Ablation of Ca\(^{2+}\) Channel β3 Subunit Leads to Enhanced N-Methyl-d-aspartate Receptor-dependent Long Term Potentiation and Improved Long Term Memory\(^*\)\(^\dagger\)

The β subunits of voltage-dependent Ca\(^{2+}\) channels (VDCCs) have marked effects on the properties of the pore-forming α\(_1\) subunits of VDCCs, including surface expression of channel complexes and modification of voltage-dependent kinetics. Among the four different β subunits, the β\(_3\) subunit (Ca\(_{β3}\)) is abundantly expressed in the hippocampus. However, the role of Ca\(_{β3}\) in hippocampal physiology and function \textit{in vivo} has never been examined. Here, we investigated Ca\(_{β3}\)-deficient mice for hippocampus-dependent learning and memory tasks. Our results suggest a possibility that, among the auxiliary subunits, the β subunits are entirely cytosolic, and they have marked effects on the properties of VDCCs α\(_1\) subunits, including trafficking of Ca\(^{2+}\) channel complexes to the plasma membrane, voltage dependence and activation/inactivation kinetics of Ca\(^{2+}\) currents (4, 5). Four β subunits (Ca\(_{β1}–4\)) have been cloned, and each Ca\(_{β}\) has distinctive properties (5), but their functional roles in the brain \textit{in vivo} are still poorly understood.

Structurally, Ca\(_{β}\) has five different domains, with the two conserved domains sharing significant homology among the β subunits. The conserved domains were revealed as an Src homology 3 domain and a guanylate kinase (GK) domain (6–9), and thus Ca\(_{β}\) is included in membrane-associated guanylate kinase family that has scaffolding functions. Interestingly, it has been suggested that Ca\(_{β}\) can bind to other molecules (10, 11). For example, Ca\(_{β}\) could directly interact with small G-proteins (Gem and Rem) and dynamin (12–14). In addition, recent studies have suggested that Ca\(_{β}\) can work without marked influence on VDCCs. For example, regulation of gene transcription by a direct interaction between a short splice variant of Ca\(_{β4}\) and a nuclear protein was shown in the cochlea (15). Ca\(_{β3}\) was also shown to regulate insulin secretion by acting on the intracellular Ca\(^{2+}\) store, whereas Ca\(^{2+}\) currents of VDCCs were not affected (16). This study suggests that Ca\(_{β}\) can function as a multifunctional protein.

Of the Ca\(_{β}\) subunits, Ca\(_{β3}\) is highly expressed in the brain, especially in the hippocampus (17). It was shown that α\(_1\) subunits of N- and L-type VDCCs were preferentially associated

\(^*\) This work was supported by the National Honor Scientist Program of Korea, grants from Korea Institute of Science and Technology, the National Creative Research Initiatives of the Ministry of Science and Technology of Korea, and Virginia Commonwealth University Medical Center Grant NEI EY12716. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby indicated this fact.

\(^\dagger\) The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

1 To whom correspondence may be addressed. Tel.: 82-2-880-7854; Fax: 82-2-872-0596; E-mail: utoh@plaza.snu.ac.kr.
2 To whom correspondence may be addressed. Tel.: 82-2-958-6931; Fax: 82-2-958-6937; E-mail: shin@kist.re.kr.

\(^1\) The abbreviations used are: VDCC, voltage-dependent Ca\(^{2+}\) channel; Ca\(_{β3}\), Ca\(^{2+}\) channels β\(_3\) subunit; EPSC, excitatory postsynaptic current; mEPSCs, miniature EPSC; LTP, long term potentiation; NMDAR, N-methyl-D-aspartate receptor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid; AMPAR, AMPA receptor; CS, conditioned stimulus; GAD, glutamate decarboxylase; AHP, after hyperpolarization; fEPSPs, field excitatory postsynaptic potentials; SH3, Src homology 3; GK, guanylate kinase; ANOVA, analysis of variance; GABA, γ-aminobutyric acid; PPF, paired-pulse facilitation; MI, meogehom; LTD, long term depression; AP, action potential; PP-LFS, paired-pulses low frequency stimulation.
A Novel Function of Ca\textsubscript{a},β3 in the Hippocampus

