Calcium sensor regulation of the $Ca_V2.1$ Ca^{2+} channel contributes to short-term synaptic plasticity in hippocampal neurons

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Short-term synaptic plasticity is induced by calcium (Ca^{2+}) accumulating in presynaptic nerve terminals during repetitive action potentials. Regulation of voltage-gated $Ca_V2.1$ Ca^{2+} channels by Ca^{2+} sensor proteins induces facilitation of Ca^{2+} currents and synaptic facilitation in cultured neurons expressing exogenous $Ca_V2.1$ channels. However, it is unknown whether this mechanism contributes to facilitation in native synapses. We introduced the IM-AA mutation into the IQ-like motif (IM) of the Ca^{2+} sensor binding site. This mutation does not alter voltage dependence or kinetics of $Ca_V2.1$ currents, or frequency or amplitude of spontaneous miniature excitatory postsynaptic currents (mEPSCs); however, synaptic facilitation is completely blocked in excitatory glutamatergic synapses in hippocampal autaptic cultures. In acutely prepared hippocampal slices, frequency and amplitude of mEPSCs and amplitudes of evoked EPSCs are unaltered. In contrast, short-term synaptic facilitation in response to paired stimuli is reduced by ∼50%. In the presence of EGTA-AM to prevent global increases in free Ca^{2+}, the IM-AA mutation completely blocks short-term synaptic facilitation, indicating that synaptic facilitation by brief, local increases in Ca^{2+} is dependent upon regulation of $Ca_V2.1$ channels by Ca^{2+} sensor proteins. In response to trains of action potentials, synaptic facilitation is reduced in IM-AA synapses in initial stimuli, consistent with results of paired-pulse experiments; however, synaptic depression is also delayed, resulting in sustained increases in amplitudes of later EPSCs during trains of 10 stimuli at 10–20 Hz. Evidently, regulation of $Ca_V2.1$ channels by CaS proteins is required for normal short-term plasticity and normal encoding of information in native hippocampal synapses.

calcium channel | calcium sensor proteins | synaptic facilitation | calmodulin | hippocampus

Modification of synaptic strength in central synapses is highly dependent upon presynaptic activity. The frequency and pattern of presynaptic action potentials regulates the postsynaptic response through diverse forms of short- and long-term plasticity that are specific to individual synapses and depend upon accumulation of intracellular Ca^{2+} (1–4). Presynaptic plasticity regulates neurotransmission by varying the amount of neurotransmitter released by each presynaptic action potential (1–5). P/Q-type Ca^{2+} currents conducted by voltage-gated $Ca_V2.1$ Ca^{2+} channels initiate neurotransmitter release at fast excitatory glutamatergic synapses in the brain (6–9) and regulate short-term presynaptic plasticity (3, 10). These channels exhibit Ca^{2+}-dependent facilitation and inactivation that is mediated by the Ca^{2+} sensor (CaS) protein calmodulin (CaM) bound to a bipartite site in their C-terminal domain composed of an IQ-like motif (IM) and a CaM binding domain (CBD) (11–14). Ca^{2+}-dependent facilitation and inactivation of P/Q-type Ca^{2+} currents correlate with facilitation and rapid depression of synaptic transmission at the Calyx of Held (15–18). Elimination of $Ca_V2.1$ channels by gene deletion prevents facilitation of synaptic transmission at the Calyx of Held (19, 20). Cultured sympathetic ganglion neurons with presynaptic expression of exogenous $Ca_V2.1$ channels harboring mutations in their CaS regulatory site have reduced facilitation and slowed depression of postsynaptic responses because of reduced Ca^{2+}-dependent facilitation and Ca^{2+}-dependent inactivation of $Ca_V2.1$ currents (21). The CaS proteins Ca^{2+}-binding protein 1 (CaBP-1), visinin-like protein-2 (VILIP-2), and neuronal Ca^{2+} sensor-1 (NCS-1) induce different degrees of Ca^{2+}-dependent facilitation and inactivation of channel activity (22–26). Expression of these different CaS proteins with $Ca_V2.1$ channels in cultured sympathetic ganglion neurons results in corresponding bidirectional changes in facilitation and depression of the postsynaptic response (25, 26). Therefore, binding of CaS proteins to $Ca_V2.1$ channels at specific synapses can change the balance of CaS-dependent facilitation and inactivation of $Ca_V2.1$ channels, and determine the outcome of synaptic plasticity (27). Currently, it is not known whether such molecular regulation of $Ca_V2.1$ by CaS proteins induces or modulates synaptic plasticity in native hippocampal synapses.

