Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 L-type calcium channels operate in a similar voltage range but show different coupling to Ca\textsuperscript{2+}-dependent conductances in hippocampal neurons

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Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 L-type calcium channels operate in a similar voltage range but show different coupling to Ca\textsuperscript{2+}-dependent conductances in hippocampal neurons. Am J Physiol Cell Physiol 306: C1200–C1213, 2014. First published April 24, 2014; doi:10.1152/ajpcell.00329.2013.—In the central nervous system, L-type voltage-gated calcium channels (LTCCs) come in two isoforms, namely Cav1.2 and Cav1.3 channels. It has been shown previously that these channels differ in biophysical properties, in subcellular localization, and in the coupling to the gene transcription machinery. In previous work on rat hippocampal neurons we have identified an excitatory cation conductance and an inhibitory potassium conductance as important LTCC coupling partners. Notably, a stimulus-dependent interplay of LTCC-mediated Ca\textsuperscript{2+} influx and activation of these Ca\textsuperscript{2+}-dependent conductances was found to give rise to characteristic voltage responses. However, the contribution of Cav1.2 and Ca\textsubscript{v}1.3 to these voltage responses remained unknown. Hence, the relative contribution of the LTCC isoforms therein was the focus of the current study on hippocampal neurons derived from genetically modified mice, which either lack a LTCC isoform (Ca\textsubscript{v}1.3 knockout mice) or express a dihydropyridine-insensitive LTCC isoform (Ca\textsubscript{v}1.2DHP\textsuperscript{−} knockin mice). We identified common and alternate ion channel couplings of Cav1.2 and Ca\textsubscript{v}1.3 channels. Whereas hyperpolarizing Ca\textsuperscript{2+}-dependent conductances were coupled to both Cav1.2 and Ca\textsubscript{v}1.3 channels, an afterdepolarizing potential was only induced by the activity of Cav1.2 channels. Unexpectedly, the activity of Cav1.2 channels was found at relatively hyperpolarized membrane voltages. Our data add important information about the differences between Cav1.2 and Ca\textsubscript{v}1.3 channels that furthers our understanding of the physiological and pathophysiological neuronal roles of these calcium channels. Moreover, our findings suggest that Ca\textsubscript{v}1.3 knockout mice together with Ca\textsubscript{v}1.2DHP\textsuperscript{−} knockin mice provide valuable models for future investigation of hippocampal LTCC-dependent afterdepolarizations.

knockout; dihydropyridine; voltage-gated calcium channel; gene deletion

L-TYPE VOLTAGE-GATED CALCIUM channel (LTCC)-targeting drugs have a long history in the treatment of cardiovascular diseases. However, it emerged from studies published within about the last decade that LTCCs also play important roles in higher brain functions such as learning and memory, fear conditioning, as well as mood and addictive behavior (9, 11, 15, 38, 50, 51, 54). In addition, evidence accumulated that malfunction of LTCCs may be crucially involved in various neurological diseases such as bipolar disorder, autism, age-dependent memory deficits, Morbus Alzheimer, Morbus Parkinson, and epilepsy (1, 2, 3, 7, 30, 36, 59, 61). What is more, there is now evidence that the implication of LTCCs in these pathogenic conditions may be specifically due to one of the two brain LTCC isoforms, e.g., Ca\textsubscript{v}1.3 channels in Parkinson’s disease and Ca\textsubscript{v}1.2 in Alzheimer’s disease, bipolar-disorder, and autism, respectively (2, 36, 61). Therefore, isoform specific blockers of LTCCs are currently being developed for therapeutic applications (29). To shed light on the pathological mechanisms of LTCC malfunction, it is necessary to obtain a deeper understanding of the functional differences between Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 channels.

On the cellular level, the neuronal role of LTCCs has been demonstrated to lie in gene regulation (e.g., excitation-transcription coupling), synaptic plasticity (e.g., long-term potentiation), differentiation, and control of electrical excitability (10, 38, 40, 60). Differences between Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 have been identified with respect to biophysical properties and subcellular localization (32), but both LTCC isoforms appear to be involved in the above-mentioned functions, although isoform-specific properties were identified with respect to the coupling to the gene transcription machinery (65, 74).

In our previous work we focused on LTCCs in hippocampal neurons to address physiological details of these channels in excitability (18). We identified an excitatory cation conductance and an inhibitory potassium conductance as important LTCC coupling partners. Notably, a stimulus-dependent interplay of LTCC-mediated Ca\textsuperscript{2+} influx and activation of these Ca\textsuperscript{2+}-dependent conductances gives rise to characteristic voltage responses. In addition to current-induced depolarizations in tetrodotoxin-silenced neurons, we could also provide evidence that both couplings play a role in normal (18) and abnormal discharge activities (49). However, the contribution of the two central nervous system LTCC isoforms Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 to the coupling-dependent voltage responses has not been addressed so far. Hence, this was the focus of the current study on hippocampal neurons derived from genetically modified mice, which either lack an LTCC isoform [Ca\textsubscript{v}1.3-knockout mice (46)] or express a dihydropyridine-insensitive LTCC isoform variant [Ca\textsubscript{v}1.2DHP\textsuperscript{−} knockin mice (24); also known as Ca\textsubscript{v}1.2DHP\textsuperscript{−}/Ca\textsubscript{v}1.3-knockout mice (54)]. We provide evidence that the two LTCC isoforms have alternate coupling to Ca\textsuperscript{2+}-dependent conductances in hippocampal neurons, whereas the voltage range of operation seems less differential than commonly thought. Our data add to the current knowledge of the respective roles of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 channels in neuronal functions, which is of considerable importance in the light of ongoing
assiduous efforts to employ isoform-specific LTCC modulators in neurological therapy (2, 28, 29, 71).