with Ca\textsubscript{a},β3 in the hippocampus (18–20). In addition, N- and L-type VDCCs have been strongly implicated in activity-dependent long-lasting synaptic changes, such as LTP, as well as in learning and memory (21, 22). Therefore, we examined the Ca\textsubscript{a},β3-deficient mice (23) for hippocampus-dependent learning and memory and synaptic plasticity. Interestingly, long-term memory and NMDAR-dependent LTP were increased in the Ca\textsubscript{a},β3-deficient mice, whereas there was no significant change in Ca\textsuperscript{2+} currents. Furthermore, the mutant mice showed increased NMDAR-mediated synaptic responses and an increased NR2B level in the hippocampus. These results reveal Ca\textsuperscript{2+} channel-independent functions of Ca\textsubscript{a},β3 in the hippocampus.

EXPERIMENTAL PROCEDURES

Animals—The generation of mice lacking Ca\textsubscript{a},β3 was described in our previous study (23). Ca\textsubscript{a},β3 heterozygous (Ca\textsubscript{a},β3+/−) mice were backcrossed into two inbred backgrounds, C57BL/6J and 129S4/SvJae, each over 18 generations. Ca\textsubscript{a},β3 wild-type (Ca\textsubscript{a},β3+/+) and Ca\textsubscript{a},β3-deficient (Ca\textsubscript{a},β3−/−) mice used for analysis were obtained from interbreeding Ca\textsubscript{a},β3+/− mice of the two backgrounds. Animal care and handling were carried out according to the institutional guidelines. The mice were maintained with free access to food and water under a 12:12-h light/dark cycle. Behavioral experiments were performed on 8–12-week-old mice. All experiments were performed in a blind manner with respect to the genotype.

Contextual and Cued Fear Conditioning—The fear conditioning was carried out as described in our previous study (24). A fear-conditioning shock chamber (19 × 20 × 33 cm) containing a stainless steel rod floor (5 mm diameter, spaced 1 cm apart) and a monitor was used (WinLinc behavioral experimental software, Coulbourn Instruments). For conditioning, mice were placed in the fear-conditioning apparatus chamber for 2 min, and then a 28-s acoustic conditioned stimulus (CS) was delivered. Following the CS, a 0.5-mA shock of unconditioned stimulus was immediately applied to the floor grid for 2 s. This protocol was performed twice at 60-s interval. To assess contextual learning, the animals were placed back into the training context 24 h after training, and then freezing behavior was observed for 4 min. To assess cued learning, the animals were placed in a different context (a novel chamber, odor, floor, and visual cues) 24 h after training, and their behaviors were monitored for 5 min. During the last 3 min of this test, animals were exposed to the tone. Fear response was quantified by measuring the length of the time when the animal showed freezing behaviors, which was defined as lack of movements with a crouching position, except for respiratory movements (25). Foot-shock intensity was evaluated by placing naive animals in the conditioning chamber used for fear conditioning. Animals were subjected to a 1-s series of gradually increasing mild foot-shock amperage at 20-s intervals as follows: 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, and 0.6 mA. The shock intensity that evoked initial sensation responses (flinching and running), vocalization, and jumping was recorded for each mouse.

Novel Object Recognition Memory Task—The task was performed as described (24, 26, 27). The mice were individually habituated to an open-field box (40 × 40 × 40 cm) for 3 days. During the training trial, two objects were placed in the box, and animals were allowed to explore them for 5 min. A mouse was considered to be exploring the object when its head was facing the object within 1-inch distance. Following retention intervals (1 or 24 h), animals were placed back into the box with two objects in the same locations, but one of the familiar objects was replaced by a novel object, and mice were then allowed to explore the two objects for 5 min. The preference percentage, percentage of the time spent exploring the novel object over the total time spent exploring both objects, was used to quantitate the recognition memory.

