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

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The β subunits of voltage-dependent Ca$^{2+}$ channels (VDCCs) have marked effects on the properties of the pore-forming α subunits of VDCCs, including surface expression of channel complexes and modification of voltage-dependent kinetics. Among the four different β subunits, the β3 subunit (Caβ3) is abundantly expressed in the hippocampus. However, the role of Caβ3 in hippocampal physiology and function in vivo has never been examined. Here, we investigated Caβ3-deficient mice for hippocampus-dependent learning and memory and synaptic plasticity at hippocampal CA3-CA1 synapses. Interestingly, the mutant mice exhibited enhanced performance in several hippocampus-dependent learning and memory tasks. However, electrophysiological studies revealed no alteration in the Ca$^{2+}$ current density, the frequency and amplitude of miniature excitatory postsynaptic currents and NMDAR-dependent long term potentiation were significantly increased in the mutant. Protein blot analysis showed a slight increase in the level of NMDAR-2B in the mutant hippocampus. On the other hand, however, N-methyl-d-aspartate receptor (NMDAR)-mediated synaptic currents and NMDAR-dependent long term potentiation were significantly increased in the mutant. Protein blot analysis showed a slight increase in the level of NMDAR-2B in the mutant hippocampus. Our results suggest a possibility that, unrelated to VDCCs regulation, Caβ3 negatively regulates the NMDAR activity in the hippocampus and thus activity-dependent synaptic plasticity and cognitive behaviors in the mouse.

Voltage-dependent Ca$^{2+}$ channels (VDCCs) play important roles in the regulation of diverse neuronal functions by mediating Ca$^{2+}$ entry into cells. VDCCs have multiple subunit structures consisting of a major pore-forming subunit (α) and several auxiliary subunits (αδ, β, and γ). Additional subunits are entirely cytosolic, and they have marked effects on the properties of VDCCs in vivo. 4 Four subunits of Caβ (Caβ1–4) have been cloned, and each Caβ has distinctive properties, but their functional roles in the brain in vivo are still poorly understood.

Structurally, Caβ 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 (SH3) domain and a guanylate kinase (GK) domain (6–9), and thus Caβ is included in membrane-associated guanylate kinase family that has scaffolding functions. Interestingly, it has been suggested that Caβ can bind to other molecules (10, 11). For example, Caβ could directly interact with small G-proteins (Gem and Rem) and dynamin (12–14). In addition, recent studies have suggested that Caβ can work without marked influence on VDCCs. For example, regulation of gene transcription by a direct interaction between a short splice variant of Caβ4 and a nuclear protein was shown in the cochlea (15). Caβ3 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 Caβ can function as a multifunctional protein.

Of the Caβ subunits, Caβ3 is highly expressed in the brain, especially in the hippocampus (17). It was shown that αi subunits of N- and L-type VDCCs were preferentially associated with NMDARs and Caβ3 (18, 19). Among the four different α subunits of VDCCs, including surface expression of channel complexes and modification of voltage-dependent kinetics. Among the auxiliary subunits, the β subunits are entirely cytosolic, and they have marked effects on the properties of VDCCs in vivo. 4 Four subunits of Caβ (Caβ1–4) have been cloned, and each Caβ has distinctive properties, but their functional roles in the brain in vivo are still poorly understood.

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The abbreviations used are: VDCC, voltage-dependent Ca$^{2+}$ channel; Caβ3, Caβ$^{2+}$ channel β3 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-isoxazolopropionic 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-amino butyric acid; PPF, paired-pulse facilitation; MIΩ, megohm; LTD, long term depression; AP, action potential; PP-LFS, paired-pulse low frequency stimulation.
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with Caαβ3 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 Caαβ3-deficient mice (23) for hippocampus-dependent learning and memory and synaptic plasticity. Interestingly, long term memory and NMDAR-dependent LTP were increased in the Caαβ3-deficient mice, whereas there was no significant change in Ca2+ currents. Furthermore, the mutant mice showed increased NMDAR-mediated synaptic responses and an increased NR2B level in the hippocampus. These results reveal Ca2+ channel-independent functions of Caαβ3 in the hippocampus.