MATERIALS AND METHODS

Origin of wild-type and genetically modified mice strains. C57Bl/6N mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Ca\(_{\text{1.3}}^{\text{−/−}}\) mice (46) and Ca\(_{\text{1.2DHP}}^{\text{−/−}}\) mice (54) were obtained from Joerg Striessnig (Department of Pharmacology and Toxicology, University of Innsbruck, Austria). Before the start of the investigations, mice were sanitized by embryo transfer at the Division for Laboratory Animal Science and Genetics of the Medical University of Vienna (Himberg, Austria). Mice breeding pairs were kept in the in-house laboratory animal facility and were checked daily for litter. Newborn mice were used immediately after birth, because long-term culture of primary hippocampal neurons requires isolation of the cells at this early stage. This ruled out the use of conditional knockout models (e.g., Refs. 38, 66), which have been developed to circumvent the lethality of CNCA1C gene deletion.

Since our approach is based on the use of dihydropyridine-type LTCC modulators (18), Ca\(_{\text{1.2DHP}}^{\text{−/−}}\) mice represented a suitable alternative experimental model. In the modified channel, a threonine to tyrosine mutation in segment IIIS5 of the channel protein eliminates high dihydropyridine sensitivity through a steric mechanism, see Ref. 24 and Drugs.

Cell culture of primary hippocampal neurons. Hippocampi were dissected after the mice were euthanized by decapitation. Genotypes of hippocampal neurons were prepared in the same manner as described previously (18). Hence, all experiments were performed ex vivo. The keeping and killing of the animals was done in full accordance with all rules of the Austrian animal protection law and the Austrian animal experiment law.

Electrophysiology. Perforated patch measurements were performed using 500 μg/ml amphotericin B (from Streptomyces sp., compound purchased from Sigma-Aldrich, Vienna, Austria) added to the pipette solution which contained the following (in mM): 120 potassium gluconate, 1.5 sodium gluconate, 3.5 NaCl, 1.5 CaCl\(_2\), 0.25 MgCl\(_2\), 10 HEPES, 10 glucose, and 5 EGTA. pH was adjusted to 7.3 by KOH. Experiments were started only after the series resistance had dropped to the lowest achievable level (e.g., to between 20 and 30 MΩ), which usually occurred within 15–30 min. Membrane voltage was recorded using a Multiclamp 700B amplifier (Axon Instruments) in the current clamp mode exactly as described by us previously with the same electrodes as well as pipette and external solutions (49). To assure that

![Fig. 1. L-type voltage-gated calcium channel (LTCC)-mediated voltage responses in mouse hippocampal neurons.](http://ajpcell.physiology.org/)
only viable cells were used, the following inclusion criteria had to be met: a membrane voltage of at least $-50$ mV and the capability of generating overshooting action potentials, which was always tested before the recordings. Experiments were performed at room temperature, and cells were superfused continuously with external solution containing the following (in mM): 140 NaCl, 3 KCl, 2 CaCl$_2$, 2 MgCl$_2$, 10 HEPES, and 20 glucose (pH was adjusted to 7.4 by NaOH). LTCC activity was modulated by application of the dihydropyridines isradipine (LTCC antagonist) and Bay K8644 (BayK; LTCC agonist), both at 3 µM in all experiments. Activation of LTCCs was provoked by incremental current injections (typically 5 injections of equally increasing amplitude, e.g., injection 1 of 75 pA to injection 5 of 375 pA in a typical experiment, separated by 30-s intervals) to depolarize the neurons experimentally beyond the LTCC activation threshold. Duration of current injection was 8 s in most of the experiments, but pulse duration was varied in some experiments, as indicated. Unless stated otherwise, the recordings were made in the presence of 500 nM tetrodotoxin (TTX) in the external solution. As in earlier work of our group (18, 49), bona fide LTCC effects were identified as being inhibited by isradipine and augmented by BayK. In neurons of all strains, the dihydropyridine-type modulators did not affect the resting membrane potential [e.g., $V_m = -68.8 \pm 5.5$ mV (means ± SD) in dimethyl sulfoxide (DMSO; control, see Drugs), $-68.5 \pm 6.0$ mV in BayK, and $-69.7 \pm 7.4$ mV in isradipine, as determined from 28 wild-type neurons used in this study]. Membrane resistance close to the resting potential also remained unaffected, which enabled onset analysis of LTCC-mediated active voltage responses.

**Drugs.** BayK, DMSO, isradipine, and bulk chemicals were purchased from Sigma-Aldrich. Since some of these drugs were dissolved in DMSO, the concentration of this solvent was kept constant at 0.3% in all solutions. Control solution contained 0.3% DMSO only, whereas DMSO-soluble compounds were diluted from concentrated stock solutions so as to obtain the same final concentration of DMSO.

In the majority of the experiments, BayK and isradipine were used at lower micromolar concentrations (e.g., 3 µM). It has been demonstrated (54) that in this concentration range, Ca$_{v,1.2}$DHP$^{-/-}$ channels are not potentiated by BayK but can be partially inhibited by isradipine (this difference in residual sensitivity may be due to the more complex structural requirements that are required for effective stimulation by dihydropyridine agonists than for the action of antagonists, see Ref. 64). In our experiments, 3 µM isradipine typically sufficed to eliminate distinct active voltage responses; only responses that are presumably caused by pronounced LTCC-mediated Ca$^{2+}$-elevations, e.g., sag responses (18), were noted in some instances to persist in the presence of isradipine in Ca$_{v,1.2}$DHP$^{-/-}$ neurons (data not shown).