Social Transmission of Food Preference Task—This task was performed as described previously (21, 28, 29), with slight modifications. “Demonstrator” mice were given a distinctively scented food (cinnamon or cocoa) for 2 h and then immediately allowed to interact with “observer” mice for 30 min. Either 1 or 24 h later, observers were given a choice between two scented foods: either the same scented food that the demonstrators had eaten (cued) or another distinctively scented food (non-cued). Half of the observers in each genotype was subjected to interaction with the demonstrators that had eaten cinnamon as cued food and the other half with those that had eaten cocoa as cued food to control for the possibility of food preference bias.

Whole-cell Patch Clamp Recording on Acutely Isolated CA1 Pyramidal Neurons and on Hippocampal Slices—All experiments were performed on 2–3-week-old mice. Preparation of and recording from hippocampal slices (400 μm thick) were as described in our previous study (21, 30). Hippocampal slices were prepared in oxygenated, cold ACSF (124 mM NaCl, 3.5 mM KCl, 1.25 mM NaH2PO4, 2.5 mM CaCl2, 1.3 mM MgSO4, 26 mM NaHCO3, and 10 mM glucose, pH 7.4). For the measurement of Ca\textsuperscript{2+} currents, acutely isolated CA1 pyramidal neurons were prepared from hippocampal slices, as described in our previous study (30). The recorded CA1 neurons were voltage-clamped at −60 mV using glass pipette electrodes (3–5 MΩ series resistance <20 MΩ) and the I-V curve was generated in a stepwise fashion: +10-mV increments from −60 to +40 mV. Internal pipette solution contained the following, 130 mM CsCl, 10 mM EGTA, 4 mM HEPES, 2 mM MgATP, 0.3 mM Tris-GTP, 5 mM tetraethylammonium chloride, and was brought to pH 7.4 with NaOH. Extracellular solution contained the following, 25 mM tetraethylammonium chloride, 5 mM 4-aminopyridine, 20 mM HEPES, 3 mM KCl, 5 mM CaCl2, 2 mM MgCl2, 100 mM NaCl, 0.001 mM tetrodotoxin, and was brought to pH 7.4 with NaOH. For the measurement of after hyperpolarization (AHP) currents, visually guided CA1 pyramidal neurons in hippocampal slice were held at −55 mV, and currents were evoked by depolarizing voltage commands to 20 mV for 200 ms followed by a return to −55 mV for 10 s. During recording, the slices were superfused with ACSF at room temperature. Glass pipettes (3–5 MΩ) were filled with solution containing 140 mM KMeSO4, 8 mM NaCl, 1 mM MgCl2, 10 mM HEPES, 2 mM Mg-ATP, 0.4 mM Na2-GTP, and 0.02 mM EGTA (pH 7.3, 290 mosm). In addition, action potentials (APs) were triggered under current clamp mode by depolarizing current injection (from +30 to +90 pA), and the number of AP (from threshold to the peak) and AP durations (width at half-height) were measured. The internal solution for mEPSC (miniature excitatory...
A Novel Function of Cav3 in the Hippocampus

RESULTS

Normal Gross Morphology of the Hippocampus in the Cav3−/− Mice—We first examined the cytoarchitectonic divisions in the brain of the Cav3−/− mice, especially in the hippocampus. The Cav3−/− mice exhibited normal hippocampal divisions, including CA1, CA2, CA3, and dentate gyrus. No expression of Cav3 was observed in the Cav3−/− hippocampus (Fig. 1A), whereas Cav3 was abundant in the wild-type hippocampus as was shown previously (17). The immunoreactivities and the expression patterns of S100b (a neurofilament protein) (Fig. 1B) and GAD (GABA-synthesizing enzyme) (Fig. 1C) were normally observed in the hippocampus of the Cav3−/− mice as in the Cav3+/+ mice. In addition, Nissl staining of the coronal brain sections revealed no gross abnormalities in the hippocampus of the Cav3−/− mice (Fig. 1D).