**EXPERIMENTAL PROCEDURES**

**Animals**—The generation of mice lacking Caαβ3 was described in our previous study (23). Caαβ3 heterozygous (Caαβ3+/−) mice were backcrossed into two inbred backgrounds, C57BL/6j and 129S4/SvJae, each over 18 generations. Caαβ3 wild-type (Caαβ3+/+) and Caαβ3-deficient (Caαβ3−/−) mice used for analysis were obtained from interbreeding Caαβ3+/− 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 control 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 (US) 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 Ca2+ 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, 10 mM HEPES, 4 mM MgCl2, 4 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-aminoypyridine, 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
postsynaptic currents) recording was filled with the following buffer, 135 mM potassium glutonate, 5 mM KCl, 2 mM MgCl₂, 5 mM EGTA, 10 mM HEPES, 0.5 mM CaCl₂, 5 mM Mg-ATP and 0.3 mM Na-GTP, and was brought to pH 7.4 with KOH. The experiment was performed in the presence of tetrodotoxin (1 μM) and bicuculline (10 μM, a GABA type A receptor antagonist). The recorded CA1 pyramidal neurons were voltage-clamped at −70 mV. The frequency and amplitude of mEPSCs were analyzed with MiniAnalysis (Synaptosoft). The recorded CA1 pyramidal neurons were voltage-clamped at −70 mV. The frequency and amplitude of mEPSCs were analyzed with MiniAnalysis (Synaptosoft) (21). For the measurement of AMPAR- and NMDAR-mediated synaptic currents in visually guided CA1 pyramidal neurons, pipettes (3−5 MΩ) were filled with the internal solution (130 mM cesium glutonate, 5 mM KCl, 0.1 mM CaCl₂, 2.0 mM MgCl₂, 5 mM EGTA, 10 mM HEPES, 10 mM QX-314, 4 mM Na-ATP, and 0.4 mM Na-GTP, brought to pH 7.3 with CsOH). The currents were measured in the presence of bicuculline (10 μM) and CGP 55845 (5 μM, a GABA type B receptor antagonist). The synaptic currents were evoked by a bipolar tungsten electrode that was placed in the stratum radiatum. NMDAR- and AMPAR-mediated responses were discriminated based on their distinct kinetics and voltage dependence; the NMDAR-mediated currents were measured at +40 mV, 100 ms after the response onset, whereas the AMPAR-mediated currents were taken as the peak amplitude response recorded at −70 mV (31). D-AP5 (50 μM) blocked the late component of the currents recorded at +40 mV, whereas CNQX (10 μM), an AMPA receptor blocker, eliminated the currents recorded at −70 mV. Whole-cell patch clamp current were recorded and digitized with a MultiClamp 700A amplifier and a Digidata 1320 or 1322A (Axon Instruments, CA), and acquired data were analyzed with the pCLAMP version 9.2 (Axon Instruments) and the Mini Analysis Program (Synaptosoft).

Extracellular Recording on Hippocampal Slices—Preparation of hippocampal slices and the method of field excitatory postsynaptic potentials (fEPSPs) recording have been described previously (21, 24). Hippocampal slices (400 μm) were prepared from 7−8-week-old mice, as described above. Slices were then placed in a warm, humidified (32 °C, 95% O₂, 5% CO₂) recording chamber containing oxygenated ACSF and maintained for 1.5 h prior to experiments. A bipolar stimulating electrode was placed in the stratum radiatum in the CA1 region, and extracellular field potentials were also recorded in the stratum radiatum using a glass microelectrode (borosilicate glass, 3−5 MΩ, filled with 3 M NaCl). Test responses were elicited at 0.033 Hz. Base-line stimulation was delivered at an intensity that evoked a response that was ~40% of the maximum evoked response. LTP was induced electrically by one of the following protocols: 1) 100-Hz LTP was induced for 100 ms, 300 ms, or 1 s; 2) 200-Hz LTP was induced by 10 trains of 200-ms stimulation at 200 Hz delivered every 5 s. LTD was elicited by paired-pulses low frequency stimulation (PP-LFS) (50-ms pulse interval at 1 Hz for 15 min). Drugs were added to the perfusion medium at least 30 min before recording.