To understand the functional role of regulation of $Ca_V2.1$ channels by CaS proteins in synaptic plasticity in vivo, we generated knock-in mice with paired alanine substitutions for the isoleucine and methionine residues in the IM motif (IM-AA) in their C-terminal domain. Here we investigated the effects of mutating this CaS regulatory site on hippocampal neurotransmission and synaptic plasticity. This mutation had no effect on basal Ca^{2+} channel function or on basal synaptic transmission. However, we found reduced short-term facilitation in response to paired stimuli in autaptic synapses in hippocampal cultures and in Schaffer collateral (SC)-CA1 synapses in acutely prepared hippocampal slices. Moreover, synaptic facilitation in mutant SC-CA1

Significance

Information processing in the brain is mediated through synaptic connections between neurons, where neurotransmitter molecules released from presynaptic nerve terminals stimulate postsynaptic cells. Strength of synaptic transmission is increased transiently by short-term synaptic facilitation in response to repeated stimulation of nerve fibers. Synaptic transmission is initiated by calcium influx through calcium channels in presynaptic nerve terminals, which are regulated by calcium sensor proteins. We found that genetically modified mice in which we introduced a mutation in the binding site for calcium sensor proteins in presynaptic calcium channels have substantially altered facilitation in hippocampal synapses in response to pairs or trains of repetitive high-frequency stimuli. Our results show that disruption of calcium channel regulation by calcium sensor proteins impairs short-term facilitation in native synapses.

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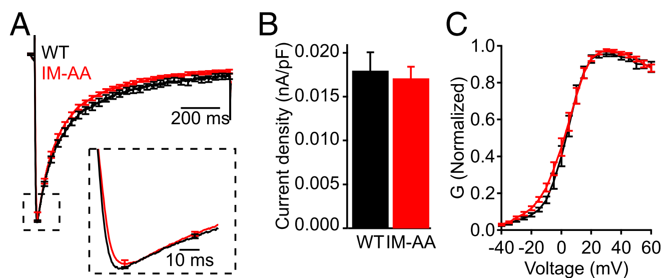

Fig. 1. IM-AA mutation in $Ca_V2.1$ channels does not alter the kinetics of P/Q Ca^{2+} currents in cultured hippocampal neurons. (*A*) Mean-normalized $Ca_V2.1$-mediated I_{Ca} from WT (black, $n = 23$) and IM-AA (red, $n = 27$) evoked by 1-s depolarizing test pulse from −80 to +10 mV. The traces shown are averages ± SEM. Enlarged time scale of the peak $Ca_V2.1$-mediated I_{Ca} is shown in a dashed-line box. (*B*) Average peak $Ca_V2.1$-mediated I_{Ca} current density from WT (black, $n = 23$) and IM-AA (red, $n = 27$). (*C*) Voltage dependence of activation of I_{Ca} from WT (black, $n = 23$) and IM-AA (red, $n = 27$) tail currents measured from a holding potential of −80 mV to the indicated potential. *n* indicates the number of cells recorded in each genotype.

synapses developed and decayed more slowly during trains of stimuli. These results identify a critical role for modulation of $Ca_V2.1$ channels by CaS proteins in short-term synaptic plasticity, which is likely to have important consequences for encoding and transmitting information in the hippocampus.

Results

Short-Term Synaptic Plasticity in Hippocampal Autapses from IM-AA Mice. As a first step in analyzing IM-AA mice, we evaluated the effect of this mutation on endogenous $Ca_V2.1$ channels. We recorded $Ca_V2.1$-mediated P/Q-type Ca^{2+} currents from hippocampal pyramidal cell bodies from WT and IM-AA mice in autaptic single neuron microcultures (28) after blocking currents conducted by Ca_V1, $Ca_V2.2$, and $Ca_V2.3$ channels. We found that P/Q-type Ca^{2+} currents were indistinguishable in neurons from WT and IM-AA mice. The IM-AA mutation did not affect the voltage-dependence of activation, rate of activation, peak current amplitude, or rate of inactivation of the P/Q-type Ca^{2+} current (Fig. 1).

We next examined spontaneous neurotransmitter release at hippocampal autaptic synapses by analyzing spontaneous miniature excitatory postsynaptic currents (mEPSCs) from WT and IM-AA synapses (Fig. 2 *A*–*C*). The amplitude and frequency of mEPSCs was similar in WT and IM-AA synapses. Thus, the IM-AA mutation of endogenous $Ca_V2.1$ channels does not alter spontaneous neurotransmitter release. These results indicate that the presynaptic neurotransmitter release machinery and the postsynaptic response to release of single vesicles of glutamate are unchanged in IM-AA mice.

Superior cervical ganglion neurons that transiently express mutant $Ca_V2.1$/IM-AA channels have reduced synaptic facilitation (21); therefore, we compared short-term synaptic plasticity in hippocampal autaptic synapses from WT and IM-AA mice (Fig. 2 *D*–*F*). Macroscopic EPSCs were evoked by pairs of depolarizing stimuli at interstimulus intervals (ISI) ranging from 50 to 200 ms. We found that IM-AA synapses had significantly reduced paired-pulse ratio (PPR; mean amplitude of the second EPSC/mean amplitude of the first EPSC) compared with WT synapses (Fig. 2 *D* and *F*). WT synapses showed clear facilitation (PPR > 1) at ISI from 50 to 100 ms (Fig. 2*F*), which declined to baseline for longer intervals. In contrast, no significant facilitation was observed for IM-AA synapses (Fig. 2*F*).

