33), but the underlying mechanism remains unclear. We investigated whether membrane potential ( $V_m$ ) changes in the subthreshold range affect the glutamate release and, if so, whether LTCCs are involved in this mechanism. Autaptic cultured neurons allowed us to assess the relationship between  $V_m$  and spontaneous release by manipulating  $V_m$  in two ways: shifting the HP of patched neurons and changing the external  $\text{K}^+$  concentration ( $[\text{K}^+]_e$ ). Lowering HP from  $-70$  mV to  $-80$  mV reduced mEPSC frequency (Fig. 2 *A1* and *B*,  $0.82 \pm 0.02$ ,  $N = 12$ ), while elevating HP from  $-70$  mV to  $-60$  mV increased mEPSC frequency (Fig. 2 *A1* and *B*,  $1.49 \pm 0.06$ ,  $N = 11$ ), indicating that  $V_m$  around the RMP dynamically impacts spontaneous glutamate release. Reduction of the mEPSC amplitude by depolarization was detected (*SI Appendix, Fig. S6*;  $-80$  mV,  $17.66 \pm 0.68$ ;  $-70$  mV,  $15.98 \pm 0.54$ ; and  $-60$  mV,  $14.2 \pm 0.56$  pA), which possibly reflects decreased driving force for nonselective cation currents.  $V_m$ -dependent changes in mEPSC frequency were similarly observed when  $V_m$  was altered by changing the RMP with different  $[\text{K}^+]_e$  (Fig. 2 *A2* and *B*). At 2.5 mM  $[\text{K}^+]_e$ ,  $V_m$  was  $-73.4 \pm 1.37$  mV (orange,  $N = 7$ ), and mEPSC frequency normalized to the data at  $-70$  mV was  $0.93 \pm 0.04$  ( $N = 7$ ). Normalized mEPSC frequency at 1 mM  $[\text{K}^+]_e$  ( $V_m = -85.33 \pm 1.9$  mV, light orange) was reduced to  $0.72 \pm 0.03$  ( $N = 6$ ), while the frequency at 5 mM  $[\text{K}^+]_e$  ( $V_m = -54.67 \pm 2.72$  mV, dark

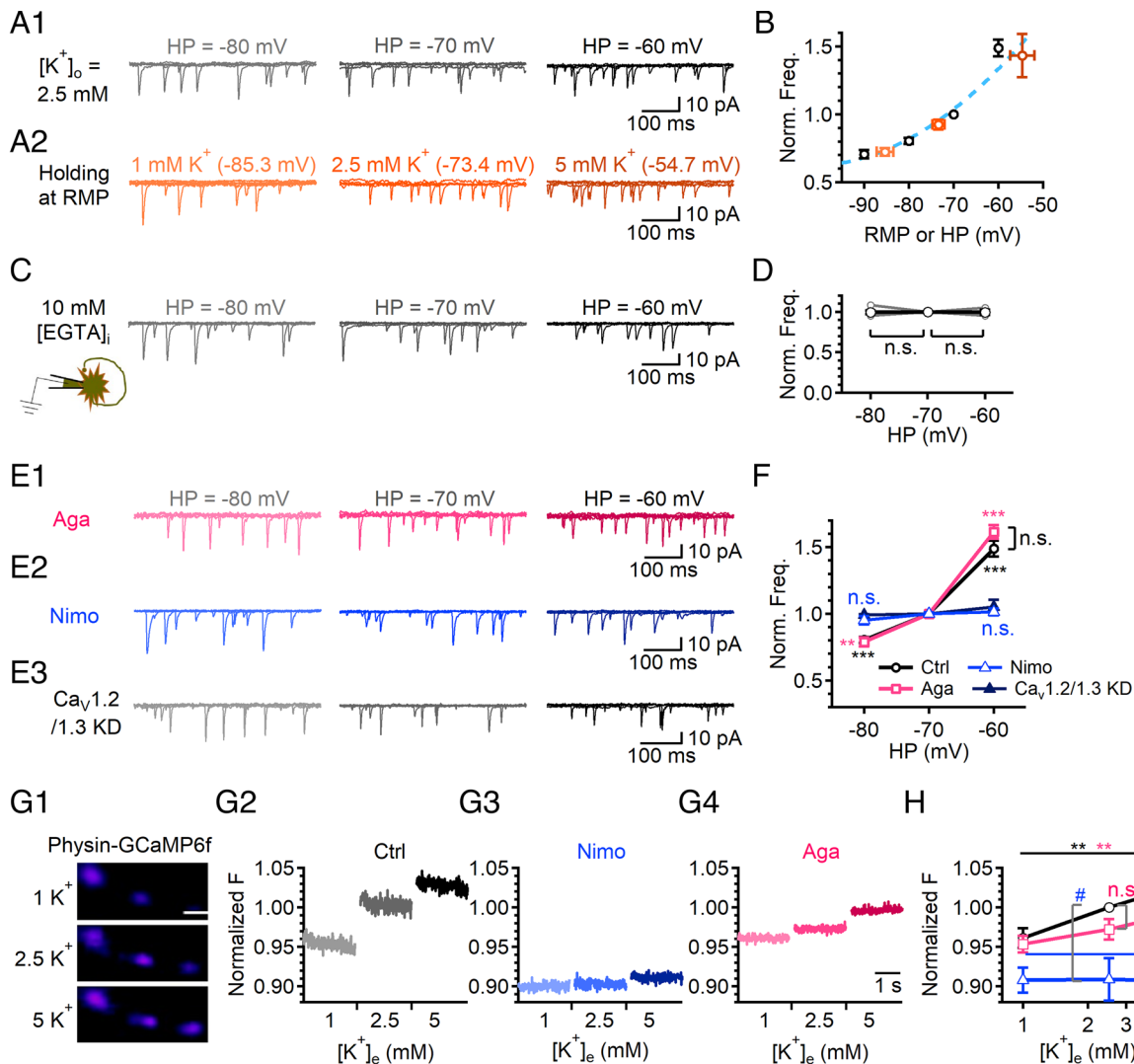


**Fig. 1.** LTCCs regulate both spontaneous and evoked glutamate release. (A, Top) There are representative traces of mEPSCs in control, Nimo (A1, blue), and Bay K (A2, green). Five 500-ms-long mEPSC traces were overlaid. (Bottom) Average time courses of the normalized mEPSC frequency. The data were normalized by the mean mEPSC frequency of control. A dashed gray line indicates the control level. (B) A bar graph of average values of the normalized mEPSC frequency in different conditions. (C) A bar graph of the average values of the normalized mEPSC amplitude. (D, Top) There are representative traces of eEPSCs in the control, Nimo (D1), and Bay K (D2) conditions. The gray dashed line indicates the control first eEPSC peak amplitude. (Bottom) Average time courses of the normalized first eEPSC amplitude. (E) A bar graph of the average values of the normalized first eEPSC amplitude in different conditions compared to control. (F) A bar graph of the average values of the PPR in different conditions. (G) Average time courses of the normalized mEPSC frequency (circle) and first eEPSC amplitude (triangle) in  $Ca_v1.2$  and  $Ca_v1.3$  double knockdown autapses in the presence of Nimo (G1) and Bay K (G2). (H) A bar graph of the average values of the normalized mEPSC frequency in different conditions compared to control. (I) A bar graph of the average values of the normalized first eEPSC amplitude in different conditions compared to control. (J1, Top) A schematic image of autaptic pyramidal neuron containing 10 mM EGTA internal patch pipette solution. (Bottom) Average time courses of the normalized mEPSC frequency (Nimo, circle; Bay K, triangle). (J2) A bar graph of the average values of the normalized mEPSC frequency in different conditions compared to control. (K1) Average time courses of the normalized first eEPSC amplitude. (K2) A bar graph of the average values of the normalized first eEPSC amplitude in different conditions compared to control. The individual raw values are described in *SI Appendix, Table S1*.

orange) increased to  $1.43 \pm 0.16$  ( $N = 7$ ). The relationship between  $V_m$  and the mEPSC frequency shown in Fig. 2B indicates that this relationship is not affected by the method of changing  $V_m$ .