Immunohistology and Western Blot—Immunostaining was performed as described previously (32, 33). Animals were anesthetized and perfused through the heart with 50 ml of cold saline and 50 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were then removed and were post-fixed over-night. Coronal sections containing hippocampus were stained with the following primary antibodies: anti-β₃ subunit (anti-Caβ₃, Alomone Labs), anti-SMI-32, and anti-GAD. A biotinylated secondary antibody and the avidin/biotin system were used for each antibody followed by a 3,3’-diaminobenzidine reaction. Some of the DAB reactions incorporated a nickel intensification procedure. For gross morphology of the hippocampus, Nissl staining was used. For Western blot analysis, total hippocampal proteins were prepared as described previously (34). 25 μg of protein were loaded per lane and analyzed by SDS-PAGE followed by Western blotting. The following antibodies have been described previously: NMDAR 2A/2B (35) and GluR1/2 (34). The following fusion protein was used for the generation of the following polyclonal antibody: H₆-rat NMDAR1 (amino acids 340−561; 1740 guinea pig). Antibody for α-tubulin was purchased from Sigma.

Statistical Analysis—All data are given as mean ± S.E. Two-way repeated ANOVA, one-way ANOVA, and Student’s t test were used for statistical analyses. p < 0.05 was considered statistically significant.

RESULTS

Normal Gross Morphology of the Hippocampus in the Caβ₃⁻/⁻ Mice—We first examined the cytoarchitectonic divisions in the brain of the Caβ₃⁻/⁻ mice, especially in the hippocampus. The Caβ₃⁻/⁻ mice exhibited normal hippocampal divisions, including CA1, CA2, CA3, and dentate gyrus. No expression of Caβ₃ was observed in the Caβ₃⁻/⁻ hippocampus (Fig. 1A), whereas Caβ₃ was abundant in the wild-type hippocampus as was shown previously (17). The immunoreactivities and the expression patterns of SMI-32 (a neurofilament protein) (Fig. 1B) and GAD (GABA-synthesizing enzyme) (Fig. 1C) were normally observed in the hippocampus of the Caβ₃⁻/⁻ mice as in the Caβ₃⁺/+ mice. In addition, Nissl staining of the coronal brain sections revealed no gross abnormalities in the hippocampus of the Caβ₃⁻/⁻ mice (Fig. 1D).

Enhanced Contextual Fear Conditioning in the Caβ₃⁻/⁻ Mice—Because Caβ₃ 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 Caβ₃ 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 Caβ₃⁺/+ (n = 14) and Caβ₃⁻/⁻ (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 Caβ₃⁻/⁻ mice displayed more freezing behavior than the Caβ₃⁺/+ mice (F₁,26 = 8.36, p < 0.01, two-way repeated ANOVA), indicating an enhanced long term memory of the Caβ₃⁻/⁻ 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 Caβ₃⁻/⁻ mice is limited to the hippocampus-dependent fear conditioning. There was no sig-
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![Images]

FIGURE 1. Histological assessment of the hippocampus of the Cavβ3−/− mouse. A, strong immunoreactivity of Cavβ3 in the Cavβ3+/+ hippocampus indicates that it is highly expressed in the hippocampus, whereas there is no detectable signal in the Cavβ3−/−. Similar levels of SMI-32 (B) and GAD (C) immunoreactivity are observed in the hippocampus of Cavβ3+/+ and Cavβ3−/− 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 Cavβ3+/+ and Cavβ3−/− mice. The scale bars equal 100 μm (A, B, and D) and 50 μm (C). Arrowheads indicate CA1, CA2, and CA3 region in order.