Because short-term facilitation is typically inversely related to initial release probability, we measured the amplitude of evoked EPSCs in response to the first stimulus in synapses from WT and IM-AA mice. The IM-AA mutation did not significantly alter the amplitude of the first EPSC response (Fig. 2*E*) ($P = 0.32$). If it had any effect, the trend toward smaller EPSCs observed in these experiments would be expected to increase facilitation, thereby opposing the observed reduction of facilitation by the IM-AA mutation. Hence, the IM-AA mutation in $Ca_V2.1$ channels does not alter basal synaptic transmission, but instead specifically blocks paired-pulse facilitation in hippocampal autaptic synapses.

The IM-AA Mutation Reduces Short-Term Facilitation in Schaffer Collateral-CA1 Synapses. To extend our experiments to a more physiological synapse, we examined synaptic transmission and plasticity in acutely dissected hippocampal slices. We recorded from SC-CA1 synapses that have complex, but well-studied, forms of synaptic plasticity. Spontaneously occurring mEPSCs recorded in the presence of tetrodotoxin were unchanged in frequency and amplitude (Fig. 3). Because spontaneous mEPSCs result from activation of postsynaptic AMPA receptors (AMPAR) by spontaneous presynaptic release of glutamate, these data show that $Ca_V2.1$/IM-AA mutation does not affect the spontaneous release probability, the quantal size of the presynaptic release of glutamate, or the AMPAR-dependent peak of the postsynaptic response at this synapse.

We measured excitatory responses of CA1 pyramidal neurons in response to extracellular stimuli applied to SC fibers. The input–output relationship of evoked EPSCs was not changed in hippocampal slices from IM-AA mice (Fig. 4 *A* and *B*), indicating that basal synaptic transmission has not been altered by the IM-AA mutation. We then examined changes in response to paired-pulse stimuli in the presence of ω-conotoxin GVIA (ω-Ctx) to block any contribution by endogenous $Ca_V2.2$ channels to synaptic transmission at this synapse (6, 9). The percentage of block by ω-Ctx was similar for WT and IM-AA mice, suggesting that the relative contribution of these channels to synaptic transmission is the same (Fig. 4*C*). In addition, we added QX-314 in the recording pipette to block postsynaptic I_{Na} and I_{Ca} through Na_V and Ca_V channels, respectively (29). In this native synapse,

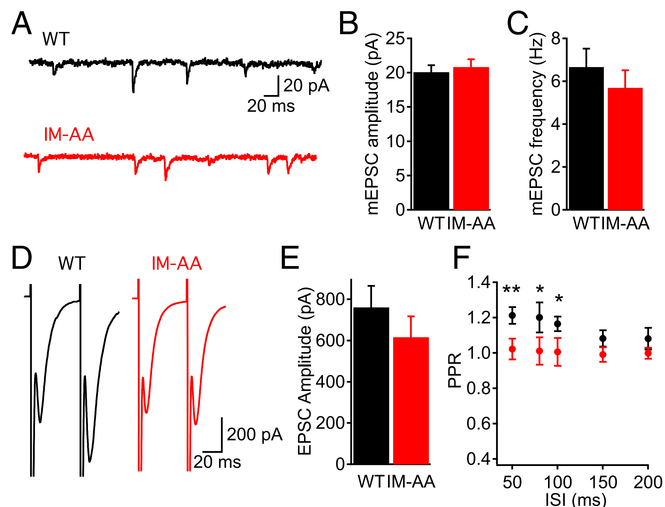

Fig. 2. IM-AA mutation in $Ca_V2.1$ channels abolishes paired-pulse facilitation but does not affect spontaneous neurotransmitter release in cultured hippocampal synapses. (*A*) Example mEPSCs from WT and IM-AA in the presence of 1 μM ω-conotoxin GVIA. (*B*) Average mEPSC amplitude from WT ($n = 36$) and IM-AA ($n = 26$). (*C*) Average mEPSC frequency from WT (black, $n = 36$) and IM-AA (red, $n = 26$). (*D*) Example paired action currents followed by EPSCs from WT (black) and IM-AA (red) hippocampal neurons evoked by 1-ms depolarizing stimuli at a 50-ms ISI in the presence of 1 μM ω-conotoxin GVIA. (*E*) Average amplitude of first EPSC from WT (black, $n = 21$) and IM-AA (red, $n = 20$). (*F*) Average PPR plotted against ISI from WT (black, $n = 21$) and IM-AA (red, $n = 20$). *n* indicates the number of cells recorded in each genotype. $^*P < 0.05$; $^{**}P < 0.01$.