We previously reported that  $Ca^{2+}$ -dependent spontaneous release in cultured hippocampal neurons is mediated by a local  $Ca^{2+}$  increase via the stochastic opening of P/Q-, N-, and R-type VGCCs (30). However, 10 mM EGTA in the pipette solution completely abolished the effect of subthreshold  $V_m$  on mEPSC frequency (Fig. 2C and D), suggesting that the  $V_m$ -dependent

increase in the spontaneous release is not mediated by a nanodomain  $Ca^{2+}$  increase induced by the increased open probability of P/Q-, N-, and R-type VGCCs. Accordingly, the  $V_m$ -dependent regulation of spontaneous release was not affected by blocking P/Q-type VGCCs using 100  $\mu$ M  $\omega$ -agatoxin (Aga, Fig. 2E1 and F). Thus, we tested the involvement of global  $Ca^{2+}$  changes, possibly attributable to other types of VGCCs such as LTCCs or T-type VGCCs.  $V_m$ -dependent changes in mEPSC frequency were completely abolished by blocking LTCCs using Nimo (Fig. 2E2



**Fig. 2.**  $V_m$ -dependent regulation of spontaneous release is mediated by LTCC-dependent changes in basal  $Ca^{2+}$ . (A) Representative traces of mEPSC frequency in each HP at 2.5 mM  $[K^+]_e$  (A1) and each  $[K^+]_e$  at the corresponding RMP (A2). (B) A graph indicating the relationship between HP (black) or RMP (orange) and the normalized mini frequency compared to control -70 mV or 2.5 mM  $[K^+]_e$  values. (C, Left) A schematic image of autapse containing 10 mM EGTA patch pipette solution. (Right) Representative traces of mEPSC frequency in each HP at 2.5 mM  $[K^+]_e$ . (D) A graph indicating the average value of the normalized mEPSC frequency in various HPs compared to -70 mV. (E) There are representative traces of mEPSCs in the Aga (E1), Nimo (E2), and shCa $_v1.2/1.3$  (E3) in each HP at 2.5 mM  $[K^+]_e$ . (F) A graph indicating the average value of the normalized mEPSC frequency in various HPs with Aga, Nimo, and shCa $_v1.2/1.3$  compared to -70 mV. (G1) Representative resting images of synaptophysin-GCaMP6f (Physin-GCaMP6f) in the control condition at different  $[K^+]_e$  in the primary cultured hippocampal synapses. Neurons transfected with Physin-GCaMP6f were applied with normal Tyrode's buffer. (Scale bar, 5  $\mu$ m.) (G2-G4) Normalized traces of Physin-GCaMP6f at rest in the various conditions of subthreshold potential in control (G2), Nimo (G3), and Aga-treated synapses (G4) compared to control at 2.5 mM  $[K^+]_e$ . (H) Normalized mean values of fluorescence intensities at resting status in the various conditions of subthreshold potential in control, Nimo, and Aga-treated neurons (Bonferroni test after one-way ANOVA). The individual raw values are described in SI Appendix, Table S1.

and F) or expression of shCa $_v1.2$  and shCa $_v1.3$  (Fig. 2 E3 and F) but not by a low concentration of NiCl $_2$  (40  $\mu$ M), which blocks T-type Ca $^{2+}$  channels (SI Appendix, Fig. S7). These results demonstrate that LTCCs specifically mediate the  $V_m$ -dependent regulation of spontaneous release. Given that  $V_m$ -dependent regulation of transmitter release by LTCCs was abolished by a high concentration of EGTA, it can be hypothesized that Ca $^{2+}$  influx through LTCCs increases global Ca $^{2+}$  levels at presynaptic terminals and indirectly modulates neurotransmitter release triggered by local Ca $^{2+}$  increases via P/Q-, N-, and R-type VGCCs. To test this hypothesis, we investigated whether presynaptic Ca $^{2+}$  levels are indeed changed by  $V_m$  and, if this is the case, whether  $V_m$ -dependent changes in Ca $^{2+}$  are affected by LTCC inhibition. To visualize presynaptic Ca $^{2+}$  levels, we expressed GCaMP6f (a genetic Ca $^{2+}$  indicator) fused to synaptophysin (a key synaptic vesicle protein) in hippocampal neuron culture (Physin-GCaMP6f, Fig. 2G1). To estimate changes in basal Ca $^{2+}$  at the resting state ( $[Ca^{2+}]_b$ ) in

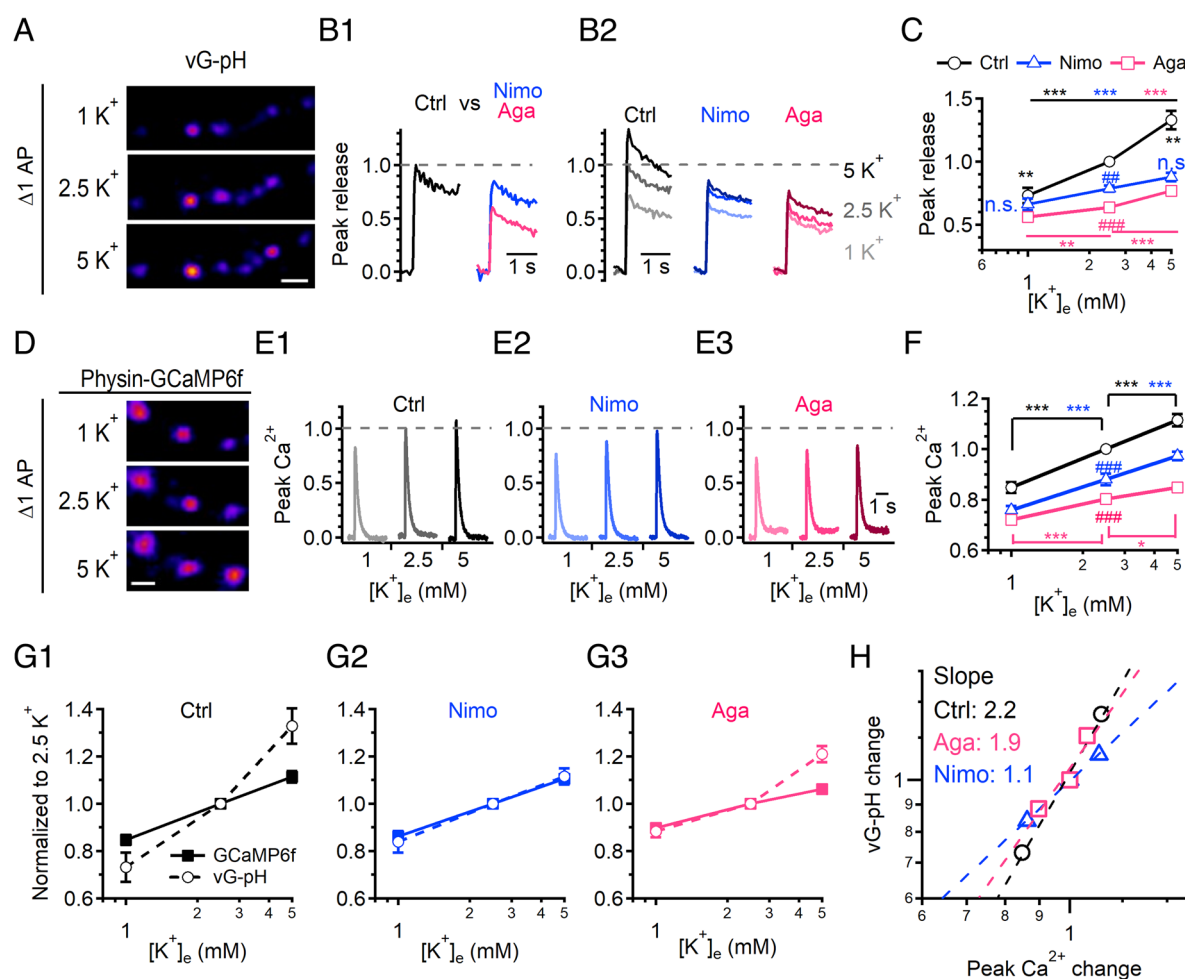
presynaptic terminals by  $V_m$  changes, the fluorescence intensity in the resting condition (F) was measured in different  $[K^+]_e$  and normalized to control values obtained in 2.5 mM  $[K^+]_e$ . F was decreased by hyperpolarization ( $[K^+]_e = 1$  mM) and increased by depolarization ( $[K^+]_e = 5$  mM) under control conditions (black, Fig. 2 G2 and H). Nimo reduced F by  $9.1 \pm 2.97\%$  at 2.5 mM  $[K^+]_e$ , and  $V_m$ -dependent changes in F were abolished by Nimo (blue, Fig. 2 G3 and H). In contrast, Aga did not significantly affect  $V_m$ -dependent changes in F (pink, Fig. 2 G4 and H). These results support the hypothesis that  $V_m$ -dependent regulation of presynaptic Ca $^{2+}$  is specifically mediated by LTCCs, which in turn regulate spontaneous transmitter release.