Enhanced Contextual Fear Conditioning in the Cav3−/− Mice—Because Cav3 is highly expressed in the hippocampus and is known to be associated with N- or L-type VDCCs, which play important roles in hippocampus-dependent learning and memory in animals (17–19), we examined whether the deletion of Cav3 affected the animal’s capacity for hippocampus-dependent learning and memory. First, we subjected the mice to the fear conditioning assay that is known to require the function of the hippocampus (36). The Cav3−/+ (n = 14) and Cav3+/− (n = 14) mice showed similar levels of freezing response during the training (Fig. 2A). In the contextual fear memory assay performed 24 h after the training, the Cav3−/− mice displayed more freezing behavior than the Cav3−/+ (F(1, 26) = 8.36, p < 0.01, two-way repeated ANOVA), indicating an enhanced long term memory of the Cav3−/− mice for contextual fear conditioning. A post hoc test (Scheffe’s test) also revealed significant differences between the two genotypes during the 2nd (p < 0.05), the 3rd (p < 0.05), and the 4th min (p < 0.05) (Fig. 2B). On the other hand, no difference was observed between the two genotypes in the cued fear conditioning assay (Fig. 2C), indicating that the enhanced memory in the Cav3−/− mice is limited to the hippocampus-dependent fear conditioning. There was no sig-
A Novel Function of Ca_{\beta3} in the Hippocampus

A

B

C

D

FIGURE 1. Histological assessment of the hippocampus of the Ca_{\beta3}^+/− mouse. A, strong immunoreactivity of Ca_{\beta3} in the Ca_{\beta3}^+/− hippocampus indicates that it is highly expressed in the hippocampus, whereas there is no detectable signal in the Ca_{\beta3}^−/−. Similar levels of SMI-32 (B) and GAD (C) immunoreactivity are observed in the hippocampus of Ca_{\beta3}^+/− and Ca_{\beta3}^−/− brain. SMI-32 staining provides Golgi-like staining of neurons/axons, and GAD immunoreactivity is restricted to small interneurons around the pyramidal layer. D, normal gross morphology of the hippocampus revealed by Nissl staining in comparable hippocampal regions of Ca_{\beta3}^+/− and Ca_{\beta3}^−/− mice. The scale bars equal 100 μm (A, B, and D) and 50 μm (C). Arrowheads indicate CA1, CA2, and CA3 region in order.

significant difference in response to variable electric intensities between Ca_{\beta3}^−/− (n = 7) and Ca_{\beta3}^+/+ (n = 9) mice, indicating comparable reactivity or sensitivity to electric foot-shock of the two genotypes (Fig. 2D).

Enhanced Novel Object Recognition Memory in the Ca_{\beta3}^−/− Mice—We next subjected the mice to the novel object recognition task that is based on the animal’s ability to discriminate a novel object from a familiar one, which requires the hippocampus (37). We first assessed the amount of time spent by the animals exploring the two objects during the training trial, and we found that both of the genotypes, Ca_{\beta3}^+/+ (n = 17) and Ca_{\beta3}^−/− mice (n = 14), explored the two objects for equal time (Fig. 2E), which indicated no preference of the animals for either object. At a 1-h retention interval, when one of the familiar objects was replaced by a novel one, both Ca_{\beta3}^+/+ (n = 8) and Ca_{\beta3}^−/− mice (n = 7) exhibited increased preference for the novel object to the familiar one (F_{(1, 13)} = 22.86, p < 0.001, two-way repeated ANOVA). No difference, however, was found between the two genotypes (F_{(1, 13)} = 0.01, p = 0.96, one-way ANOVA) (+/+, 72.90 ± 4.27%; −/−, 73.34 ± 8.83%) (Fig. 2F). At the 24-h retention test, however, Ca_{\beta3}^−/− mice (n = 7) showed increased preference for the novel object compared with Ca_{\beta3}^+/+ (n = 9) (F_{(1, 14)} = 36.14, p < 0.001, two-way repeated ANOVA, Scheffe’s post hoc test, p < 0.01) (+/+, 62.68 ± 6.26%; −/−, 88.90 ± 3.23%) (Fig. 2F), indicating that the Ca_{\beta3}^−/− mice have an enhanced performance in the object recognition memory task.