**Data analysis and statistics.** Afterpotentials were quantified by measuring the area (mV·ms) between the baseline (= membrane voltage before the current injection) and the recorded trace from the end of the current injection to the repolarization back to the baseline level. GraphPad Prism version 5.03 was used for preparation of the graphs (all data are represented as means ± SE, unless otherwise stated) and for statistical analysis, which was performed using Kruskal-Wallis one-way ANOVA with Dunn’s multiple comparison post hoc test, and with Mann Whitney test for the data shown in Fig. 10C.

**RESULTS**

LTCC activity leads to both depolarizing and hyperpolarizing voltage responses. To investigate the effect of LTCC activation on membrane voltage, we depolarized mouse hippocampal neurons in the presence of 500 nM TTX by 8-s long current injections. Depolarization was induced by five successive current injections of increasing amplitude (injections 1 to 5 as described in MATERIALS AND METHODS), so as to test for active responses at various levels of depolarization, between the resting membrane potential and about $-20$ mV. The

![Fig. 2. LTCC-mediated voltage responses in Ca$_{v,1.3}^{-/-}$ neurons. Overlays of voltage traces as in Fig. 1 (see there for a description of the labeling) but from different Ca$_{v,1.3}^{-/-}$ neurons recorded in the presence of isra (grey traces) and either DMSO (A, black traces) or BayK (B, black traces). Arrowheads indicate afterpotentials.](http://ajpcell.physiology.org/)

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recordings (n was at least 36 for each mouse strain) were made in the presence of DMSO and after modulation of the activity of LTCCs by addition of BayK (LTCC agonist) and isradipine (antagonist), respectively. Afterwards, overlays of responses obtained in the absence of LTCC activity (i.e., in the presence of isradipine, “isra”-labeled traces) with responses recorded when only solvent (DMSO traces) or when BayK were present (BayK traces) were generated to test for LTCC-mediated changes in the voltage responses. In all but 1 out of 36 neurons derived from wild-type neurons, LTCC-mediated responses were detected. Despite quantitative differences, qualitatively two major types of responses could be identified and were termed according to their appearance as either “bumps” or as “hyperpolarizing sags”: the term bump was used to describe responses where depolarizations rose above the response elicited in the presence of isradipine, irrespective of the magnitude of the difference between the two traces; the term hyperpolarizing sag was used to describe responses that, after an initial rise above the trace recorded in isradipine, hyperpolarize already during the pulse towards or even below the response elicited in the presence of the LTCC inhibitor so that the voltage response declines to or even traverses the isradipine trace in the overlays. In some neurons, the LTCC-mediated response was accompanied by oscillatory activity of variable frequency and amplitude. Figure 1 depicts prototypical examples recorded from different neurons. The exemplary traces also illustrate that bump responses were typically followed by afterdepolarizations (ADPs), whereas sag responses were typically followed by a distinct afterhyperpolarization (AHP) (these afterpotentials are marked with arrowheads in Fig. 1). The response modes could be identified before potentiation of LTCCs by BayK (see Fig. 1A, overlays of DMSO and isradipine traces) but application of BayK pronounced their appearance (Fig. 1B, overlays of BayK and isradipine traces). The bump response was seen in 44% of the neurons (16 out of 36) where it typically occurred at the lowest levels of depolarization (e.g., current injections 1 or 2, as indicated by the bold current pulse below the traces in Fig. 1A). Hyperpolarizing sags appeared when the neurons were depolarized to −35 mV or above (e.g., in current injections 4 and 5) in 75% of the neurons (27 out of 36). Oscillatory activity occurred at intermediate levels of depolarization, where afterpotentials were typically missing or small (see Fig. 1, Aii and Bii).

These LTCC-mediated response modes were identical to the ones described by us previously in rat hippocampal neurons (18). In this earlier study, we characterized the underlying conductances and provided evidence that the excitatory “bump” response involves not only LTCC-mediated Ca2+ influx but also a coupling to Ca2+-dependent sodium-permeable channels (e.g., CAN channels). On the other hand, the hyperpolarizing sag response was shown to involve activation of Ca2+-dependent potassium channels (e.g., apamin-sensitive KCa channels). Figure 1C schematically illustrates the respective ion fluxes and channel coupling that may underlie the two response modes.

Both LTCC-dependent response modes persist in CaV1.3−/− neurons. To gain insight into the involvement of specific LTCC isoforms, we performed identical experiments as above...
on hippocampal neurons derived from Ca$_{v}$.1.3$^{-/-}$ mice. Again, all types of responses could be identified, both under control conditions (Fig. 2A) and in the presence of BayK (Fig. 2B). As in wild-type neurons, bumps were followed by ADPs, whereas hyperpolarizing sags were accompanied by AHPs (marked by arrowheads in Fig. 2). Again, the bump response typically occurred at the lowest levels of depolarization (e.g., current injections 1 or 2, as indicated by the bold current pulse below the traces) and was seen in 70% of Ca$_{v}$.1.3$^{-/-}$ neurons (32 out of 46). Responses were occasionally associated with oscillatory activity as the level of depolarization was increased. Hyperpolarizing sags appeared when the neurons were depolarized to $\sim$35 mV or above (e.g., in current injections 4 and 5) and where seen in 43% of Ca$_{v}$.1.3$^{-/-}$ neurons (20 out of 46).