**LTCCs and P/Q-Type VGCCs Contribute to  $V_m$ -Dependent Regulation of Evoked Release with Different Mechanisms.** Next, we examined whether evoked release is also affected by subthreshold  $V_m$  changes. Measuring the eEPSC amplitude at

different  $V_m$  is not suitable in the autaptic cultured neuron because the eEPSC amplitude of autaptic neurons is affected not only by transmitter release from presynaptic terminals but also by the  $V_m$  of the postsynaptic compartment. To circumvent this caveat, we used a pHluorin (pH-sensitive Green Fluorescent Protein (GFP))-based assay system that visualizes the amount of vesicle exocytosis by the increase in the fluorescence intensity, while  $V_m$  was changed by changing RMP with different  $[K^+]_e$  (Fig. 3A). A single stimulus (1 AP) was applied to primary cultured hippocampal neurons expressing vGlut1-tagged pHluorin (vG-pH) by  $Ca^{2+}$  phosphate transfection, and the increase in fluorescence intensity ( $\Delta F$ ) by 1 AP was quantified as a measure of the evoked release amount. The  $\Delta F$  values obtained under experimental conditions were normalized to the values obtained under normal  $[K^+]_e$  conditions (2.5 mM). Hyperpolarization of  $V_m$  in 1 mM  $[K^+]_e$  decreased  $\Delta F$  by  $26.9 \pm 6.2\%$  ( $N = 11$ ), whereas depolarization of  $V_m$  in 5 mM  $[K^+]_e$  increased  $\Delta F$  by  $32.8 \pm 7.4\%$  ( $N = 11$ , black, Fig. 3B2 and C) showing the increased evoked release by  $V_m$  depolarization in control. At 2.5 mM  $[K^+]_e$ , Aga and Nimo decreased  $\Delta F$  by  $36.3 \pm 3.15\%$  and  $21.16 \pm 5.55\%$ , respectively (Aga,  $N = 11$ ; Nimo,

$N = 10$ ; Fig. 3B1 and C). In the presence of Aga and Nimo, the  $V_m$ -dependent effect on the evoked release was significantly attenuated. A five-fold change in  $[K^+]_e$  from 1 to 5 mM induced an increase in  $\Delta F$  by 1.82-fold in the control but 1.36-fold in Aga ( $P = 0.0464$ , Fig. 3B2 and C) and 1.33-fold in Nimo ( $P = 0.0225$ , Fig. 3B2 and C). These findings revealed that both LTCCs and P/Q-type VGCCs contribute to the  $V_m$ -dependent effect on evoked release.

To understand whether two types of VGCCs contribute to  $V_m$ -dependent regulation of evoked release with different mechanisms, we examined effects of  $[K^+]_e$  on AP-induced  $Ca^{2+}$  increase in presynaptic terminals (peak  $Ca^{2+}$ ) in Physin-GCaMP6f expressing neuron culture (Fig. 3D). A five-fold change in  $[K^+]_e$  from 1 to 5 mM induced an increase in  $\Delta[Ca^{2+}]$  by 1.31-fold in the control (Fig. 3E1 and F), which was smaller than the effect of  $[K^+]_e$  on glutamate release shown above. Both Nimo and Aga significantly reduced peak  $Ca^{2+}$ , but the effect of  $[K^+]_e$  on peak  $Ca^{2+}$  was not affected by Nimo (1.28-fold, Fig. 3E2 and F) but reduced by Aga (1.18-fold, Fig. 3E3 and F), suggesting that LTCCs did not contribute to the increase in AP-driven  $Ca^{2+}$  influx by subthreshold



**Fig. 3.** LTCCs and P/Q-type VGCCs contribute to  $V_m$ -dependent regulation of evoked release with different mechanisms. (A) Representative synapse images of vG-pH at 1 AP-evoking ( $\Delta 1$  AP) neurons in the control condition under different  $[K^+]_e$ . Neurons transfected with vG-pH were stimulated with 1 AP. (Scale bar, 5  $\mu m$ .) (B1) Ensemble average traces of vG-pH at 1 AP in the control (left, black), Nimo (right, blue), and Aga (right, pink) conditions. (B2) Ensemble average traces of vG-pH at 1 AP at different  $[K^+]_e$  conditions of subthreshold potential in control, Nimo, and Aga-treated neurons. (C) Normalized mean values of amplitudes of 1 AP responses in the various subthreshold potential of control, Nimo, or Aga-treated neurons. (D) Representative  $\Delta 1$  AP images of Physin-GCaMP6f in the control condition at different  $[K^+]_e$  in the primary cultured hippocampal synapses. (E) Ensemble average traces of Physin-GCaMP6f at 1 AP in the various conditions of subthreshold  $V_m$  in control (E1), Nimo (E2), and Aga-treated neurons (E3). (F) Normalized mean peak values of amplitudes of 1 AP responses in the various conditions of subthreshold potential in control, Nimo, and Aga-treated neurons. (G) The relationship of  $[K^+]_e$  versus the normalized GCaMP6f or vG-pH in control (G1), Nimo (G2), and Aga (G3) conditions. (H) A log-log plot for vG-pH change against peak  $Ca^{2+}$  change in control (black), Nimo (blue), and Aga (pink) conditions. The black dashed line (slope: 2.2), blue dashed line (slope: 1.1), and pink dashed line (slope: 1.9) were fitted by control, Nimo, or Aga, respectively. The individual raw values are described in *SI Appendix, Table S1*.

depolarization, while P/Q-type VGCCs did, at least in part. These results implied that the Nimo and Aga differentially affect the relationship between AP-induced glutamate release and peak  $\text{Ca}^{2+}$ . To examine this idea, we compared the effect of  $[\text{K}^+]_e$  on peak  $\text{Ca}^{2+}$  (solid line) with that on evoked release (broken line) in the control (Fig. 3G1) and in the presence of Nimo (Fig. 3G2), or Aga (Fig. 3G3), in the plots where values are normalized to the values obtained at 2.5 mM  $[\text{K}^+]_e$  in each experimental condition. The effect of  $[\text{K}^+]_e$  changes on evoked release was much larger than that on peak  $\text{Ca}^{2+}$  in the control condition (Fig. 3G1). This tendency was maintained in the presence of Aga (Fig. 3G3), but  $V_m$ -dependent effect on release and that on  $\text{Ca}^{2+}$  were almost identical in the presence of Nimo (Fig. 3G2). A log-log plot between glutamate release and peak  $\text{Ca}^{2+}$  showed that data points obtained in the control can be fitted by the line with slope of 2.2 (black dotted line, circle; Fig. 3H), indicating a high  $\text{Ca}^{2+}$  cooperativity of the release as was reported previously in hippocampal neurons (30, 34). In the presence of Aga,  $\text{Ca}^{2+}$  cooperativity was comparable to that of control (1.9, pink dotted line, square; Fig. 3H), suggesting that P/Q-type VGCCs contribute to both AP-evoked  $\text{Ca}^{2+}$  increase and the release but do not significantly affect the relationship between the two. In the presence of Nimo, however, the slope decreased to 1.1 (blue dotted line, triangle; Fig. 3H), suggesting that LTCC-dependent mechanisms contribute to the high  $\text{Ca}^{2+}$  cooperativity of the release. Considering the specific role of LTCCs in  $V_m$ -dependent regulation of basal  $\text{Ca}^{2+}$ , it can be suggested that the LTCC-mediated increase in basal  $\text{Ca}^{2+}$  contributes to the high  $\text{Ca}^{2+}$  cooperativity of AP-evoked transmitter release.