Enhanced Long Term Memory in the Social Transmission of Food Preference Task in the Ca_{\beta3}^−/− Mice—Finally, we carried out the social transmission of food preference assay, another hippocampus-dependent memory task. This task exploits the tendency of mice to prefer food that they have recently smelled on the breath of other mice (demonstrator mice), and subsequently, this tests their ability to learn and remember the information transmitted by olfactory cues during social interactions. 1 h after social interactions with demonstrator mice, both Ca_{\beta3}^+/+ (n = 7) and Ca_{\beta3}^−/− (n = 6) mice preferred the “cued” food to the “non-cued” food, and there was no significant difference between the two genotypes (+/+, 83.70 ± 3.63%; −/−, 75.87 ± 7.34%, F_{(1, 11)} = 0.72, p = 0.41, one-way ANOVA) (Fig. 2G). The amount of total food eaten was not different between geno-
types during this task (Fig. 2H). These results indicate that the mice were not deficient in olfaction or social interactions.

On the other hand, 24 h after interactions with demonstrator mice, Cavβ3−/− mice (n = 10) exhibited significantly increased preference for cued food compared with Cavβ3+/− mice (n = 10) (+/+; 71.61 ± 4.72%; −/−, 71.61 ± 3.65%, F(1, 18) = 7.10, p < 0.05, one-way ANOVA) (Fig. 2G). There was no significant difference between genotypes in the amount of total food that was eaten (Fig. 2H). These results suggest that Cavβ3−/− mice displayed an enhanced memory in the social transmission of food preference task.

No Change in Ca2+ Currents in the Caβ3−/− CA1 Pyramidal Neurons—Next we examined whether Ca2+ currents (I_{Ca}) are altered or not in the Cavβ3−/− neurons by whole-cell patch clamp recordings in CA1 pyramidal neurons. Total Ca2+ currents were activated by step depolarizations (+10 mV increments) from a holding potential of −60 mV (Fig. 3A). In CA1 neurons from both Cavβ3−/− and Cavβ3+/− mice, Ca2+ currents reached their maximum amplitudes at −30 mV (Fig. 3B). Unlike previous studies that showed a reduced Ca2+ current density in Cavβ3−/− neurons (superior cervical ganglion neurons (23), dorsal root ganglion neurons (38), and olfactory sensory neurons (39)), there was no significant difference in the Ca2+ current density between Cavβ3+/− and Cavβ3−/− CA1 pyramidal neurons (+/+, 35.46 ± 2.94 pA/pF; n = 18, at 0 mV; −/−, 34.80 ± 3.06 pA/pF, n = 21, p = 0.88, Student’s t test) (Fig. 3B). Furthermore, there was no difference in the Ca2+ current divided by maximum values of the Ca2+ current (I/I_{max}) (Fig. 3C), and in the time constant (τ) of Ca2+ current decay (+/+, 82.70 ± 9.75 ms; −/−, 77.70 ± 12.58 ms, p = 0.76, Student’s t test) (Fig. 3D), indicating no changes in voltage dependence and inactivating kinetics in the Cavβ3−/− CA1 neurons.

Normal Intrinsic Firing Properties and AHP Currents in the Cavβ3−/−—As a close coupling was reported by co-immuno-precipitation between Cavβ3 and N- or L-type VDCCs in hippocampal neurons (18–20), we measured N- or L-type VDCCs-mediated cellular properties in CA1 neurons. Ca2+ influx
A Novel Function of Cavδ3 in the Hippocampus

First we produced AP discharges by a depolarizing current (AHP), and thus can modulate firing properties (40). A mutation did not affect intrinsic firing behaviors of hippocampal neurons (Fig. 5). These results show that the Cavδ mutation had no significant effect upon the synaptic function and the presynaptic short term plasticity in hippocampal CA3-CA1 synapses.