Both LTCC-dependent response modes can be activated by BayK in Ca$_{v}$.1.2DHP$^{-/-}$ neurons. The presence of bumps and hyperpolarizing sags as well as ADPs and AHPs in Ca$_{v}$.1.3$^{-/-}$ neurons suggests that these LTCC-induced voltage effects were all mediated by Ca$_{v}$.1.2 channels. To explore this possibility, we used neurons derived from knockin mice, which express a Ca$_{v}$.1.2 channel mutant that lacks high dihydropyridine sensitivity (Ca$_{v}$.1.2DHP$^{-/-}$ neurons, see MATERIALS AND METHODS) and took advantage of the pronounciation of the LTCC responses upon application of the channel agonist BayK. Because Ca$_{v}$.1.2DHP$^{-/-}$ channels cannot be potentiated by BayK even in the low micromolar range (see Drugs), BayK-induced effects on voltage can only be due to Ca$_{v}$.1.3 channel activity. On the other hand, 3 μM isradipine is a concentration high enough to inhibit a considerable fraction of Ca$_{v}$.1.2DHP$^{-/-}$ channels (54) and was thus again used for reference recordings. As expected, both bumps and hyperpolarizing sags could be identified under control conditions (Fig. 3A). However, in the presence of BayK LTCC-mediated voltage responses appeared more pronounced even in Ca$_{v}$.1.2DHP$^{-/-}$ neurons, with bumps occurring in $\sim$45% of the neurons (17 out of 38) and hyperpolarizing sags in 71% of the neurons (27 out of 38) (Fig. 3B). This suggests that Ca$_{v}$.1.3 channels can also induce both bumps and sag responses. To corroborate this notion, we made direct comparisons of voltage responses in Ca$_{v}$.1.2DHP$^{-/-}$ neurons evoked before addition of BayK (when only DMSO was present) and after addition of the dihydropyridine agonist. Figure 4 shows examples from three neurons (representative of 10 similar observations) where under control conditions (DMSO) LTCC-mediated voltage responses were absent or very small (note the largely overlapping traces in the three examples in Fig. 4A), so that it can be clearly seen that only BayK induced the characteristic LTCC-mediated voltage responses in this subset of neurons (Fig. 4B). As in wild-type and Ca$_{v}$.1.3$^{-/-}$ neurons, responses were occasionally associated with oscillatory activity as the level of depolarization was increased.

Differences and similarities in the coupling of Ca$_{v}$.1.2 and Ca$_{v}$.1.3 channels to afterpotential-mediating conductances. Taking together the results described so far, it can be concluded that bump and hyperpolarizing sag responses can be induced by Ca$^{2+}$ influx irrespective of whether it occurs through Ca$_{v}$.1.2 or Ca$_{v}$.1.3 channels. According to the data obtained in rat neurons on the mechanisms underlying the two opposing voltage responses (see scheme in Fig. 1C), this would also suggest that both LTCC isoforms couple to depolarizing

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Fig. 4. Ca$_{v}$.1.3-mediated voltage responses. The induction of active voltage response by BayK in Ca$_{v}$.1.2DHP$^{-/-}$ neurons is demonstrated by a comparison of traces recorded in 3 neurons in the presence of solvent (DMSO) only (A) with traces recorded from the same neurons after addition of BayK (B). All responses are shown in overlays with the corresponding trace obtained when isradipine was present. Ai and Bi, Aii and Bii, Aiii and Biii are from the same neuron. Pronounced differences from the isra traces [bump (i), osci (ii), and sag (iii) responses] were only induced when BayK was present, implicating that they were mediated by Ca$_{v}$.1.3 channels.
CAN channels and inhibitory KCa channels. However, closer inspection of the voltage responses recorded in neurons derived from the two genetically modified mouse strains indicated that there are differences in the respective coupling modes of Cav1.2 and Cav1.3 channels: we noted that in the presence of BayK, bump responses are always associated with readily observable ADPs in Cav1.3Δ−/− neurons but not in Cav1.2DHP−/− neurons. Hence, Cav1.2-mediated Ca2+ influx appears to be required for formation of depolarizing afterpotentials. To evaluate this observation we determined the afterpotential area in recordings from neurons of all mouse strains (see MATERIALS AND METHODS for details of the determination). To warrant that only neurons with a discernible LTCC conductance were included, this was performed for recordings with a clear bump response only (Fig. 5A). The result of this analysis is shown in the graph in Fig. 5B. Large depolarizing afterpotential areas were found in both wild-type and Cav1.3Δ−/− neurons, reflecting the occurrence of ADPs as illustrated also in Figs. 1Aii, and 2Bii. Depolarizing afterpotential areas were significantly smaller in Cav1.2DHP−/− neurons, which corresponds to the lack of distinct ADPs in these neurons (see Fig. 3Bii and Fig. 4Bii). In contrast, a similar evaluation of AHPs that accompanied hyperpolarizing sag responses (see Fig. 1, Aiii and Biii, Fig. 2 Aiii and Biii, and Fig. 3, Aiii and Biii) revealed no significant difference among neurons from wild-type and genetically modified mice strains, both under control conditions (n = 10–14) and in the presence of BayK (n = 19; Fig. 6).

Previous work in our laboratory has shown a bimodal activation pattern for ADPs, which appear already with moderate depolarizations but decrease or give way to AHP potentials as the depolarizing stimulus is increased [both in terms of amplitude and duration (18)]. Hence, we used a second, considerably shorter stimulation paradigm (50 to 1,050 ms, as indicated in Fig. 7D, with current injections large enough to depolarize the neurons to at least −35 mV) to test for ADPs in neurons of the three mice strains. As illustrated in Fig. 7, ADPs were readily evoked in wild-type and Cav1.3Δ−/− neurons (Fig. 7, A1–A3 and C1–C3). Again, distinct ADPs were missing in voltage responses recorded from Cav1.2DHP−/− neurons (Fig. 7, B1–B3). The analysis of afterpotential areas in these recordings is summarized in the graph in Fig. 7E (n = 10 for each strain). Besides the significantly smaller afterpotential areas determined in recordings from Cav1.2DHP−/− neurons, it emerges that afterpotential areas in Cav1.3Δ−/− neurons are at least as large as those of ADPs from the wild-type neurons. Hence, our experiments demonstrate that the ADPs are selectively induced by Ca2+ influx via Cav1.2 channels and not by Ca2+ influx via Cav1.3 channels.