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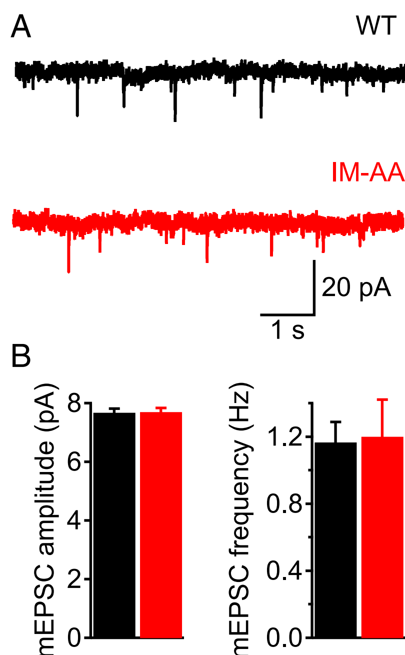

Fig. 3. IM-AA mutation in $Ca_V2.1$ channels does not alter spontaneous release in SC-CA1 synapses. (*A*) Example mEPSCs from WT (black) and IM-AA (red) in the presence of 1 μM ω-conotoxin GVIA and 1 μM tetrodotoxin. (*B*) Average mEPSC amplitude from WT (black, $n = 24$) and IM-AA (red, $n = 34$) (*Left*), and average mEPSC frequency from WT (black, $n = 24$) and IM-AA (red, $n = 34$) (*Right*). *n* indicates the number of 4-min recordings in each genotype.

facilitation in response to paired-pulse stimulation was far more pronounced than in cultured hippocampal synapses, with typical increases in synaptic strength up to threefold (Fig. 4 *D* and *E*). However, SC-CA1 synapses of WT mice had significantly larger PPRs than those of IM-AA mice at ISI from 50 to 100 ms (Fig. 4 *D* and *E*), even though the amplitude of the first EPSC was unchanged between WT and IM-AA (Fig. 4*F*). Approximately half of the synaptic facilitation observed in WT synapses was lost in IM-AA synapses, reducing the PPR from ∼3 to ∼2. In contrast to hippocampal autapses, these results indicate that native SC-CA1 synapses in hippocampal slices have at least two mechanisms that contribute to short-term facilitation. Regulation of $Ca_V2.1$ channels accounts for approximately half of synaptic facilitation, whereas other unknown processes account for the remainder. Nevertheless, in both cultured hippocampal synapses and native SC-CA1 synapses, IM-AA dramatically reduces short-term facilitation measured using the paired-pulse protocol. A similar effect of the mutation is observed despite the differing basal facilitation properties of the two types of synaptic preparations.

The IM-AA Mutation Completely Blocks Facilitation in Response to Rapid, Local Ca^{2+} Transients. To selectively study synaptic facilitation resulting from rapid, local increases in Ca^{2+}, we applied the membrane-permeant chelator EGTA-AM, which enters cells and synapses and is hydrolyzed to EGTA. The released EGTA is a slow Ca^{2+} chelator, which binds entering Ca^{2+} and eliminates global Ca^{2+} increases without affecting rapid, local Ca^{2+} transients (30). Application of EGTA-AM (100 μM) decreased evoked EPSC amplitudes in both WT and IM-AA synapses to a similar extent (Fig. 5 *A* and *B*). These results indicate that the distance and binding rate for Ca^{2+} entering through $Ca_V2.1$ channels to induce exocytosis is unchanged in IM-AA mice. In the presence of EGTA-AM, the IM-AA mutation completely abolished paired-pulse facilitation (Fig. 5*C*). This striking effect of treatment with EGTA-AM indicates that all of the facilitation in response to rapid, local Ca^{2+} transients in SC-CA1 synapses is caused by binding of local Ca^{2+} to CaS proteins bound to $Ca_V2.1$ channels through their IM motif, which results in facilitation of both P/Q-type Ca^{2+} currents and synaptic transmission.

The IM-AA Mutation Alters the Timing of Facilitation and Depression in SC-CA1 Synapses. To further probe the effects of the IM-AA mutation in hippocampal slices, we examined synaptic function in response to trains of action potentials at different frequencies, which more closely mimics the physiological function of these synapses (Fig. 6 *A* and *B*). We found that WT synapses exhibited synaptic facilitation in response to trains of stimuli at all frequencies tested (Fig. 6 *C*–*F*). This facilitation was transient at 10–50 Hz, and decayed toward baseline after four pulses. In contrast, facilitation in IM-AA synapses developed more slowly but did not decay during trains of 10 stimuli at 5, 10, or 20 Hz (Fig. 6 *C*–*E*). At 50 Hz, facilitation in IM-AA synapses decayed prominently, but to a lesser extent than in WT (Fig. 6*F*). Thus, neither facilitation nor depression was completely blocked by the