A significant difference in response to variable electric intensities between Cavβ3−/− (n = 7) and Cavβ3+/+ (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 Cavβ3−/− 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, Cavβ3+/+ (n = 17) and Cavβ3−/− 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 Cavβ3+/+ (n = 8) and Cavβ3−/− 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, Cavβ3−/− mice (n = 7) showed increased preference for the novel object compared with Cavβ3+/+ (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 Cavβ3−/− 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 Cavβ3−/− 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 Cavβ3+/+ (n = 7) and Cavβ3−/− (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-
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FIGURE 3. Normal Ca$^{2+}$ currents in hippocampal CA1 pyramidal neurons of Ca$_{\beta3}^{+/-}$/mice. A, representative Ca$^{2+}$ current traces in Ca$_{\beta3}^{+/-}$ and Ca$_{\beta3}^{-/-}$ mice. Ca$^{2+}$ currents were elicited by voltage step depolarizations (+10 mV increments) from −60 mV to +40 mV. Scale bars, 200 pA and 50 ms. B, I-V curve of total Ca$^{2+}$ currents. There are no differences in total current density between genotypes. The current density was estimated by dividing the peak amplitude by the cell capacitance (pA/pF). C, Ca$^{2+}$ current divided by maximum values of Ca$^{2+}$ current (I/I$_{\text{max}}$). D, r value was obtained by fitting current traces evoked at 0 mV to a single exponential curve.

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, Ca$_{\beta3}^{-/-}$ mice (n = 10) exhibited significantly increased preference for cued food compared with Ca$_{\beta3}^{+/-}$ mice (n = 10) (+/+, 71.61 ± 4.72%; −/−, 88.62 ± 3.56%, 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 Ca$_{\beta3}^{-/-}$ mice displayed an enhanced memory in the social transmission of food preference task.

No Change in Ca$^{2+}$ Currents in the Ca$_{\beta3}^{-/-}$/CA1 Pyramidal Neurons—Next we examined whether Ca$^{2+}$ currents (I$_{\text{Ca}}$) are altered or not in the Ca$_{\beta3}^{-/-}$ neurons by whole-cell patch clamp recordings in CA1 pyramidal neurons. Total Ca$^{2+}$ currents were activated by step depolarizations (+10-mV increments) from a holding potential of −60 mV (Fig. 3A). In CA1 neurons from both Ca$_{\beta3}^{+/-}$ and Ca$_{\beta3}^{-/-}$ mice, Ca$^{2+}$ currents reached their maximum amplitudes at ~0 mV (Fig. 3B). Unlike previous studies that showed a reduced Ca$^{2+}$ current density in Ca$_{\beta3}^{-/-}$ neurons (superior cervical ganglion neurons (23), dorsal root ganglion neurons (38), and olfactory sensory neurons (39)), there was no significant difference in the Ca$^{2+}$ current density between Ca$_{\beta3}^{+/-}$ and Ca$_{\beta3}^{-/-}$ 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 Ca$^{2+}$ current divided by maximum values of the Ca$^{2+}$ current (I/I$_{\text{max}}$) (Fig. 3C), and in the time constant (τ) of Ca$^{2+}$ 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 Ca$_{\beta3}^{+/-}$/CA1 neurons.

Normal Intrinsic Firing Properties and AHP Currents in the Ca$_{\beta3}^{-/-}$—As a close coupling was reported by co-immuno-precipitation between Ca$_{\beta}$ and N- or L-type VDCCs in hippocampal neurons (18–20), we measured N- or L-type VDCCs-mediated cellular properties in CA1 neurons. Ca$^{2+}$ influx...
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![Figure 4](image)

**FIGURE 4.** Normal firing properties and normal AHP currents of Cav\(_{\beta3}^{+/+}\) CA1 pyramidal neurons. **A.** Action potentials are generated by increasing depolarizing current injections from +30 to 90 pA. **B.** Number of APs evoked at different current injections. **C.** Interspike intervals of APs. **D.** AP duration of APs. **E.** AHP currents at different sequence of APs. The data are presented as mean ± SEM. Scale bars, 40 pA and 100 ms.