Cav1.2 and Cav1.3 channel-mediated response modes show a similar stimulus dependency. As indicated above we have previously reported on a dynamic interplay of the two LTCC

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Fig. 5. Size of ADPs in neurons of wild-type and LTCC-gene modified mice. A: overlays of traces recorded in the presence of BayK and isradipine exemplify pronounced bump responses in neurons of wild-type (left), Cav1.2DHP−/− (middle), and Cav1.3Δ−/− mice (right) that were selected for evaluation of the ADP size. Arrowheads indicate distinct depolarizing afterpotentials. B: average area of ADPs (nV·ms) recorded in the presence of BayK in response to 8 s-long current injections is displayed for neurons from the 3 mouse strains as indicated. Boxed figures indicate the number of experiments. Statistical analysis revealed a significant difference between wild-type and Cav1.2DHP−/− data (*P < 0.05), between Cav1.2DHP−/− and Cav1.3Δ−/− data (**P < 0.01), but not between wild-type and Cav1.3Δ−/− data (n.s., not significant).

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Fig. 6. Size of AHPs in neurons of wild-type and LTCC-gene modified mice. The average area of AHPs (nV·ms) recorded in the presence of DMSO (left) and BayK (right) is displayed for neurons from the 3 mouse strains as indicated. Boxed figures indicate the number of experiments. Statistical analysis did not reveal any significant difference between AHP areas of the 3 mouse strains in both DMSO and BayK (n.s., not significant).
coupling modes giving rise to a stimulus dependency of the response modes in rat hippocampal neurons. We also observed this stimulus dependency in mice neurons: a transition of LTCC-dependent response modes could be identified in control (DMSO) experiments (not shown) but was best seen in the presence of BayK. As for wild type, transition was manifest in 10 neurons (in other neurons it was incomplete in that bumps or sag responses predominated). A representative example is illustrated in Fig. 8, which depicts four responses of the same neuron to consecutive current injections of increasing amplitude (Fig. 8, Ai–Aiv; see the bold current pulse in the pulse protocol below the traces). The change from bump to sag responses was accompanied by a switch from depolarizing to hyperpolarizing afterpotentials. As indicated in Fig. 8, the bump/ADP responses were accompanied by a switch from depolarizing to hyperpolarizing afterpotentials. As indicated in Fig. 8, the bump/ADP responses were evoked with the smallest level of depolarization (e.g., current injection 1 or 2, see the bold current pulse below the traces in Ai). In contrast, sag/AHP responses were prominent at the highest levels of depolarization (Fig. 8Aiv). The schematic drawings in B indicate the proposed switch from excitatory to inhibitory coupling that may underlie the transition between response modes together with the Ca^{2+} entry routes upon which the couplings are based according to previous (18) and above-mentioned data. However, transition from excitatory to inhibitory response mode was also seen in neurons derived from Ca_{v}1.3^{−/−} neurons and in neurons derived from Ca_{v}1.2DHP^{−/−} mice (Fig. 9). In Ca_{v}1.3^{−/−} neurons, the switch from bump to sag response during the current injection was followed by a decaying ADP that gave way to an AHP as the depolarization was increased (representative of 11 neurons), which was similar to the situation in wild-type neurons. In contrast, in Ca_{v}1.2DHP^{−/−} bumps, that were not followed by ADPs, diminished as the depolarization was increased and turned into hyperpolarizing sags, which were accompanied by AHP potentials (also representative of 11 neurons).

Cav_{1.2} and Cav_{1.3} channels operate in a similar voltage range. In our experiments, we gradually raised $V_{m}$ of the neurons to depolarized voltages. The resting membrane potential of the neurons was not affected by application of BayK or isradipine (see MATERIALS AND METHODS). As for the smallest levels of depolarization, voltage traces also did not differ when recorded under control conditions or after potentiation or block of LTCCs with dihydropyridines. However, at a certain level of depolarization, BayK traces but also DMSO traces started to diverge from the isradipine trace. Close inspection of the point of divergence (in the first out of the 5 pulses in which this occurred) was used for determination of the “onset” of LTCC-mediated voltage responses (see Fig. 10, A and B, for an

**Fig. 7. ADPs induced by brief depolarizing current injections require Ca_{v}1.2 channels.** The traces in A–C depict 3 examples each of voltage responses evoked by a series of brief current injections (duration 50 to 1,050 ms, as indicated in D) that are followed by ADPs in wild-type neurons (A1–A3) and neurons derived from Ca_{v}1.3^{−/−} neurons (C1–C3) but are missing in neurons form Ca_{v}1.2DHP^{−/−} mice (B1–B3). The grey trace in each overlay depicts the response with the largest ADP in the series. In E the average area of the largest ADPs (mV·ms) is displayed for neurons from the 3 mouse strains as indicated. Boxed figures indicate the number of experiments. Statistical analysis revealed a significant difference between wild-type and Ca_{v}1.2DHP^{−/−} data (**$P < 0.01$), between Ca_{v}1.2DHP^{−/−} and Ca_{v}1.3^{−/−} data (**$P < 0.001$), but not between wild-type and Ca_{v}1.3^{−/−} data (n.s., not significant).
DISCUSSION