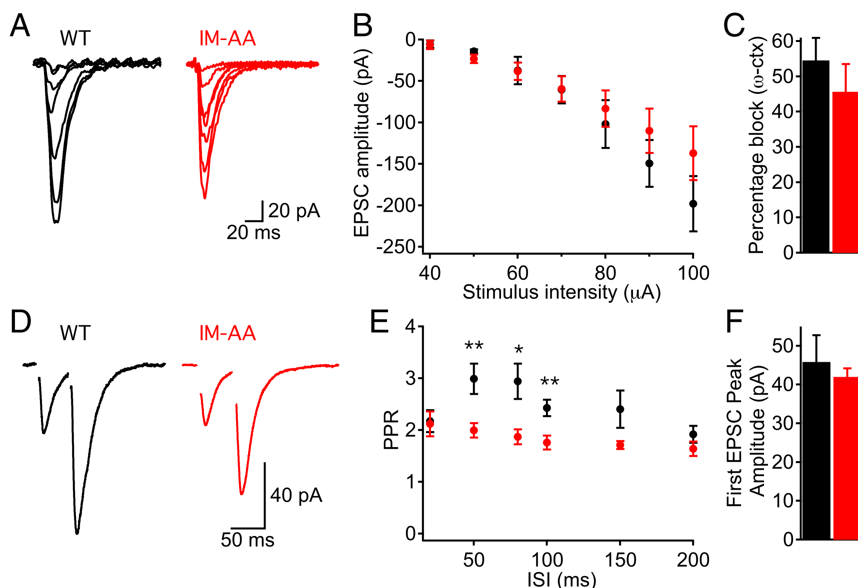

Fig. 4. IM-AA mutation reduces paired-pulse facilitation in SC-CA1 synapses. (*A*) Example evoked EPSCs from WT (black) and IM-AA (red) pyramidal neurons in response to incremental stimulation of SC fibers. (*B*) Average peak amplitude of evoked EPSCs plotted as a function of stimulus intensity from WT (black, $n = 8$) and IM-AA (red, $n = 7$). (*C*) Percentage of evoked synaptic response blocked by application of 1 μM ω-conotoxin GVIA from WT (black, $n = 10$) and IM-AA (red, $n = 9$). (*D*) Example paired EPSCs from WT (black) and IM-AA (red) pyramidal neurons at a 50-ms ISI from WT and IM-AA. Stimulus artifacts were blanked for clarity. (*E*) PPR plotted as a function of ISI from WT (black, $n = 12$) and IM-AA (red, $n = 11$). All recordings were made in the presence of 1 μM ω-conotoxin GVIA, 50 μM APV, 50 μM picrotoxin, and 10 μM CGP55845 hydrochloride. (*F*) Mean amplitudes of EPSCs in response to the first stimulus from WT (black, $n = 12$) and IM-AA (red, $n = 11$). *n* indicates the number of cells recorded in each genotype. $*P < 0.05$; $**P < 0.01$.

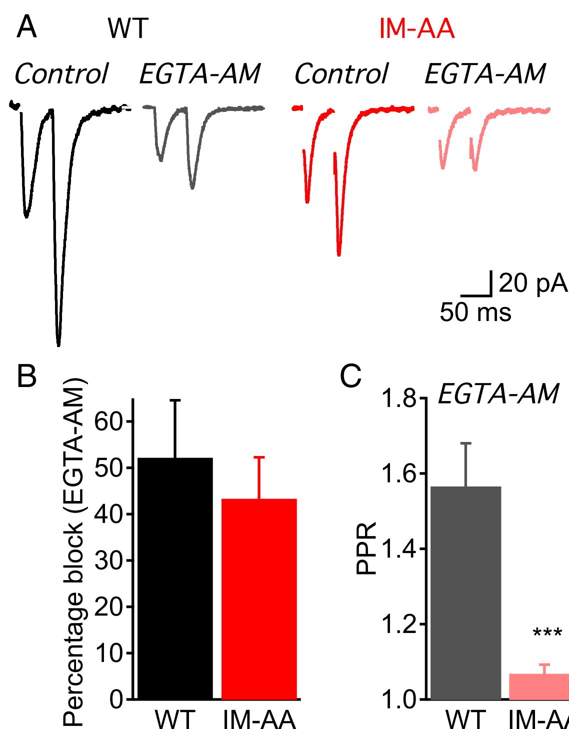

Fig. 5. IM-AA mutation abolishes paired-pulse facilitation in SC-CA1 synapses buffered with the slow Ca^{2+} chelator EGTA. (*A*) Example paired EPSCs from WT (black) and IM-AA (red) pyramidal neurons at a 50-ms ISI in the presence (EGTA-AM) and absence (Control) of the calcium chelator EGTA-AM (100 μM). Stimulus artifacts were erased for clarity. (*B*) Percentage blocked of evoked synaptic responses by application of 100 μM EGTA-AM from WT ($n = 8$) and IM-AA ($n = 8$). (*C*) PPR from WT (gray, $n = 8$) and IM-AA (pink, $n = 8$) in the presence of 100 μM EGTA-AM. All recordings were made in the presence of 1 μM ω-conotoxin GVIA, 50 μM APV, 50 μM picrotoxin, and 10 μM CGP55845 hydrochloride. *n* indicates the number of cells recorded in each genotype. $^{***}P < 0.001$.