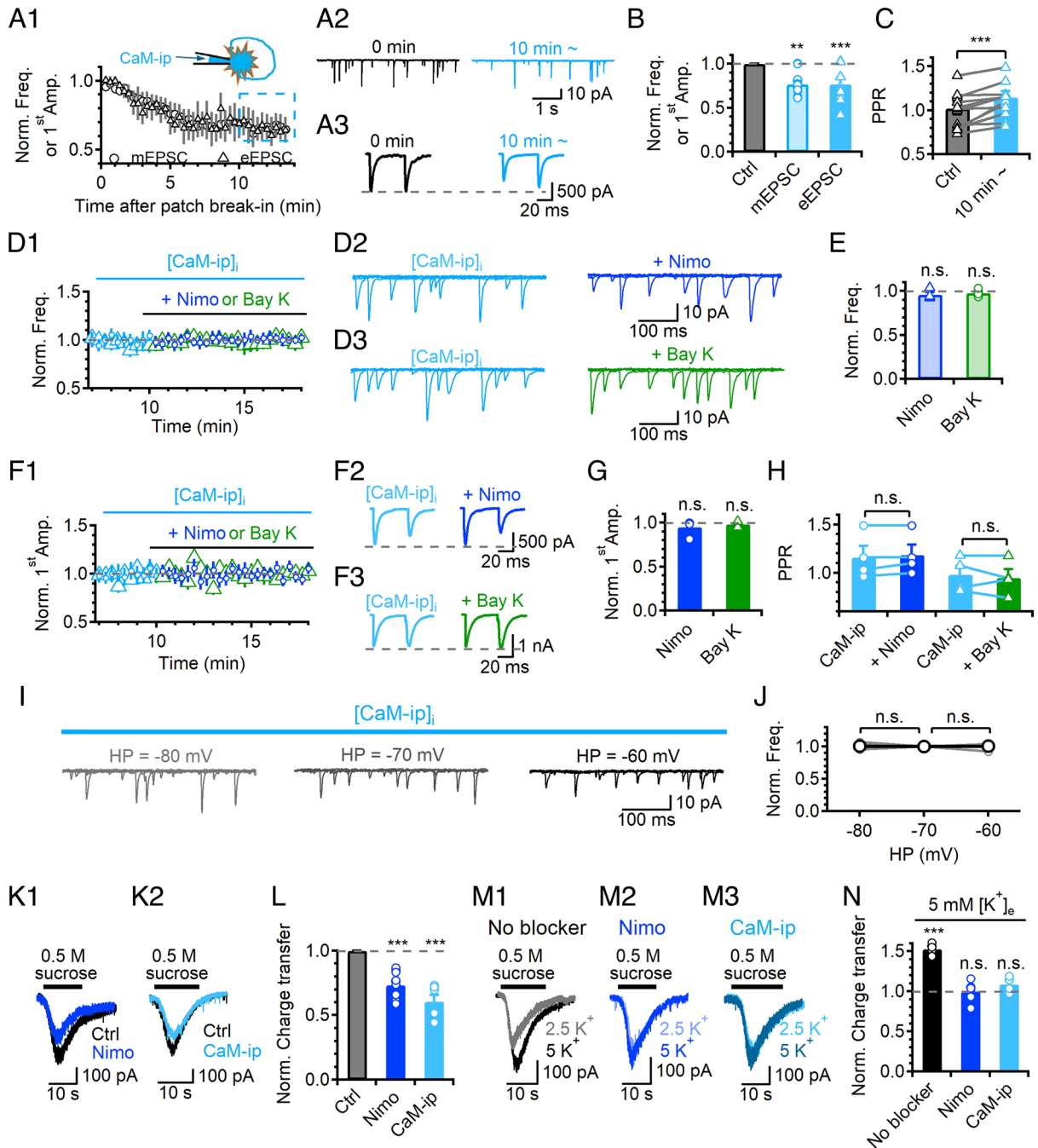
**$V_m$ - and LTCC-Dependent Regulation of Release Is Mediated by  $\text{Ca}^{2+}$ /Calmodulin Signaling.** To explore the signaling mechanism underlying LTCC-mediated regulation of transmitter release, we first investigated the role of  $\text{Ca}^{2+}$ /calmodulin (CaM)-dependent signaling using a calmodulin inhibition peptide (CaM-ip, 10  $\mu\text{M}$ ) in a pipette solution. Immediately after patch break-in, mEPSCs (Fig. 4A11 (circle) and Fig. 4A2) and eEPSCs (Fig. 4A1 (triangle) and Fig. 4A3) were recorded sequentially until the effects of CaM-ip perfusion reached a steady state. At the steady state, CaM-ip reduced the mEPSC frequency to  $0.77 \pm 0.04$  (pale bar, Fig. 4B,  $N = 11$ ) and eEPSC amplitude to  $0.77 \pm 0.07$  (solid bar, Fig. 4B,  $N = 10$ ) which was accompanied by the increased PPR (Fig. 4C), while scramble sequence of CaM-ip had no influence (SI Appendix, Fig. S8), indicating the involvement of  $\text{Ca}^{2+}$ /CaM signaling in transmitter release at physiological RMP. In the presence of CaM-ip, the application of Nimo or Bay K did not show further effects on the mEPSC frequency (Fig. 4D and E), eEPSC amplitude (Fig. 4F and G), and PPR (Fig. 4H). Furthermore,  $V_m$ -dependent changes in mEPSC frequency were also abolished (Fig. 4I and J). Taken together, these findings show that  $\text{Ca}^{2+}$ /CaM signaling mediates LTCC-dependent regulation of transmitter release.

The release probability ( $p_r$ ) and size of the readily releasable synaptic vesicle pool (RRP) are the key determinants of presynaptic neurotransmitter release. Nimo and CaM-ip decreased eEPSC amplitude in association with increased PPR (Figs. 1E and F and 4B and C), suggesting the involvement of increased  $p_r$  in LTCC-mediated facilitation of transmitter release. To investigate whether the  $V_m$ -dependent regulation of transmitter release involves changes in RRP size, we used the hypertonic sucrose solution (500 mM) application technique (35, 36), where the area of the current trace under the baseline during a brief application of sucrose ( $\sim 15$  s) can be regarded as RRP size (Fig. 4K). After patch break-in, the hypertonic sucrose solution was applied with fast-flow rate approximately  $1.5 \text{ mL min}^{-1}$ . Nimo decreased the RRP size by  $0.73 \pm 0.03$  (Fig. 4K1 and L,  $N = 8$ ), suggesting the

involvement of RRP in LTCC-mediated regulation of transmitter release. CaM-ip in the pipette solution also significantly decreased the RRP size (Fig. 4L, CaM-ip,  $0.61 \pm 0.05$ ,  $N = 6$ ). We then examined whether subthreshold depolarization affected RRP size. At 5 mM  $[\text{K}^+]_e$ , RRP size increased 1.53-fold ( $N = 4$ , Fig. 4M1 and N), and this increase was not observed in the presence of Nimo and CaM-ip (Fig. 4M and N). These results suggest that the RRP size is increased by depolarization via  $\text{Ca}^{2+}$ -dependent signaling, which underlies the  $V_m$ -dependent regulation of transmitter release identified in the present study (summarized in SI Appendix, Fig. S9).