Enhanced NMDAR-dependent LTP in the Cavδ3−/− Mice—We then investigated the mutant mice for activity-dependent long lasting synaptic changes, such as LTP and LTD, a cellular model of learning and memory (43). We tried to induce LTP by several different stimulation protocols. LTP was induced by 200-Hz tetanic stimulations. As shown in Fig. 6A, an administration of tetanus at 100 Hz for 1 s elicited a significantly increased potentiation in the Cavδ3−/− (n = 9) compared with that in the Cavδ3+/− (−/−, 169.47 ± 7.33% of base line at 60 min, n = 9; +/+), 144.75 ± 6.10%, n = 9, p < 0.05, Student’s t test). With a 200-Hz tetanic stimulation, the Cavδ3−/− also exhibited more robust potentiation than the Cavδ3+/− (−/−, 231.92 ± 15.72% of base line at 60 min, n = 10; +/+ 181.72 ± 15.89%, n = 8, p < 0.05, Student’s t test) (Fig. 6B). Even at short 100-Hz stimulations for 300 ms, enhanced LTP in the Cavδ3−/− was also observed (−/−, 139.31 ± 7.35% of base line at 60 min, n = 10; +/+ 144.70 ± 8.03%, n = 8, p < 0.05, Student’s t test) (Fig. 6C). However, in the presence of D-AP5, a specific NMDAR inhibitor, the enhancement of LTP in the Cavδ3−/− disappeared under the same stimulation condition, and a similar level of potentiation was induced in the two genotypes (Fig. 6, D and E). Together, these results indicate that
the increased potentiation in the Caβ3−/− is NMDAR-dependent LTP. No significant difference was noted between the two genotypes in LTD that was induced by PP-LFS (Fig. 6F).

Increased NMDAR-mediated Synaptic Currents and NR2B Levels in the Caβ3−/− Mice—NMDAR is known to play a crucial role in LTP, as well as learning and memory (43–46). Therefore, we examined the possibility that changes in the synaptic responses mediated by NMDAR might underlie the increased LTP in Caβ3−/− mice. To evaluate this possibility, we first measured the NMDAR-mediated fEPSPs by adding CNQX (10 μM), an AMPA receptor blocker, to the buffer with reduced Mg2+ concentration (0.1 mM). A significant difference was noted between the Caβ3+/+ and the Caβ3−/− in these NMDAR-mediated field responses; the Caβ3−/− (n = 13) exhibited higher NMDAR-mediated fEPSPs than the Caβ3+/+ (n = 12) (F(1, 23) = 5.52, p < 0.05, two-way repeated ANOVA) (Fig. 7A). To assess this finding more directly, we measured the excitatory postsynaptic currents (EPSCs) evoked by stimulations at Schaffer collateral axons under the whole-cell voltage clamp conditions in CA1 neurons. It was found that there was no significant difference in the amplitude of AMPAR-mediated EPSCs at −70 mV between the two genotypes (Fig. 7B, left). However, a significant difference was noted in the NMDAR/AMPAR amplitude ratio between Caβ3+/+ (n = 15, 0.28 ± 0.04 at +40 mV) and Caβ3−/− (n = 13, 0.47 ± 0.06 at +40 mV) (p < 0.05, Student’s t test) (Fig. 7B, right). Together, these results indicate that NMDAR-mediated responses are increased in Caβ3−/− mice.

In an effort to obtain some clue for the mechanism underlying the increased NMDAR responses in the Caβ3−/− mice, we quantified the levels of NMDAR subunits by Western blot analysis. It was found that the protein level of NR2B subunit in the hippocampus of Caβ3−/− mice was relatively increased, whereas other glutamate receptors did not change. The equal amount of protein loading was confirmed by normalizing against the amount of tubulin. *, p < 0.05.

**FIGURE 7. Increased NMDAR-mediated responses and increased NR2B levels in the hippocampus of Caβ3−/− mice.** A, NMDAR-mediated synaptic potentials in the presence of CNQX (10 μM) and reduced Mg2+ (0.1 mM). The Caβ3−/− shows higher NMDAR-mediated fEPSPs than Caβ3+/+ (F(1, 23) = 5.52, p < 0.05, two-way repeated ANOVA). B, NMDAR- and AMPAR-mediated EPSCs recorded under voltage clamp. Representative traces of EPSCs evoked at −70 mV and +40 mV in Caβ3+/+ and Caβ3−/−. Scale bars, 0.1 nA and 50 ms. There are no differences in AMPAR-mediated EPSCs at −70 mV between genotypes (left bar graph), but the ratio of NMDAR/AMPAR response in the Caβ3−/− is higher than that of the Caβ3+/+ (right bar graph). NMDAR-mediated responses were taken from the amplitude of currents at +40 mA, 100 ms after EPSCs onset, whereas the AMPAR-mediated responses were taken as the peak amplitude of EPSCs recorded at −70 mV. *, p < 0.05. C, Western blot analysis. The relative levels of glutamate receptors in hippocampal proteins of Caβ3−/− mice. The NR2B level of Caβ3−/− mice was relatively increased, whereas other glutamate receptors did not change. The equal amount of protein loading was confirmed by normalizing against the amount of tubulin. *, p < 0.05.
A Novel Function of Cav_3 in the Hippocampus

demonstrates a previously unidentified outcome of the deletion of Cav_3 in the adult brain.