IM-AA mutation in response to trains of stimuli; instead, development of these processes was shifted to longer times and higher frequencies. The slower development of facilitation during the initial stimuli in trains is consistent with our results from paired-pulse experiments (Fig. 4), in that facilitation is reduced in response to the second pulse of the trains at 10 Hz and 20 Hz. However, the slower development and longer persistence of facilitation during high-frequency trains of stimulation in IM-AA synapses demonstrate that the IM-AA mutation alters the frequency-dependent encoding of information in response to trains of action potentials in the hippocampus, rather than simply blocking or diminishing short-term facilitation as observed in paired-pulse experiments.

Discussion

Our working hypothesis is that regulation of presynaptic Ca^{2+} channels contributes substantially to short-term synaptic plasticity. A key step in synaptic transmission and short-term presynaptic plasticity is Ca^{2+} influx through $Ca_V2.1$ channels (3, 10, 20, 21, 27). Short-term facilitation and depression of synaptic transmission in transfected neurons relies upon Ca^{2+}-dependent facilitation and inactivation of the P/Q-type Ca^{2+} current through exogenously expressed $Ca_V2.1$ channels, which are mediated by binding of CaM and CaS proteins to the C terminus of $Ca_V2.1$ channels at the IM motif and CBD, respectively (21). Our experiments with IM-AA mice show that CaM/CaS action upon $Ca_V2.1$ channels at the IM motif is a key molecular determinant of short-term plasticity at hippocampal synapses in cell culture and acute hippocampal slices. Our results forge a direct, causal link in native hippocampal synapses between regulation of $Ca_V2.1$ channels at the CaS regulatory site and short-term synaptic plasticity.

Normal Basal Function of IM-AA Synapses. Synaptic transmission depends critically upon the amplitude and timing of the presynaptic Ca^{2+} transient and the response of the synaptic release machinery, including synaptotagmin, soluble NSF attachment protein receptor (SNARE) proteins, and their interacting protein partners, to the entering Ca^{2+}. Our control experiments show that IM-AA synapses have normal basal synaptic transmission. In both autaptic synapses in single neuron microcultures and SC-CA1 synapses in hippocampal slices, the frequency and amplitude of mEPSCs generated by spontaneous release of individual synaptic vesicles are of normal amplitude and frequency. These results indicate that the presynaptic release machinery and the postsynaptic AMPA receptors that respond to released glutamate are unaltered by this mutation. Similarly, in both autaptic synapses and SC-CA1 synapses, the kinetics and amplitude of single EPSCs in IM-AA synapses are unaltered from WT synapses. Because the kinetics and amplitude of single EPSCs are highly sensitive to the rate, amplitude, and localization of Ca^{2+} entry, these results indicate that normal, highly localized Ca^{2+} transients are generated by $Ca_V2.1$ channels in IM-AA synapses, and they eliminate the possibility that compensatory changes in Ca^{2+} buffers or other Ca^{2+} signaling proteins have altered presynaptic Ca^{2+} handling in a way that significantly affects our results. Because the basal function of these synapses is normal by these stringent criteria, IM-AA mice provide an ideal preparation in which to explore the significance of regulation of $Ca_V2.1$ channels by CaS proteins in short-term synaptic plasticity.

Impairment of Synaptic Facilitation in IM-AA Synapses. Based on previous results in transfected superior cervical ganglion neurons, it was expected that the IM-AA mutation would abolish facilitation at native hippocampal synapses when synaptic transmission is controlled by $Ca_V2.1$ channels (21). Consistent with this expectation, the IM-AA mutation completely abolished synaptic facilitation assayed with a paired-pulse protocol in hippocampal autaptic synapses in cell culture. These results support a substantial role for regulation of $Ca_V2.1$ channels by CaS proteins in synaptic facilitation in hippocampal synapses.

Whereas synaptic facilitation was absent in cultured autaptic IM-AA synapses, the IM-AA mutation reduced facilitation at the native SC-CA1 synapse in hippocampal slices by ∼50%. These results also support a substantial role for regulation of $Ca_V2.1$ channels by CaS proteins in this native hippocampal synapse. However, because facilitation was still observed in the SC-CA1 synapse from IM-AA mice, our results indicate that other regulatory mechanisms must also contribute to the larger short-term synaptic facilitation observed in SC-CA1 synapses.