We recapitulated the presence and role of LTCCs in the  $V_m$ -dependent regulation of glutamate release in mossy fiber (MF)-CA3 synapses in acute brain slices. GABAergic currents were blocked by picrotoxin. In CA3-PCs, mEPSCs were recorded in the presence of tetrodotoxin (TTX), whereas eEPSCs were recorded by stimulating MF every 20 s with the electrode placed in the stratum lucidum. According to the sensitivity to DCG-IV (37) and rise time analysis (38, 39), EPSCs recorded in CA3-PCs were identified to be mainly attributed to MF-CA3 synapses (SI Appendix, Methods and Fig. S10). In the control, Bay K increased mEPSC frequency (Fig. 5C2, green,  $1.73 \pm 0.06$ ,  $N = 4$ ), whereas Nimo significantly decreased mEPSC frequency (Fig. 5A2 and C2, blue,  $0.72 \pm 0.04$ ,  $N = 15$ ). Calmidazolium (CMZ, 10  $\mu\text{M}$ ) caused a significant decrease in mEPSC frequency (Fig. 5A3 and C2, CMZ, brown,  $0.55 \pm 0.05$ ,  $N = 12$ ). The effects of Bay K, Nimo, and CMZ on evoked release were similar to those obtained for spontaneous release. Nimo and CMZ significantly decreased eEPSC amplitude (Fig. 5B and D2, Nimo,  $0.6 \pm 0.04$ ,  $N = 4$ ; and CMZ,  $0.62 \pm 0.07$ ,  $N = 6$ ), which was accompanied by the increased PPR (Fig. 5E), whereas Bay K increased eEPSC amplitude (Fig. 5D2,  $1.5 \pm 0.09$ ,  $N = 5$ ) which was accompanied by the decreased PPR (Fig. 5E). To examine the possible involvement of postsynaptic LTCCs in the effects of LTCC targeting drugs on mEPSCs and eEPSCs, we tested the effects of Bay K, while postsynaptic LTCCs were inhibited by the internal patch pipette solution containing 1 mM verapamil, which was shown to block LTCCs when intracellularly applied (40). We confirmed the blocking effect of intracellular verapamil on the postsynaptic LTCCs (SI Appendix, Fig. S11A–D). In the presence of verapamil in CA3-PCs, Bay K still enhanced the eEPSC amplitude (SI Appendix, Fig. S11F2,  $1.87 \pm 0.17$ ,  $N = 7$ ) and the mEPSC frequency (SI Appendix, Fig. S11E2,  $1.78 \pm 0.03$ ,  $N = 5$ ), excluding the possible involvement of postsynaptic LTCCs. We then examined whether mEPSCs and eEPSCs at MF-CA3 synapses are regulated by subthreshold depolarization induced by increasing  $[\text{K}^+]_e$ , and LTCCs and CaM are involved in this regulation. We observed the 1.76-fold increase in mEPSCs frequency by depolarization through increasing  $[\text{K}^+]_e$  to 5 mM from 2.5 mM (Fig. 5F and H,  $N = 7$ , black), and this increase was almost completely abolished in the presence of Nimo (Fig. 5F and H,  $N = 10$ , blue) or CMZ (Fig. 5F and H,  $N = 7$ , brown). The amplitude of eEPSC was increased by 1.86-fold in 5 mM  $[\text{K}^+]_e$ , and this increase was almost completely abolished in the presence of Nimo or CMZ (Fig. 5G and I). The increased eEPSC amplitude in 5 mM  $[\text{K}^+]_e$  was accompanied by the decreased PPR (Fig. 5J), supporting the involvement of presynaptic mechanism in the depolarization-induced increase in transmitter release. Collectively, these results suggest that  $\text{Ca}^{2+}$ /CaM-dependent signaling activated by basal  $\text{Ca}^{2+}$  increase via presynaptic LTCCs contributes to the  $V_m$ -dependent regulation of transmitter release at MF-CA3 synapses in acute hippocampal slices.

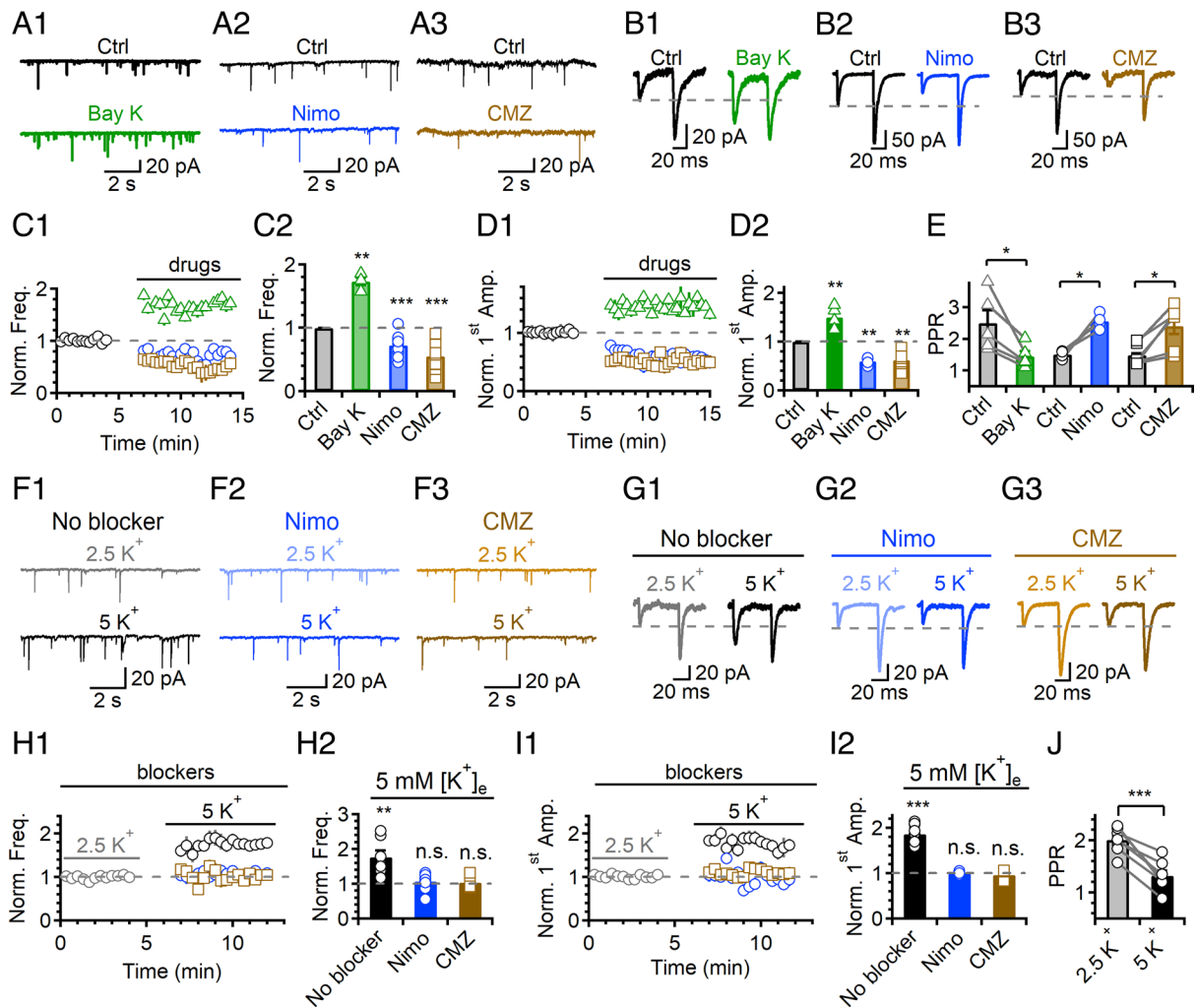
**Contribution of LTCCs to Glutamate Release Is Developmentally Regulated.** It has been suggested that LTCCs do not participate in neurotransmitter release in most neurons (21, 22). A previous study



**Fig. 4.**  $V_m$ - and LTCC-dependent regulation of release is mediated by  $Ca^{2+}$ /calmodulin signaling. (A, A1, Top) A schematic image of CaM-ip diffusion through the autaptic cell body to the axon terminal. Bottom: Average time courses of the normalized mEPSC frequency (circle) or first eEPSC amplitude (triangle) after patch break-in. The data were normalized by the mean frequency or mean amplitude of the first eEPSCs of the initial 1 min after patch break-in. The light blue dashed line box indicates the steady-state average effects, which were used for the bar graphs. (A2 and A3) There are representative traces of mEPSCs (A2) and eEPSCs (A3) immediately patch break-in and 10 min after patch in the presence of 10  $\mu$ M CaM-ip (light blue). The gray dashed line indicates the control first eEPSC peak amplitude. (B) A bar graph showing the normalized mEPSC frequency (pale) and the first eEPSC amplitude (solid) 10 min after patch in the presence of CaM-ip compared to immediately after patch break-in. A dashed gray line indicates the control level. (C) A bar graph of the average values of the PPR. (D1 and F1) An average time course of the normalized mEPSC frequency (D1) and the normalized first eEPSC amplitude (F1) in the presence of CaM-ip followed by Nimo (circle) or Bay K (triangle). (D2 and F2) Representative traces of mEPSC or eEPSC 10 min after patch in the presence of CaM-ip followed by Nimo (D2 and F2) or Bay K (D3 and F3), respectively. (E and G) A bar graph of the average value of the normalized mEPSC frequency (E) and the first eEPSC amplitude (G) in the presence of CaM-ip followed by Nimo or Bay K compared to 10 min after patch break-in. (H) A bar graph of average values of the PPR in different conditions. (I) There are representative traces of mEPSCs in each HP 10 min after patch in the presence of CaM-ip. (J) A graph showing the average value of the normalized mEPSC frequency in various HPs in the presence of CaM-ip compared to  $-70$  mV. (K) Representative traces of the hypertonic sucrose solution application in the presence of each blocker (K1, Nimo; K2, CaM-ip). Top solid line in each trace indicates sucrose application periods. (L) A bar graph of the average values of the normalized charge transfer in different conditions. (M) Representative traces of the hypertonic sucrose solution application for the comparison of 2.5 mM and 5 mM  $[K^+]_e$  in the pretreatment of each blocker (M1, no blocker; M2, Nimo; M3, CaM-ip). (N) A bar graph of the average values of the normalized charge transfer in pretreatment of each blocker in 5 mM  $[K^+]_e$  compared to 2.5 mM  $[K^+]_e$ . The individual raw values are described in *SI Appendix, Table S1*.