Yet the Cav subunits of VDCCs have been known to be associated with VDCCs and regulate Ca^{2+} influx through VDCCs by modulating the properties of VDCCs α_s subunits, including trafficking of channel complexes to the plasma membrane, Ca^{2+} current densities, and voltage-dependent activation or inactivation (4, 5). Of the Cav subtypes, the Cav_3 is the predominant form in the brain (17), and its role in several neurons has been revealed by studies carried out using mice lacking the Cav_3. In superior cervical ganglion neurons, the Cav_3−/− showed reduced N- and L-type Ca^{2+} currents relative to the Cav_3+/+ and shifting of voltage-dependent activation in P/Q-type Ca^{2+} currents (23). In dorsal root ganglion neurons, the Cav_3−/− mice showed a reduced expression of N-type VDCCs and functional alterations of Ca^{2+} currents, which was thought to be involved in the reduced pain responses of the Cav_3−/− mice (38). In olfactory sensory neurons, the Cav_3−/− mice also exhibited decreased protein expressions and Ca^{2+} currents of L-type and N-type VDCCs, leading to increased olfactory neuronal activities (39). These reduced expressions of proteins or Ca^{2+} currents of VDCCs might be considered to mostly result from deficiency in trafficking of channel complexes to the plasma membrane.

However, although the Cav_3 is known to be highly expressed in the hippocampus (17) and has been shown to associate with 42% of the α_s subunits of L-type VDCCs in the hippocampus (18), we could not observe a change in nifedipine-sensitive L-type Ca^{2+} currents in hippocampal CA1 pyramidal neurons of the Cav_3−/− mice (supplemental Fig. 1). In addition, there were no clear differences in the patterns of the immunohistological labeling for the α_1C (CA_1.2 and the α_1D (CA_1.3) subunits of L-type VDCCs in the hippocampus, between the two genotypes (supplemental Fig. 2). Furthermore, although Cav_3 in the brain was shown to associate with about 52% α_1B subunit of N-type VDCCs that play a crucial role in neurotransmitter release at hippocampal CA3-CA1 synapses (19–21, 47, 48), the basal synaptic transmission, including mEPSCs, was not altered at hippocampal CA3-CA1 synapses of the Cav_3−/− mice. Therefore, some compensation by other Cav_3 isoforms might have occurred for the deletion of Cav_3 in the hippocampus of the Cav_3−/− mice, as was reported in olfactory sensory neurons of the Cav_3−/− mice (39).

Instead, however, we found an increased LTP at hippocampal CA3-CA1 synapses in the Cav_3−/− mice. The induction of LTP by a tetanic stimulation at 100 Hz is known to be dependent on NMDAR, and 200-Hz LTP requires both NMDAR and L-type VDCCs at hippocampal CA3-CA1 synapses (49). When NMDAR was blocked by D-AP5, the enhancement in 100-Hz and 200-Hz LTP of the Cav_3−/− mice was obliterated. This indicates that the increased potentiation in the Cav_3−/− is of the NMDAR-dependent component in LTP, rather than L-type VDCC-dependent. The increased LTP and long term memory in the Cav_3−/− mice could be analogous to other cases where an alteration of NMDAR-mediated synaptic responses resulting from the increased levels of NR2B was shown (45, 46).

Although the Ca^{2+} currents and mEPSCs were measured from 2- to 3-week-old mice, basal synaptic transmission and LTP were recorded in 7- to 8-week-old mice. Thus, no alteration in Ca^{2+} currents of at least N- and L-type VDCCs could be expected in the adult Cav_3−/− mice, because they showed normal responses in basal synaptic transmission and NMDA-independent LTP, in which N- and L-type VDCCs have a crucial role, respectively (21, 22, 47–49).