Facilitation of $Ca_V2.1$ channels is mediated by CaM or another CaS protein that is localized near the source of Ca^{2+} entry at the intracellular mouth of the channel, such that facilitation is unaffected by a slow Ca^{2+} chelator like EGTA (30). Even though EGTA has high affinity for Ca^{2+}, its slow rate of Ca^{2+} binding can prevent slow increases in Ca^{2+} at a distance in the range of 100 nm from the source of entering Ca^{2+}, but it cannot buffer fast local increases in Ca^{2+} near the intracellular mouth of the $Ca_V2.1$ channel. To isolate synaptic facilitation that is caused by rapid, local facilitation of $Ca_V2.1$ channels, we studied the effect of EGTA on synaptic facilitation in IM-AA synapses. In IM-AA synapses, EGTA completely prevented facilitation elicited by paired-pulse stimuli. These results indicate that local regulation of $Ca_V2.1$ channels by Ca^{2+} entry and binding to CaM and other CaS proteins is entirely responsible for short-term synaptic plasticity that depends on local Ca^{2+} transients in hippocampal SC-CA1 synapses. The remaining half of synaptic facilitation in IM-AA synapses evidently depends on more global changes in Ca^{2+} concentration that cause facilitation because of changes in endogenous Ca^{2+} buffers, regulation of SNARE protein function,

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Fig. 6. IM-AA mutation shifts the timing of facilitation and depression during high-frequency trains in SC-CA1 synapses. (*A* and *B*) Examples of WT (*A*, black) and IM-AA (*B*, red) EPSCs recorded during a 20-Hz stimulus train. The shaded gray (WT) and red (IM-AA) areas represent asynchronous release (31). Stimulus artifacts were blanked for clarity. (*C*–*F*) Average normalized peak amplitude of evoked EPSCs during trains from WT (black) and IM-AA (red). (*C*) 5 Hz: WT, $n = 13$; IM-AA, $n = 9$. (*D*) 10 Hz: WT, $n = 13$; IM-AA, $n = 10$. (*E*) 20 Hz: WT, $n = 14$; IM-AA, $n = 16$. (*F*) 50 Hz: WT, $n = 9$; IM-AA, $n = 9$. All recordings were made in the presence of 1 μM ω-conotoxin GVIA, 50 μM APV, 50 μM picrotoxin, and 10 μM CGP55845 hydrochloride. *n* indicates the number of cells recorded in each genotype. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

or docking synaptic vesicles closer to $Ca_V2.1$ channels (1, 3, 4, 31–36).

Altered Timing and Pattern of Short-Term Synaptic Plasticity in IM-AA Synapses. At the SC-CA1 synapse in hippocampal slices from IM-AA mice, the remaining short-term synaptic plasticity exhibited striking changes in timing and temporal pattern. During trains of stimuli, facilitation in IM-AA synapses develops slowly but is unusually prolonged. The rapid phase of synaptic depression in IM-AA synapses requires higher-frequency stimulation to be evident, and it is delayed when it occurs. These effects on the timing of synaptic facilitation and depression would be expected to have important consequences for synaptic encoding of information in the frequency and pattern of action-potential trains (2, 37). Indeed, the frequency range in which these changes in short-term synaptic plasticity occur overlaps the frequencies of γ and θ rhythms that are critical for information processing and transmission in the hippocampus. These alterations in timing of the two phases of synaptic plasticity may arise through effects of the IM-AA mutation on $Ca_V2.1$ channel facilitation and inactivation. The opposing actions of CaS proteins that enhance synaptic facilitation like CaM, VILIP-2, and NCS-1 (11, 12, 24, 26, 38) versus those that oppose facilitation and favor depression, like CaBP1 (22, 39), would all be altered by the IM-AA mutation, leading to the new balance of facilitation and depression of synaptic transmission observed in our studies. Considering the importance of the timing and pattern of postsynaptic responses during trains of stimulation for information processing in the hippocampus, it will be of great interest to study spatial learning and memory in IM-AA mice.

Materials and Methods

Animals. Mice with a mutation in the IM motif of $Ca_V2.1$ channels, in which Ile1913-Met1914 were changed to Ala1913-Ala1914 via conversion of the nucleotide sequence from ATCATG to GCCGCT, were generated by Ingenious Targeting Laboratory (Ronkonkoma, NY). The mutation (within exon 40) was generated by PCR mutagenesis and confirmed by sequencing. Traditional blastocyst injection of ES cells expressing the targeting vector resulted in chimeric mice. These chimeric mice were mated first to generate heterozygotes, which were then back-crossed for 10 generations with C57BL/6J to generate homozygous IM-AA mutant mice in a pure genetic background. All experiments were performed according to the guidelines for the care and use of animals approved by the Animal Care and Use Committee at the University of Washington.