showed the role of P/Q-, N-, and R-type VGCCs in spontaneous glutamate release at synapses of cultured hippocampal neurons, but the contribution of LTCCs was not recognized (41). Since

the neurons that they used were younger than ours (8 to 11 d vs. more than 3 wk after plating), it is likely that the contribution of LTCC may be developmentally regulated, such that the effect



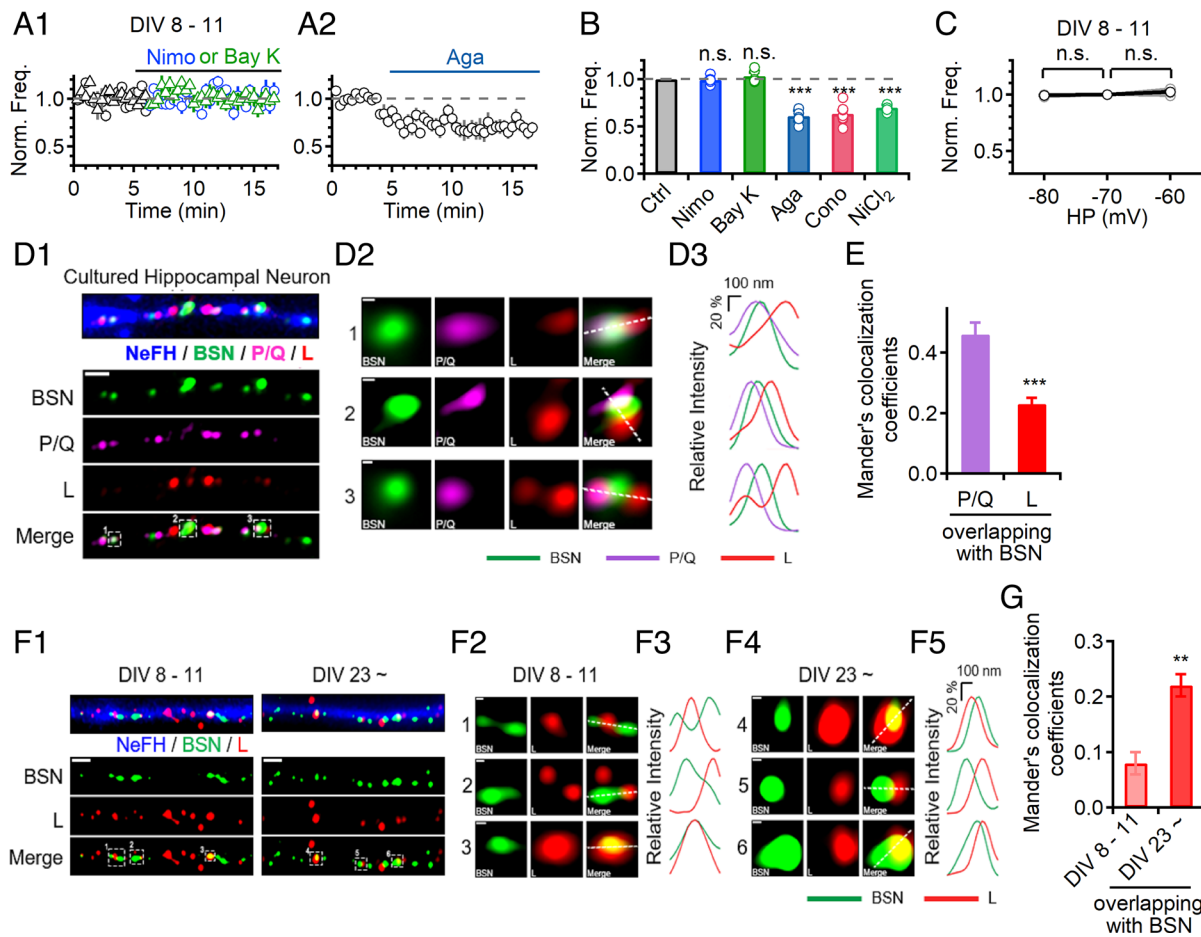
**Fig. 5.** LTCC-mediated  $V_m$ -dependent regulation of glutamate release at the hippocampal MF-CA3 synapses. (A) There are representative traces of mEPSCs in the control, Bay K (A1, green), Nimo (A2, blue), and CMZ (A3, brown) conditions. (B) There are representative traces of eEPSCs in the control, Bay K (B1), Nimo (B2), and CMZ (B3) conditions. The gray dashed line indicates the control first eEPSC peak amplitude. (C1) Average time courses of the normalized mEPSC frequency. A dashed gray line indicates the control level. (C2) A bar graph of the average values of the normalized mEPSC frequency in the presence of each drug in 2.5 mM  $[K^+]_e$  compared to 2.5 mM  $[K^+]_e$  control. (D1) Average time courses of the normalized first eEPSC amplitude. (D2) A bar graph of the average values of the normalized first eEPSC amplitude in the presence of each drug in 2.5 mM  $[K^+]_e$  compared to 2.5 mM  $[K^+]_e$  control. (E) A bar graph of average values of the PPR in different conditions. (F) Representative traces of mEPSC for the comparison of 2.5 mM and 5 mM  $[K^+]_e$  in the pretreatment of each blocker (F1, no blocker; F2, Nimo; F3, CMZ). (G) Representative traces of eEPSC for the comparison of 2.5 mM and 5 mM  $[K^+]_e$  in the pretreatment of each blocker (G1, no blocker; G2, Nimo; G3, CMZ). (H1) Average time courses of the normalized mEPSC frequency. (H2) A bar graph of the average values of the normalized mEPSC frequency in pretreatment of each blocker in 5 mM  $[K^+]_e$  compared to 2.5 mM  $[K^+]_e$ . (I1) Average time courses of the normalized first eEPSC amplitude. (I2) A bar graph of the average values of the normalized first eEPSC amplitude in pretreatment of each blocker in 5 mM  $[K^+]_e$  compared to 2.5 mM  $[K^+]_e$ . (J) A bar graph of average values of the PPR. The individual raw values are described in *SI Appendix, Table S1*.

of LTCC did not appear in immature neurons. Therefore, we examined the contribution of each VGCC, including LTCC, to mEPSC frequency in hippocampal neurons on days in vitro (DIV) 8 to 11 in autaptic cultured neurons. We found that Nimo (Fig. 6A1 and B,  $N = 6$ ) and Bay K ( $N = 4$ ) did not affect mEPSC frequency, whereas Aga, Cono, or 100  $\mu$ M NiCl<sub>2</sub> significantly decreased the frequency (Fig. 6A2 and *SI Appendix, Fig. S12 A and B*). Consistent with the lack of LTCC contribution, changing the HP between  $-80$  and  $-60$  mV did not affect mEPSC frequency in immature neurons (Fig. 6C and *SI Appendix, Fig. S12C*,  $N = 6$ ), further supporting the critical role of LTCCs in regulating transmitter release by  $V_m$ .

To examine whether the lack of LTCCs contribution to spontaneous release in immature neurons is due to low levels of LTCCs expression in immature neurons, we tested the effect of Nimo on  $Ca^{2+}$  currents elicited by depolarization from  $-70$  mV to 0 mV (*SI Appendix, Fig. S13A*). A decrease in the peak inward  $Ca^{2+}$

current by Nimo observed in immature neurons (*SI Appendix, Fig. S13 C and D*,  $0.39 \pm 0.05$ ,  $N = 6$ ) was not significantly different from that in mature neurons ( $0.35 \pm 0.04$ ,  $N = 11$ , *SI Appendix, Fig. S13D*). These results suggest that LTCCs are already present in immature neurons, but their localization to presynaptic terminals may occur later. To examine this idea, we compared the presynaptic expression level of LTCC between immature neurons (DIV 8-11) and mature neurons (DIV 23-) by analyzing the extent of overlap between LTCCs and the presynaptic marker. We first examined the subcellular localization of LTCC and P/Q-type VGCC along with Neurofilament H (NeFH, an axonal marker) and Bassoon (BSN, an active zone marker) in adult neurons. Airyscan superresolution (SR) imaging revealed that immunofluorescent signals of P/Q-type VGCC overlapped considerably with those of BSN, whereas the LTCC signals did not fully overlap with those of BSN, and most of their localization were in the juxtasyaptic sites (Fig. 6 D and E), as previously