Our results suggest a possibility that Cav_3 can be a multifunctional protein as was shown for other Cav isoforms. The studies of crystal structures revealed that Cav_3 subunits belong to membrane-associated guanylate kinase family that has scaffolding functions, suggesting that the Cav_3 can play a role in scaffolding multiple signaling pathways by protein-protein interactions through SH3 and GK domains (6, 8, 9). Recently, it was suggested that the Cav_3 could directly interact with other proteins, and furthermore it could function without marked influences on the property of VDCCs (10, 11, 50). The physiological unbinding of the Cav_3 from the VDCCs complex has already been demonstrated from the inactivation heterogeneity of VDCCs and reversibility of the interaction with α_s subunits (51, 52). It was reported that Cav_3 could directly bind to Gem and Rem, small G-proteins that have a GTPase activity, and this interaction inhibited the surface expression and the activity of VDCCs (12, 13). In addition, it was also shown that Cav_3 could promote endocytosis of VDCCs by interaction with dynamin (14). A short splice variant of Cav_3 could directly interact with CHCB2, a nuclear protein, and then translocate into the nucleus for the subsequent regulation of gene transcription in the cochlea (15). In this study, it was found that the Cav_3 could function independently from VDCCs without marked influences on the surface expression and voltage-dependent properties of VDCCs. Furthermore, inositol 1,4,5-trisphosphated-mediated signaling was enhanced in Cav_3-deficient pancreatic β cells, whereas Ca^{2+} currents of VDCCs were not affected (16). Similarly, Cav_3 were found to internalize Shaker K^+ channels by association with dynamin (14). These activities of Cav_3 are considered to be completely independent of VDCCs regulation, and thus indicate that Cav_3 can function as a multifunctional protein by interactions with other proteins.

In this light, it might be possible that the Cav_3 directly or indirectly associate with NR2B.

Although our results showed a modest increase of NR2B in the mutant, it is not clear whether this increase can totally explain how the NMDAR activities are enhanced. In the meantime, it was discovered that the C-terminal tail region of Cav_1.3 L-type VDCC bound to the SH3 domain of Shank, a postsynaptic scaffolding protein (53–55). Shank is also known to associate with GKAP-PSD95-NR2B through postsynaptic density-95 (PSD-95)/Discs large/zona occludens-1 domain (56). One of the binding sites of Cav_3 is the C-terminal tail region of α_1 subunits of VDCCs (6, 8, 9, 57). In this light, the removal of Cav_3 might have an influence on the interaction of VDCCs and their partners and then could lead to an alteration in the NMDAR activity. Alternatively, we cannot rule out the possibility that a compensatory increase of other Cav_3 isoforms or other developmental compensation, which may have occurred in the Cav_3−/− hippocampus, could also be linked to the alteration in the NMDAR activity. In addition, previously described behavioral alterations from the changes in dorsal root ganglion
or olfactory neuronal activities in the Cav$_{a}$3$^{-/-}$ mice (38, 39) could contribute to the phenotypes shown in our results.

Initially, we started investigating the role of the Cav$_{a}$3 in synaptic transmission and hippocampus-dependent learning and memory because of its known relationship with N- or L-type VDCCs. Interestingly, we found that the ablation of Cav$_{a}$3 led to enhanced LTP and capacity for learning and memory in the animal. These phenotypes appear to be due to the increased NMDAR activity with increased NR2B levels in the Cav$_{a}$3$^{-/-}$ mice. Even though the precise mechanism of the enhancement of the NMDAR activity in the Cav$_{a}$3$^{-/-}$ mice is not yet completely understood, our experiments may reveal a potentially novel function of Cav$_{a}$3, unrelated to a role associated with VDCCs. Further studies of the relationship, including direct or indirect protein-protein interactions, between Cav$_{a}$3 and NMDAR will be needed to confirm this role of Cav$_{a}$3 in the adult brain.

Acknowledgments—We thank Dr. Minjeong Sun, Seungeun Lee, and Sangwoo Kim for help in behavioral experiments and Jeremy Mills and Erick Green for help with histology.

REFERENCES