**Differences in the coupling of Cav1.2 and Cav1.3 channels to Ca^{2+}-dependent conductances.** The response modes previously described in rat primary hippocampal neurons could also be identified in identical form in primary neurons isolated from mouse hippocampus. Moreover, all response modes could be evoked or augmented by BayK in both Cav1.3^-/-^ neurons and in Cav1.2DHP^-/-^ neurons. Earlier (18) we provided evidence that these voltage responses are due to differential coupling of LTCC-mediated Ca^{2+} influx to de (CAN channels)- or hyperpolarizing conductances (e.g., K_{Ca} channels). In Cav1.3^-/-^ neurons, LTCC-mediated Ca^{2+} influx is through Cav1.2 channels, whereas induction by BayK in Cav1.2DHP^-/-^ neurons means that the activating Ca^{2+} influx is provided by influx via Cav1.1 channels. Hence both LTCC isoforms can activate the ion fluxes that underlie the described voltage responses. However, in Cav1.2DHP^-/-^ neurons BayK did not induce the distinct ADPs that were seen routinely in wild-type and in Cav1.3^-/-^ neurons. In contrast, AHPs were evoked or enhanced by BayK in both Cav1.3^-/-^ neurons and Cav1.2DHP^-/-^ neurons, suggesting that both LTCC isoforms have functional coupling to AHP-mediating channels.
the voltage trajectories within the neurons of each strain [compare for example the bumps responses of wild-type neurons shown in Figs. 1Bi, 5A, and 8Ai: a similar variability was seen in hyperpolarizing sag responses (not shown)], which may be due to the fact that we used primary cultures of dissociated neurons and thus sampled the data from all neuronal types and all subregions of the hippocampus. However, our data show that throughout the hippocampus, all major LTCC-mediated response modes can be elicited irrespective of whether Ca\(^{2+}\) influx occurs via Ca\(_{\text{v}}\)1.2 or Ca\(_{\text{v}}\)1.3 channels. Hence, we found no other phenotypic difference than the lack of BayK-induced ADPs in Ca\(_{\text{v}}\)1.2DHP\(^{−/−}\) neurons.

Evidence for isoform-specific roles in the induction of afterpotentials is rare, although there have been reports that AHPs in hippocampal neurons are evoked by Ca\(^{2+}\) influx via Ca\(_{\text{v}}\)1.3 channels rather than Ca\(_{\text{v}}\)1.2 channels (17, 37). This is in contrast to our observation that both channel isoforms can couple to AHP-mediating channels. Probably in certain experimental settings activation of depolarizing Ca\(^{2+}\)-dependent channels by Ca\(_{\text{v}}\)1.2 channel-mediated Ca\(^{2+}\) influx is capable of masking simultaneously occurring coupling to AHP-mediating channels. Alternatively, only loss of Ca\(_{\text{v}}\)1.3 channels may enable effective coupling of Ca\(_{\text{v}}\)1.2 channels to these channels. At present we cannot distinguish between these two possibilities. Other studies are also inconclusive regarding compensatory changes of Ca\(_{\text{v}}\)1.2 channels. For example, Clark et al. (8) reported on a lack of effect of Ca\(_{\text{v}}\).3 gene deletion on Ca\(_{\text{v}}\)1.2 protein levels in whole brain preparations, whereas Jurkovičová-Tarabová et al. (26) found increased expression levels of Ca\(_{\text{v}}\)1.2 in the lateral superior olive of Ca\(_{\text{v}}\)1.3\(^{−/−}\) mice.

In hippocampal neurons depolarizing effects were also noted for Ca\(_{\text{v}}\)1.2 channel activity by Lacinova et al. 2008 (33) but to date not for Ca\(_{\text{v}}\)1.3 channels. Hence, our study for the first time demonstrates Ca\(_{\text{v}}\)1.2-specific activation of ADP potentials and that AHPs can be evoked by Ca\(^{2+}\) influx through both Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 channels. We also provide evidence that Ca\(_{\text{v}}\)1.3 channels can cause neuronal depolarization in a direct manner that is independent of any coupling to an afterpotential-mediating Ca\(^{2+}\)-dependent conductance (see Relation between Ca\(_{\text{v}}\)1.2- and Ca\(_{\text{v}}\)1.3-mediated voltage responses and afterpotentials).

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Fig. 9. Transition between response modes in Ca\(_{\text{v}}\)1.3\(^{−/−}\) and in Ca\(_{\text{v}}\)1.2DHP\(^{−/−}\) neurons. The overlays illustrate the transition from bump to sag responses in a Ca\(_{\text{v}}\)1.3\(^{−/−}\) neuron (A) and in a Ca\(_{\text{v}}\)1.2DHP\(^{−/−}\) neuron (B) in the same manner as described in Fig. 8 for wild-type neurons. Both illustrations are representative for 11 similar observations.
Nature of the ADP-mediating conductance. With respect to rat hippocampal neurons, we have previously obtained the following data regarding the nature of the conductance underlying the above described ADP (18): 1) it is activated upon Ca\(^{2+}\)-influx via LTCCs, 2) it is due to Na\(^+\) but not Ca\(^{2+}\) ion flux, and 3) it is sensitive to flufenamic acid (\(\approx 100 \mu M\)). Other studies provided evidence pointing to a relation between such afterpotentials and transient receptor potential (TRP) channels: 1) ADPs in central neurons are due to an ADP-generating conductance (\(I_{\text{CAN}}\)), which is carried by Ca\(^{2+}\)-activated monovalent cation current channels (21, 22, 63). 2) TRPM4b and TRPM5 share many of the hallmarks of \(I_{\text{CAN}}\) channels, e.g., single-channel conductances between 20 and 35 pS, Ca\(^{2+}\)-activation, selectivity for monovalent cations, and low permeability to Ca\(^{2+}\). Other than TRPM4b and TRPM5, there are no other channels described with these features (23). 3) TRPM4 and 5 are the only TRP channels shown to be directly gated by increases in intracellular Ca\(^{2+}\) concentration. 4) In contrast to all other TRPs, both TRPM4 and 5 form ion channels permeable for monovalent cations but impermeable to Ca\(^{2+}\) [see the review by Pedersen et al. (42), for 3 and 4]. Hofmann et al. (23), therefore, noted in 2003 that these two TRP channels are currently the only molecular candidates that could account for \(I_{\text{CAN}}\); to our best knowledge, nothing has changed this view in recent years. Hence, TRPM channels can be considered as the most likely candidates for the ion channels that mediate Ca\(^{1,2}\)-dependent ADPs in hippocampal neurons, although the lack of “specific” inhibitors obviously precludes final proof of this notion.