**Fig. 6.** Contribution of LTCCs to glutamate release is developmentally regulated. (A) Average time courses of the normalized mEPSC frequency in different conditions at immature autaptic neurons (A1, blue, Nimo; green, Bay K; A2, Aga). A dashed gray line indicates the control level. (B) A bar graph of average values of the normalized mEPSC frequency in different conditions at immature autaptic neurons compared to control. (C) A graph indicating the average value of the normalized mEPSC frequency in various HPs compared to  $-70$  mV. (D1) Representative SR images of cultured rat hippocampal neurons at DIV 23 immunostained with NeFH (blue), BSN (green), P/Q-type (magenta), and L-type (red) calcium channels. (Scale bar,  $1 \mu\text{m}$ .) (D2) Magnified axonal bouton images of rectangular areas in D1. (Scale bar,  $100 \text{ nm}$ .) (D3) Fluorescent intensity profiles of the respective proteins across the line in axonal boutons in D2. (E) Mander's colocalization coefficients of each calcium channel overlapped with BSN. (F1) Representative SR images of cultured hippocampal neurons at DIV 8 to 11 (Left) and DIV 23 (Right) immunostained with NeFH, BSN, and L-type calcium channel. (Scale bar,  $1 \mu\text{m}$ .) (F2 and F4) Magnified axonal bouton images of rectangular areas in F1. (Scale bar,  $100 \text{ nm}$ .) (F3 and F5) Fluorescent intensity profiles of the respective proteins across the line in axonal boutons in F2 and F4, respectively. (G) Mander's colocalization coefficients of the LTCC overlapped with BSN. The individual raw values are described in *SI Appendix, Table S1*.

reported (29). Developmental changes in LTCC density at presynaptic terminals were further confirmed in independent sets of experiments using cultured rat hippocampal neurons, and SR imaging showed that the degree of colocalization of LTCC with BSN was significantly higher in adult neurons than in immature neurons (Fig. 6 F and G), which is consistent with the results of the electrophysiological data in this study. These results indicate that the presynaptic localization of LTCC is developmentally regulated, which may be why appropriate LTCC-mediated  $\text{Ca}^{2+}$  signaling to regulate vesicular exocytosis appears in adult neurons.

## Discussion

The molecular mechanisms of neurotransmitter release are well established, but their fine-tuned modulation remains unclear. The present study examined how subthreshold potential changes modulate two classes of synaptic transmission: AP-evoked transmitter release and spontaneous release. First, we found that subthreshold potential changes affected spontaneous and AP-evoked release through LTCCs at presynaptic terminals. Second,  $\text{Ca}^{2+}$  influx through LTCCs did not directly contribute to  $\text{Ca}^{2+}$ -triggered transmitter release but contributed to the global  $\text{Ca}^{2+}$  changes in

presynaptic terminals that regulate release probability and RRP size via CaM. Third, we demonstrated that the presynaptic localization and the role of LTCCs in synaptic transmission are developmentally regulated. In the early stages of development, LTCCs hardly colocalized with presynaptic proteins, but their colocalization increased significantly at a more mature stage. Thus,  $V_m$ -dependent regulation of glutamate release requires localization of LTCCs at presynaptic terminals, which appear in the mature state of neurons.

This study raises several interesting questions. The first is how  $V_m$ -dependent changes in presynaptic  $\text{Ca}^{2+}$  levels in the basal state affect  $\text{Ca}^{2+}$ -triggered transmitter release. Molecular mechanisms mediating  $\text{Ca}^{2+}$ -triggered transmitter release are now well established, in that synaptotagmins bind  $\text{Ca}^{2+}$  via two C2 domains, and transduce the  $\text{Ca}^{2+}$  signal into nanomechanical activation of the membrane fusion machinery, the SNARE complex (42). However, it is not well understood how changes in basal  $\text{Ca}^{2+}$ , which are far smaller than AP-evoked local  $\text{Ca}^{2+}$  increases, regulate transmitter release. Recently, the  $\text{Ca}^{2+}$  dependence of vesicle priming, fusion, and replenishment was studied in cerebellar mossy fiber boutons, revealing that the number of RRP strongly depends on basal  $\text{Ca}^{2+}$  between  $30$  and  $180 \text{ nM}$  (43). As a potential

signaling molecule that regulates  $\text{Ca}^{2+}$ -dependent vesicle priming, the interaction of diacylglycerol/PLC or  $\text{Ca}^{2+}$ /phospholipids with Munc13s (10, 16, 44–46) has been proposed (43). In the present study, we demonstrated that  $V_m$ -induced changes in transmitter release are mediated by basal  $\text{Ca}^{2+}$  changes and involve CaM activation (Figs. 2 and 4); the increases in RRP size and release probability may underlie increased transmitter release (Fig. 4).

The role of basal  $\text{Ca}^{2+}$  changes has been investigated with the aim of explaining synaptic facilitation, leading to the “residual  $\text{Ca}^{2+}$  hypothesis” (47, 48). Considering that the residual  $\text{Ca}^{2+}$  induced by high-frequency stimulation and the basal  $\text{Ca}^{2+}$  increase by  $V_m$  depolarization have different  $\text{Ca}^{2+}$  sources, namely mitochondrial  $\text{Ca}^{2+}$  release for the former (49, 50) and LTCCs for the latter (Fig. 2*G*), it would be intriguing to know whether they share common downstream mechanisms. Mitochondria-dependent residual  $\text{Ca}^{2+}$  during posttetanic potentiation was shown to increase the release probability but not RRP size (50). The  $\text{Ca}^{2+}$ –CaM–Munc13-1 complex was suggested to play a pivotal role in short-term synaptic plasticity by regulating the recovery of RRP but with no changes in RRP size or  $p_r$  at the resting state (9, 10). These differences may imply a difference in downstream mechanisms between  $V_m$ -dependent global  $\text{Ca}^{2+}$  changes and activity-dependent increases in residual  $\text{Ca}^{2+}$ . However, further studies are required to elucidate the detailed mechanisms. The involvement of distinct  $\text{Ca}^{2+}$  sensors that detect basal  $\text{Ca}^{2+}$  changes is of interest. Recently, the involvement of synaptotagmin 7, a high-affinity  $\text{Ca}^{2+}$  sensor (51), in synaptic facilitation has been suggested (52, 53). Future studies should investigate whether synaptotagmin 7 is also involved in the  $V_m$ -induced changes in transmitter release.

The second question is how presynaptic  $\text{Ca}^{2+}$  changes that mediate the  $V_m$ -dependent regulation of transmitter release are specifically attributable to LTCCs. L- and T-type VGCCs can open at or near the RMP in CA1 hippocampal neurons (54, 55). According to their biophysical properties, T-type VGCCs, low-voltage-activated  $\text{Ca}^{2+}$  channels, appear to be better suited for contributing to changes in presynaptic  $\text{Ca}^{2+}$  levels by subthreshold  $V_m$  changes. However, we found that the blockade of T-type  $\text{Ca}^{2+}$  channels did not significantly affect the  $V_m$ -dependent regulation of transmitter release (*SI Appendix, Fig. S7*). This result suggests that the contribution of VGCCs to the  $V_m$ -dependent regulation of transmitter release does not simply depend on the activation range of VGCCs. Possibly, presynaptic terminals are highly compartmentalized so the localization of the  $\text{Ca}^{2+}$  source is critical for its function. In fact, the differential roles of L- and T-type  $\text{Ca}^{2+}$  channels have been demonstrated in the postsynaptic compartment in hippocampal neurons, where L-type, but not T-type,  $\text{Ca}^{2+}$  channels contribute to metabotropic glutamate receptor 5-induced PLC activation, although L- and T-type  $\text{Ca}^{2+}$  channels equally contribute to depolarization-induced  $\text{Ca}^{2+}$  increase (56). It was thus suggested that LTCCs, but not T-type  $\text{Ca}^{2+}$  channels, may form a signaling complex with PLC so that LTCC-induced  $\text{Ca}^{2+}$  microdomains effectively activate PLC (56). The results of the present study suggest a close link between LTCCs and CaM in presynaptic terminals. It is well known that coupling between P/Q-, N-, or R-type VGCCs with low-affinity vesicular  $\text{Ca}^{2+}$  sensors is critical for  $\text{Ca}^{2+}$ -triggered vesicular fusion (41). Additionally, our study suggests that coupling between LTCCs and unidentified high-affinity  $\text{Ca}^{2+}$  sensors or  $\text{Ca}^{2+}$ -sensitive signaling proteins such as CaM may be important for the regulation of release by basal  $\text{Ca}^{2+}$  changes. Developmental changes in the LTCC contribution to the regulation of transmitter release (Fig. 6*A–C*) and presynaptic localization (Fig. 6*D–G*) shown in the present

study also highlight the importance of accurate localization for its function.