Enhanced NMDAR-dependent LTP in the Cav\(_{\beta3}^{-/-}\) 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. We tried to induce LTP by several different stimulation protocols. LTP was induced by 100-Hz (300 ms and 1 s) or 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 Cav\(_{\beta3}^{-/-}\) compared with that in the Cav\(_{\beta3}^{+/+}\) (−/−; 169.47 ± 7.33% of baseline 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 Cav\(_{\beta3}^{-/-}\) also exhibited more robust potentiation than Cav\(_{\beta3}^{+/+}\) (−/−, 231.92 ± 15.72% of base line at 60 min, \(n = 10\); +/+, 181.74 ± 17.58%; \(n = 8\), \(p < 0.05\), Student’s \(t\) test) (Fig. 6B). Even at short 100-Hz stimulations for 300 ms, enhanced LTP in the Cav\(_{\beta3}^{-/-}\) was also observed (−/−, 139.31 ± 7.35% of base line at 60 min, \(n = 10\); +/+, 114.70 ± 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 Cav\(_{\beta3}^{-/-}\) disappeared under the same stimulation condition, and a similar level of potentiation was induced in the two genotypes (Fig. 6D and E). Together, these results indicate that synaptic transmission was not altered in Cav\(_{\beta3}^{-/-}\) mice (+/+, \(n = 10\); −/−, \(n = 12\)). We next studied the effect of the Cav\(_3\) mutation on paired-pulse facilitation (PPF), a presynaptic form of short term plasticity. PPF is a transient enhancement of neurotransmitter release induced by two closely spaced stimulations. This increase in release is usually attributed to intracellular Ca\(^{2+}\) concentration in the presynaptic terminal following the first stimulus (41, 42). There were no significant differences in all tested interpulse intervals between the Cav\(_{\beta3}^{+/+}\) (\(n = 7\)) and the Cav\(_{\beta3}^{-/-}\) (\(n = 9\)) (Fig. 5D). Taken together, these results indicate that the Cav\(_3\) mutation had no significant effect upon the basal synaptic function and the presynaptic short term plasticity in hippocampal CA3–CA1 synapses.
the increased potentiation in the Caβ3−/− is NMDA-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 AMPA-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 hippocampal proteins 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.

DISCUSSION

In this study, we analyzed the Caβ3-deficient mice with respect to their capacity for learning/memory and synaptic plasticity. Although there was no change in VDCCs currents and basal synaptic transmission, we found that the deletion of Caβ3 caused an increase of NR2B expression and NMDAR activities, including currents and LTP, in the hippocampus and an enhanced capacity for learning and memory. This study
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demonstrates a previously unidentified outcome of the deletion of Ca_{\beta3} in the adult brain.

Yet the Ca_{\beta} subunits of VDCCs have been known to be associated with VDCCs and regulate Ca^{2+} influx through VDCCs by modulating the properties of VDCCs \( \alpha_i \) subunits, including trafficking of channel complexes to the plasma membrane, Ca^{2+} current densities, and voltage-dependent activation or inactivation (4, 5). Of the Ca_{\beta} subtypes, the Ca_{\beta3} 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 Ca_{\beta3}. In superior cervical ganglion neurons, the Ca_{\beta3^{-/-}} showed reduced N- and L-type Ca^{2+} currents relative to the Ca_{\beta3+/+} and shifting of voltage-dependent activation in P/Q-type Ca^{2+} currents (23). In dorsal root ganglion neurons, the Ca_{\beta3^{-/-}} 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 Ca_{\beta3^{-/-}} mice (38). In olfactory sensory neurons, the Ca_{\beta3^{-/-}} 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 Ca_{\beta3} is known to be highly expressed in the hippocampus (17) and has been shown to associate with 42% of the \( \alpha_i \) 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 Ca_{\beta3^{-/-}} mice (supplemental Fig. 1). In addition, there were no clear differences in the patterns of the immunohistological labeling for the \( \alpha_iC \) (Ca_{1.2}) and the \( \alpha_iD \) (Ca_{1.3}) subunits of L-type VDCCs in the hippocampus, between the two genotypes (supplemental Fig. 2). Furthermore, although Ca_{\beta3} in the brain was shown to associate with about 52% \( \alpha_{1B} \) 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 Ca_{\beta3^{-/-}} mice. Therefore, some compensation by other Ca_{\beta} isotypes might have occurred for the deletion of Ca_{\beta3} in the hippocampus of the Ca_{\beta3^{-/-}} mice, as was reported in olfactory sensory neurons of the Ca_{\beta3^{-/-}} mice (39).