Relation between Ca\(^{1,2}\)- and Ca\(^{1,3}\)-mediated voltage responses and afterpotentials. Previous results, for example, correlation analysis, indicated that bumps involve the same conductance that mediates depolarizing afterpotentials, with ADPs presumably being caused by a protracted deactivation of the channel contributing to the enhanced depolarization during the current injection (18). Here we show that bumps were induced by BayK also in Ca\(^{1,2}\)-DHP receptor (BayK) LTCC channels. Boxed figures indicate the onset of the LTCC-mediated response. C: box plot of onsets determined for neurons derived from the 3 mouse strains under conditions of unaltered (DMSO) and potentiated (BayK) LTCC channels. Boxed figures indicate the number of experiments. Statistical analysis revealed that there is a highly significant difference between onsets recorded in the presence of DMSO and BayK in neurons of all mouse strains (*\(p < 0.01\) and ***\(p < 0.001\)). However, no statistical difference was observed when onsets were compared between mouse strains, and this was true for both control conditions (DMSO) and in the presence of BayK.

Fig. 10. Ca\(^{1,2}\) and Ca\(^{1,3}\) channels operate in an overlapping voltage range. Overlay of traces recorded in the presence of isradipine with those recorded when either solvent (DMSO; left) or BayK (right) was present from Ca\(^{1,3}\)-neurons (A) and Ca\(^{1,2}\)-DHP-/-neurons (B). Responses were evoked with moderate depolarizations that led to bump responses, as shown for wild-type neurons derived from the 3 mouse strains under conditions of unaltered (DMSO) and potentiated (BayK) LTCC channels. Boxed figures indicate the divergence of the 2 overlaid traces, which was taken as an indication of the onset of the LTCC-mediated response.
information processing, for example, providing short-term memory or, by shunting of those excitatory postsynaptic potential that follow the plateau-inducing ones, in establishing low pass filter characteristics (5, 12, 44, 76). Similarly, ADPs were suggested to act in synaptic integration, for example when back-propagating ADP potentials affect coincidentally arriving dendritic synaptic inputs (27, 72). In contrast, the proposed role of an ADP-generating conductance in PDS suggests an involvement in neuropathological processes, because these abnormal electrical events have been suggested to play a role in epileptogenesis (57, 58).

The LTCC-ICAN coupling is one out of several mechanisms how an ADP can be generated, and this kind of ADPs has been linked to bistability in motoneurons of neonatal rats (4), burst firing in dopaminergic neurons (35, 45), and information processing in hippocampal neurons (68). As ADPs in hippocampal neurons were found in this study to depend on the coupling of Ca\textsubscript{1.2} channels to an ADP-generating conductance, this may offer one explanation for the role of Ca\textsubscript{1.2} channels in learning and memory (25, 34, 38, 66). Indeed, in hippocampal CA1 networks Ca\textsubscript{1.2} but not Ca\textsubscript{1.3} channels have been found to enable a N-methyl-D-aspartate receptor-independent form of long-term potentiation (8, 38). In contrast, although proposed earlier on in original articles and textbooks (e.g., Refs. 55, 56), the contribution to PDS has not received experimental proof so far. Hence, the two mouse models identified in this study to have either a prominent coupling of neuronal LTCCs to an ADP-generating conductance (ICAN; Ref. 18), i.e., Ca\textsubscript{1.3\textsuperscript{−/−}} mice, or to lack LTCC-agonist stimulated ADPs, i.e., Ca\textsubscript{1.2DHP\textsuperscript{−/−}} mice, can be envisaged to greatly aid in future analysis of the physiological and pathophysiological implications of LTCC-induced ADPs.

However, long-lasting depolarizations may also arise from activation of Ca\textsubscript{1.3} channels, because these LTCC subtype was shown to have a lower tendency to inactivate than Ca\textsubscript{1.2} channels and may carry significant window current (60, 70). Indeed, LTCC-mediated plateau potentials that were solely attributed to Ca\textsubscript{1.3} channels have been described previously (43, 52). Here we observed Ca\textsubscript{1.3}-mediated long lasting depolarizations in rat hippocampal neurons, i.e., BayK-induced bump responses in Ca\textsubscript{1.2DHP\textsuperscript{−/−}} neurons. Hence, similar functions (for example generation of plateau potentials) may be carried out by both Ca\textsubscript{1.2} and Ca\textsubscript{1.3} channels, yet with differing underlying mechanisms. It is possible that such mechanistical differences endow plateau potentials with different dependences on cytoplasmic calcium or membrane voltage or with different “off” kinetics. Note that Ca\textsubscript{1.3}-mediated long-lasting depolarizations observed in this study, i.e., BayK-induced bump responses in Ca\textsubscript{1.2DHP\textsuperscript{−/−}} neurons, were not accompanied by ADP potentials, which indicates that Ca\textsuperscript{2+} influx was rapidly terminated after switching off the current injection. In contrast, the Ca\textsubscript{1.2}-CAN coupling only terminates with a significant delay (thus giving rise to the ADPs), a feature that is most likely related to the time course of the intracellular Ca\textsuperscript{2+} elevation.