One of the important points revealed in the present study is that the mechanisms involved in the  $V_m$ -dependent regulation of transmitter release are surprisingly similar between evoked and spontaneous release. Since spontaneous release occurs at basal  $\text{Ca}^{2+}$  levels in a  $\text{Ca}^{2+}$ -dependent manner, it is generally thought that the  $\text{Ca}^{2+}$ -dependent mechanism underlying spontaneous release is different from that underlying AP-triggered evoked release that occurs in response to a large increase in local  $\text{Ca}^{2+}$  levels (57). As a candidate for the high-affinity  $\text{Ca}^{2+}$  sensor responsible for spontaneous release, Doc2 was suggested (58, 59), although the  $\text{Ca}^{2+}$  dependency of Doc2 is controversial (60). However, several studies have shown that the  $\text{Ca}^{2+}$ -dependent component of spontaneous release is not mediated by basal  $\text{Ca}^{2+}$  but by the local  $\text{Ca}^{2+}$  increase induced by VGCC openings (30, 41, 61) or  $\text{Ca}^{2+}$  release from the internal stores (30, 62). If the local  $\text{Ca}^{2+}$  increase induced by stochastic activation of  $\text{Ca}^{2+}$  source is sufficient to activate low-affinity  $\text{Ca}^{2+}$  sensors that mediate AP-evoked transmitter release, spontaneous release may not require high-affinity  $\text{Ca}^{2+}$  sensors. Consistent with this idea, synaptotagmin-1, a low-affinity  $\text{Ca}^{2+}$  sensor with high  $\text{Ca}^{2+}$  cooperativity for fast-evoked release, was shown to mediate spontaneous release in cortical neurons (63). Furthermore, several studies have shown that the apparent  $\text{Ca}^{2+}$  cooperativity for spontaneous release is not as low as previously thought but similar to that for evoked release (30, 63). Taken together, our study supports the idea that  $\text{Ca}^{2+}$ -dependent mechanisms that operate in the range of presynaptic  $\text{Ca}^{2+}$  levels in the resting state are not for mediating spontaneous release but for regulating both spontaneous and evoked release. However, this conclusion cannot be generalized to all synapses because  $\text{Ca}^{2+}$ -dependent mechanisms for spontaneous release vary depending on the cell type and developmental stage (30). At synapses where spontaneous release is not dependent on VGCCs, such as excitatory synapses in CA1 hippocampal neurons (30) and neocortical neuron cultures (64), spontaneous and evoked release may be regulated by distinct mechanisms.

The RMP is a key component of the physiology of excitable cells, including neurons. The RMP varies widely in different types of neurons and can fluctuate within a subthreshold range under different physiological conditions, including changes in  $\text{K}^+$  concentration, ion channel activity, and synaptic activity. Accumulating evidence demonstrates the importance of neuronal resting  $\text{Ca}^{2+}$  signaling in the regulation of synaptic efficacy and neuronal homeostasis (65). Basal  $\text{Ca}^{2+}$ -dependent enhancement of transmitter release by subthreshold depolarization has been previously reported in MF-CA3 synapses (2), calyx of Held synapses, and cerebellar interneurons (3). These observations highlight the functional importance of subthreshold potential changes that regulate AP-evoked transmitter release. However, the underlying mechanism of how subthreshold depolarization increases presynaptic  $\text{Ca}^{2+}$ , which leads to enhancement of transmitter release, was elusive. Furthermore, it was unknown whether subthreshold potential changes influence spontaneous and evoked release via the same mechanism. In the present study, we demonstrated that LTCCs are responsible for the increased presynaptic  $\text{Ca}^{2+}$  by subthreshold depolarization and that  $\text{Ca}^{2+}$ -dependent signaling, such as CaM, increases RRP size, resulting in the enhancement of both evoked and spontaneous release. Our study highlights the role of LTCC as a key player in the regulation of transmitter release by coupling subthreshold potential changes with the activation of  $\text{Ca}^{2+}$ -dependent signaling molecules that regulate transmitter release.

## Methods

**Autaptic Hippocampal Neuron Culture and Slice Preparation.** Primary cultures of rat autaptic hippocampal neurons were prepared as described previously with slight adaptations (30, 66). Hippocampal slices were prepared from P20 to 30 Sprague–Dawley rats. After anesthetizing by inhalation with 5% isoflurane, rats were decapitated, and the brain was quickly removed and chilled in an ice-cold high-magnesium cutting solution. All preparations were carried out under the Animal Welfare Guidelines of the Seoul National University (SNU) and approved by the IACUC of the SNU. The detailed processes are described in *SI Appendix, Materials and Methods*.

**Electrophysiology.** Autaptic cultured neurons were visualized using an Olympus IX70 inverted microscope and continuously perfused with an extracellular solution. Electrophysiological recordings were performed at room temperature. For recordings from hippocampal slices, slices were transferred to an immersed recording chamber continuously perfused with oxygenated aCSF using a peristaltic pump (Gilson). Temperature was maintained at  $35 \pm 1$  °C. CA3 pyramidal cells were visualized using an upright microscope equipped with differential interference contrast optics (BX51WI; Olympus). Whole-cell voltage- or current-clamp recordings were performed as described in detail in *SI Appendix, Materials and Methods*.

**Dissociated Hippocampal Neuron Culture and Optical Image Using vGlut1-pHluorin and Synaptophysin–GCaMP6f.** Hippocampal CA1–CA3 regions were dissected and dissociated from P0 to P1 SD rats and plated onto poly-ornithine-coated glass, as previously described (67). All constructs were transfected 8 days after plating and further incubated for 17 to 25 d in culture media (Minimum Essential Media (MEM), 0.5% glucose, 0.01% transferrin, 0.5 mM GlutaMAX-I, 4  $\mu$ M 1- $\beta$ -D-cytosine-arabinofuranoside, 2% B27, and 5% Fetal Bovine Serum (FBS)). For presynaptic terminal live imaging to examine synaptic transmission or synaptic  $Ca^{2+}$  levels, vGlut1-pHluorin (vG-pH) or synaptophysin–GCaMP6f (Physin–GCaMP6f) constructs were utilized. For details, please see *SI Appendix, Materials and Methods*. The detailed experimental procedures were described in *SI Appendix, Materials and Methods*.

**Immunocytochemistry and Antibodies.** For immunocytochemistry, dissociated cultured hippocampal neurons were fixed in 4% paraformaldehyde in 4% sucrose-containing PBS for 15 min and permeabilized for 5 min in 0.25% Triton X-100 at room temperature. Anti-chicken Neurofilament H (TA309177, Origine),

anti-rabbit Bassoon (141 002; Synaptic Systems), anti-guinea pig Cav2.1 (152 205; Synaptic Systems), and anti-mouse Cav1.2 (MA5-27717, Invitrogen) were used in the experiments, and Alexa Fluor secondary antibodies were purchased from Thermo Fisher Scientific. The detailed processes are described in *SI Appendix, Materials and Methods*.

**Drugs.**  $\omega$ -Agatoxin-IVA,  $\omega$ -conotoxin GVIA, and TTX were purchased from Alomone Labs. Bay K8644 and calmidazolium were purchased from Tocris. Calmodulin inhibitory peptide and calmodulin inhibitory peptide scramble were purchased from Calbiochem (Darmstadt, Germany). All other chemicals were purchased from Sigma (St. Louis, MO, USA). Toxin stock solutions were made at 1,000-fold concentration with distilled water or DMSO and stored at  $-20$  °C.

**Statistical Analysis.** Data were expressed as the mean  $\pm$  SEM, where  $N$  represents the number of cells studied. Statistical analysis was performed using Igor Pro (version 6.1, WaveMetrics, Lake Oswego, OR, USA) and OriginPro (version 9.0, OriginLab Corp., Northampton, MA, USA). Significant differences between the experimental groups were analyzed using independent or paired Student's  $t$  tests. All data are represented as the mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ ; n.s. = not significant.

**Data, Materials, and Software Availability.** All study data are included in the article and/or *SI Appendix*.

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