Instead, however, we found an increased LTP at hippocampal CA3-CA1 synapses in the Ca_{\beta3^{-/-}} 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 Ca_{\beta3^{-/-}} mice was obliterated. This indicates that the increased potentiation in the Ca_{\beta3^{-/-}} is of the NMDAR-dependent component in LTP, rather than L-type VDCC-dependent. The increased LTP and long term memory in the Ca_{\beta3^{-/-}} 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 Ca_{\beta3^{-/-}} 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 Ca_{\beta3} can be a multifunctional protein as was shown for other Ca_{\beta} isotypes. The studies of crystal structures revealed that Ca_{\beta} subunits belong to membrane-associated guanylate kinase family that has scaffolding functions, suggesting that the Ca_{\beta} 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 Ca_{\beta} 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 Ca_{\beta} from the VDCCs complex has already been demonstrated from the inactivation heterogeneity of VDCCs and reversibility of the interaction with \( \alpha_i \) subunits (51, 52). It was reported that Ca_{\beta} 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 Ca_{\beta} could promote endocytosis of VDCCs by interaction with dynamin (14). A short splice variant of Ca_{\beta4} 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 Ca_{\beta} could function independently from VDCCs without marked influences on the surface expression and voltage-dependent properties of VDCCs. Furthermore, inositol 1,4,5-trisphosphate-mediated signaling was enhanced in Ca_{\beta3}-deficient pancreatic \( \beta \) cells, whereas Ca^{2+} currents of VDCCs were not affected (16). Similarly, Ca_{\beta} were found to internalize Shaker K+ channels by association with dynamin (14). These activities of Ca_{\beta} are considered to be completely independent of VDCCs regulation, and thus indicate that Ca_{\beta} can function as a multifunctional protein by interactions with other proteins. In this light, it might be possible that the Ca_{\beta3} can 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 Ca_{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 Ca_{\beta} is the C-terminal tail region of \( \alpha_i \) subunits of VDCCs (6, 8, 9, 57). In this light, the removal of Ca_{\beta3} 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 Ca_{\beta} isotypes or other developmental compensation, which may have occurred in the Ca_{\beta3^{-/-}} 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$_{\beta3}^{-/-}$ mice (38, 39) could contribute to the phenotypes shown in our results.

Initially, we started investigating the role of the Cav$_{\beta3}$ 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$_{\beta3}$ led to enhanced LTP and capacity for learning and memory in the animal. These phenotypes appear to be due to the increased NMDAR activity in the Cav$_{\beta3}^{-/-}$ mice. Even though the precise mechanism of the enhancement of the NMDAR activity in the Cav$_{\beta3}^{-/-}$ mice is not yet completely understood, our experiments may reveal a potentially novel function of Cav$_{\beta3}$, unrelated to a role associated with VDCCs. Further studies of the relationship, including direct or indirect protein-protein interactions, between Cav$_{\beta3}$ and NMDAR will be needed to confirm this role of Cav$_{\beta3}$ in the adult brain.

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REFERENCES


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