Ca\textsubscript{1.2} and Ca\textsubscript{1.3} operate in a similar voltage range. The onset of LTCC activity in Ca\textsubscript{1.3\textsuperscript{−/−}} neurons, which must be due to activation of Ca\textsubscript{1.2} channels, occurred at potentials negative to −50 mV. This is not significantly different from BayK-induced responses in Ca\textsubscript{1.2DHP\textsuperscript{−/−}} neurons, which are mediated by Ca\textsubscript{1.3} channels. Notably, the onset of LTCC activity in Ca\textsubscript{1.3\textsuperscript{−/−}} neurons occurred at potentials more negative than the relatively depolarized threshold of activation currently thought to characterize Ca\textsubscript{1.2} channels. In heterologous expression systems, several studies indicated considerable differences between neuronal Ca\textsubscript{1.2} and Ca\textsubscript{1.3} channels in their voltage dependence of activation [see, for example, Ref. 67 (human neuronal alpha1D) and Ref. 62 (rat brain alpha 1C), discussed by Carlin et al. (6)]. Such observations were supported by direct comparison in the laboratories of J. Striessnig (31) and D. Lipscombe (70), with Ca\textsubscript{1.3} channels [from human pancreas (J. Striessnig) and rat peripheral neurons (D. Lipscombe, respectively)] being activated at considerably less depolarized potentials than rabbit cardiac Ca\textsubscript{1.2} channels (both studies). This appears at odds with our finding that central Ca\textsubscript{1.2} and Ca\textsubscript{1.3} channels operate in the same negative voltage range in hippocampal neurons. It should be noted that the biophysical properties of LTCCs are subject to modulation by transcriptional (e.g., splice variation) and posttranslational (e.g., phosphorylation) mechanisms and may also depend on the exact composition of the calcium channel complex, e.g., association with auxiliary subunits and regulatory proteins (14, 20, 53, 73). Hence, it is possible that while activation by moderate depolarizations is an intrinsic property of Ca\textsubscript{1.3} channels, Ca\textsubscript{1.2} channels may be tuned to operate in a comparable voltage range by regulatory mechanisms.

Our observation of Ca\textsubscript{1.2} channel activity at unexpectedly negative voltages is in line with a recent report by Radzicki et al. (47), who showed that Ca\textsubscript{1.2} channels were active at hyperpolarized potentials in hippocampal slices (e.g., at potentials negative to −50 mV), although in their study activation required elevation of the ambient temperature. Moreover, although not specifically noted by the authors, unrelated work on Ca\textsubscript{1.2DHP\textsuperscript{−/−}} in lateral superior olive neurons indicates the existence of Ca\textsubscript{1.2} channels operating at voltages more negative than −40 mV (26). In gene deletion studies, the question arises whether a loss of Ca\textsubscript{1.3\textsuperscript{−/−}} channels may trigger transcriptional or posttranslational modifications of at least a
subpopulation of Ca\textsubscript{v}1.2 channels, thereby changing their voltage-dependence so as to compensate for the elimination of negatively activating Ca\textsubscript{v}1.3 channels. The difference between onset medians determined in the presence of DMSO and in the presence of BayK was notably smaller in Ca\textsubscript{v}1.3\textsuperscript{-/-} neurons (3.5 mV) than in both wild-type neurons and Ca\textsubscript{v}1.2DHP\textsuperscript{-/-} neurons (\approx 12.5 mV; see Table 1). Thus the ability of LTCC potentiation to shift the average onset to more hyperpolarized voltages was less expressed in neurons lacking Ca\textsubscript{v}1.3 channels, which may be indicative of an increase in a subpopulation of rather low voltage-gated Ca\textsubscript{v}1.2 channels. However, ADPs (here identified to require Ca\textsubscript{v}1.2 activity) were evoked by small depolarizations not only in Ca\textsubscript{v}1.3\textsuperscript{-/-} neurons but also in wild-type neurons (see for example Figs. 1B and Fig. 8A, top traces), suggesting that activation of Ca\textsubscript{v}1.2 channels at relatively negative voltages also occurs in neurons that do express the Ca\textsubscript{v}1.3 LTCC isoform. Taken together, recent data challenge the view that only Cav1.3 but not Cav1.2 channels can operate at relatively low voltages also occurs in neurons that do express the Ca\textsubscript{v}1.3 LTCC isoform. Hence, our study adds important pieces of information to our current knowledge of both LTCC isoforms having the capability to operate at relatively low voltages, which may aid in further studies of the plasticity of L-type voltage-gated Ca\textsuperscript{2+} channels.

Conclusion. In the current study we identified common and alternate ion channel couplings of the central LTCC isoforms Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3. Additionally, we provide evidence that both LTCC isoforms have the capability to operate at relatively hyperpolarized membrane voltages. Hence, our study adds important pieces of information to our current knowledge of the differences between neuronal Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 channels. Furthermore, our data suggest that neurons of Ca\textsubscript{v}1.3\textsuperscript{-/-} mice may ease the investigation of Ca\textsubscript{v}1.2-mediated hippocampal ADPs, because both depolarizing effects resulting directly from Ca\textsubscript{v}1.3-channel mediated Ca\textsuperscript{2+} influx and membrane potential changes related to Ca\textsubscript{v}1.3 channel coupling to hyperpolarizing conductances are eliminated. On the other hand, neurons of Ca\textsubscript{v}1.2DHP\textsuperscript{-/-} may aid in further studies of the role of ADPs under conditions of enhanced LTCC activity (49). Hence, these genetically modified mouse strains may provide valuable models for further investigation of hippocampal LTCC-dependent ADPs but potentially also in testing of other LTCC-ADP-related electrophysiological phenomena such as plateau potentials and depolarization shifts in normal and abnormal LTCC activities (49, 75).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.H. and L.G. performed experiments; J.H., L.G., and H.K. analyzed data; J.H., L.G., S.B., and H.K. edited and revised manuscript; J.H., L.G., S.B., and H.K. approved final version of manuscript; S.B. and H.K. interpreted results of experiments; S.B. and H.K. drafted manuscript; H.K. conception and design of research; H.K. prepared figures.

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