Electrophysiology in Cultures of Dissociated Hippocampal Neurons. All experiments were performed in the presence of 1 μM ω-conotoxin GVIA to block N-type Ca^{2+} currents, 3 μM nimodipine to block L-type Ca^{2+} currents, and 400 μM SNX-482 to block R-type Ca^{2+} currents. mEPSCs were recorded at −60 mV in the presence of 1 μM tetrodotoxin in hippocampal neurons cultured on microislands after 17–21 d in vitro (28). mEPSCs were recorded using an extracellular solution containing: 119 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 30 mM glucose, 20 mM Hepes, and 1 μM glycine; the intracellular solution pipettes contained 148.5 mM K-gluconate, 9 mM NaCl,

1 mM $MgCl_2$, 10 mM Hepes, and 0.2 mM EGTA. To elicit evoked EPSCs, the membrane potential was held at −60 mV, and synaptic responses were evoked by a 1-ms depolarizing step from −60 mV to +30 mV to trigger unclamped "action currents." Ca^{2+} currents were recorded from single neurons under whole-cell voltage clamp with a step-pulse depolarization from −80 mV to +10 mV using a P/4 protocol and filtered at 2.9 kHz. Experiments were performed using an EPC10 patch clamp amplifier controlled by PULSE software (HEKA Elektronik). The internal solution contained: 30 mM CsCl, 20 mM TEA Cl, 50 mM Cs methanesulfonate, 0.2 mM EGTA, 30 mM Hepes, 1 mM $MgCl_2$, 5 mM Tris-ATP, 10 mM Tris phosphocreatine, 0.3 mM NaGTP (pH 7.2, 295 mOsm). The external solution for recording of whole-cell Ca^{2+} currents was the same as that used for recording EPSCs but was supplemented with 1 μM tetrodotoxin to block voltage-gated Na^+ channels. Series resistance was monitored, and only cells with stable series resistance were used for data analysis. Series resistance was compensated 75–85%. All averaged data represent the mean ± SEM. Statistical significance was determined using Student's *t* test.

Electrophysiology in Hippocampal Slices. Wild-type and IM-AA mice 16- to 21-d-old were anesthetized with isoflurane. Brains were rapidly removed and placed in ice-cold, high sucrose cutting solution containing: 75 mM sucrose, 25 mM $NaHCO_3$, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 87 mM NaCl, 7 mM $MgCl_2$, and 0.5 mM $CaCl_2$. Acute transverse hippocampal slices (400 μm) were cut on a 1,000 Plus Vibratome in the high-sucrose cutting solution, and transferred immediately to an incubation chamber containing artificial cerebrospinal fluid, composed of: 125 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose, saturated with 95% (vol/vol) O_2 and 5% (vol/vol) CO_2. The slices were allowed to recover at 37 °C for 45 min and then were maintained at room temperature for at least 30 min before recording.

Slices were transferred to a submerged recording chamber mounted on a Nikon microscope (E600FN) equipped for infrared differential interference contrast microscopy and were perfused with artificial cerebrospinal fluid at a rate of 1.5 mL/min at room temperature. All experiments were performed in the presence of the $GABA_A$ receptor blocker picrotoxin (50 μM), $GABA_B$ blocker CGP55845 hydrochloride (10 μM), and $Ca_V2.2$ channel blocker ω-conotoxin GVIA. In addition, a cut was made between CA1 and CA3 to prevent the propagation of epileptiform activity. Evoked postsynaptic responses were recorded from CA1 pyramidal cells, which were visualized by infrared differential interference contrast. EPSCs were induced by stimulating SCs in the stratum radiatum by a concentric bipolar stimulating electrode (FHC). Whole-cell recording pipettes (4–6 MΩ) were filled with a solution containing: 145 mM Cs-gluconate, 2 mM $MgCl_2$, 10 mM Hepes, 0.5 mM EGTA, 2 mM Tris-ATP, 0.2 mM Na_2GTP, and 5 mM QX-314. Data were collected with a MultiClamp 700A amplifier (Axon Instruments), filtered at 2 kHz, and digitized at 10 kHz. Multiple-step depolarizations were given at the beginning of every experiment to induce block of Na^+ and Ca^{2+} currents in the CA1 pyramidal cells by QX-314. Cells were held at −60 mV to record AMPAR EPSCs in the presence of the NMDA blocker AP-V (100 μM). PPRs were recorded using intervals of 20, 50, 80, 100, 150, and 200 ms. We also recorded evoked AMPA-mediated currents in response to trains at different frequencies (5, 10, 20, 50 Hz) and quantified the normalized synchronous release. We also recorded mEPSCs at −60 mV in the presence of 1 μM tetrodotoxin. The detection threshold for mEPSCs was set at 7 pA. The averaged mEPSC amplitude and frequency for each cell was calculated by collecting all mEPSCs recorded during the initial 4-min period after whole-cell access was obtained.

Data were analyzed using Clampfit (Axon) and Igor Pro software (Wavemetrics). All data are presented as means ± SEMs. Statistical significance was calculated using a Student's *t* test.

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