Facilitation versus depression in cultured hippocampal neurons determined by targeting of Ca\textsuperscript{2+} channel Ca\textsubscript{\textalpha}β\textsubscript{4} versus Ca\textsubscript{\textalpha}β\textsubscript{2} subunits to synaptic terminals

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Ca\textsuperscript{2+} channel β subunits determine the transport and physiological properties of high voltage–activated Ca\textsuperscript{2+} channel complexes. Our analysis of the distribution of the Ca\textsubscript{\textbeta} subunit family members in hippocampal neurons correlates their synaptic distribution with their involvement in transmitter release. We find that exogenously expressed Ca\textsubscript{\textbeta} and Ca\textsubscript{\textbeta} subunits distribute in clusters and localize to synapses, whereas Ca\textsubscript{\textbeta} and Ca\textsubscript{\textbeta} are homogenously distributed. According to their localization, Ca\textsubscript{\textbeta} and Ca\textsubscript{\textbeta} subunits modulate the synaptic plasticity of autaptic hippocampal neurons (i.e., Ca\textsubscript{\textbeta} induces depression, whereas Ca\textsubscript{\textbeta} induces paired-pulse facilitation [PPF] followed by synaptic depression during longer stimuli trains). The induction of PPF by Ca\textsubscript{\textbeta} correlates with a reduction in the release probability and cooperativity of the transmitter release. These results suggest that Ca\textsubscript{\textbeta} subunits determine the gating properties of the presynaptic Ca\textsuperscript{2+} channels within the presynaptic terminal in a subunit-specific manner and may be involved in organization of the Ca\textsuperscript{2+} channel relative to the release machinery.

Introduction

High voltage–activated Ca\textsuperscript{2+} channels in neurons consist of several subunits, a pore-forming α subunit (Ca\textsubscript{\textalpha}+), and several auxiliary subunits, including γ\textsubscript{3} and β (Ca\textsubscript{\textbeta}; Catterall, 2000). Ca\textsubscript{\textbeta} subunits are involved in transport of the pore-forming α subunit to the plasma membrane (Dolphin, 2003; Herlitze et al., 2003). Ca\textsubscript{\textbeta} subunits shield an ER retention signal on the α subunit, thereby guiding the pore-forming subunit to the target membrane (Bichet et al., 2000).

Ca\textsubscript{\textbeta} subunits also determine the biophysical properties of the Ca\textsuperscript{2+} channel. The effects of the Ca\textsubscript{\textbeta} subunit family members on the biophysical properties are complex. Four family members have been described (Ca\textsubscript{\textbeta}). P/Q-type channels assembled with Ca\textsubscript{\textbeta} and β subunits in heterologous expression systems are fast inactivating in comparison with Ca\textsubscript{\textbeta} and β\textsubscript{2}-assembled channels (Stea et al., 1994; Fellin et al., 2004; Luvisetto et al., 2004). Ca\textsubscript{\textbeta} has the most dramatic effects on the channel properties, causing the channel to inactivate very slowly. In addition, the Ca\textsubscript{\textbeta} subunit is unique because this subunit can be attached to the plasma membrane via its palmitoylated N-terminal protein domain (Chien et al., 1998).

Several studies also suggest that at least certain Ca\textsubscript{\textbeta} subunit family members can target and function independently of the Ca\textsubscript{\textalpha} subunits at the plasma membrane and other intracellular structures such as the nucleus. For example, these subunits may be involved in gene transcription (Hibino et al., 2003) and the regulation of Ca\textsuperscript{2+} oscillations and insulin secretion (Berggren et al., 2004).

Recently, the crystal structures of the Ca\textsubscript{\textbeta} core domains and the interaction domain between Ca\textsubscript{\textbeta} and Ca\textsubscript{\textalpha} have been determined (Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004). These studies revealed that the Ca\textsubscript{\textbeta} subunits belong to the membrane-associated guanylate kinase family containing Src homology type 3 and guanylate kinase domains (Hanlon et al., 1999; Richards et al., 2004; Rouset et al., 2005). A mutagenesis study of the Src homology type 3 and guanylate kinase domains showed that these domains regulate the inactivation of these Ca\textsuperscript{2+} channels (McGee et al., 2004) but also suggested that Ca\textsubscript{\textbeta} subunits are involved in scaffolding and in the precise localization of Ca\textsuperscript{2+} channel complexes to defined subcellular domains. Indeed, deletion of the nonconserved N and C termini of the Ca\textsubscript{\textbeta} subunit results in a loss of synaptic localization and presynaptic function.
In addition, the isolated N terminus of Cavβ4a is capable of interacting with proteins of the vesicle release machinery (Vendel et al., 2006). All Cavβ subunits are expressed in the brain. Their subcellular distribution within neurons reveals that they are localized to neuronal cell bodies and dendrites. In addition, Cavβ has been suggested to be localized to synaptic terminals (Herlitze and Mark, 2005). However, its precise function for determining synaptic transmission and, in particular, synaptic plasticity is unclear. Therefore, the goal of this study is to analyze the distribution of endogenously and exogenously expressed Cavβ subunits in hippocampal neurons and to correlate their distribution with their effects on synaptic transmission. Our results suggest that Cavβ2a and Cavβ4b subunits are targeted to presynaptic terminals, where they determine whether synapses facilitate or depress.

**Results**

**Distribution of endogenous Cavβ subunits in hippocampal neurons**

We first investigated whether hippocampal neurons in culture express endogenous Cavβ subunits, as would be predicted by the presence of the endogenous high voltage–activated Ca^{2+} channels (Reid et al., 1998; Wittemann et al., 2000). We produced a peptide-derived antibody, which recognizes all β-subunit family members (pan-β antibody). As indicated in Fig. 1A, the antibody recognized specifically Cavβ subunits in hippocampal neurons, as demonstrated by antagonistic action of the epitope peptide (not depicted). Many, but not all, of the puncta colocalize with the synaptic markers synaptobrevin 2 (Fig. 1, A–C) and synapsin 1 (Fig. 1D). The subunits are expressed throughout the neuron with high and uniform staining detected in the soma and proximal dendrites, with more clustered distribution in synaptic areas. We next analyzed whether we could detect Cavβ subunit–specific mRNAs in these neurons and whether we could see quantitative differences among the four different Cavβ mRNAs. As a positive control, we used 18S RNA. Real-time PCR revealed the highest mRNA levels for the Cavβ3 subunits and lower mRNA levels for Cavβ1,2,4 (Cavβ1 ≥ Cavβ4 ≥ Cavβ2; Fig. 1E). The results indicate that all four Cavβ subunits are expressed in hippocampal neurons in culture, which localize to the soma and to synapses.

**Distribution of exogenously expressed Cavβ subunits in hippocampal neurons**

We next analyzed whether the exogenous expression of the Cavβ members resembles the endogenous distribution of the Caβ subunits as determined in Fig. 1 and whether Cavβ subunits can target to synaptic sites when expressed alone in neurons (Fig. 2).

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**Figure 1.** Endogenous distribution and expression of Caβ subunits in cultured hippocampal neurons. (A–C) (A, green) Confocal pictures of the endogenous Caβ subunits detected with a pan-β antibody reveal punctate staining. (B, red) Hippocampal neurons were stained with an anti-synaptobrevin-II antibody and visualized with an AlexaFluo546-coupled secondary antibody. (C) Overlay of A and B demonstrates that the endogenous Caβ subunits are partially colocalized with the synaptic vesicle marker synaptobrevin-II. (right) Boxed areas show that several pan-staining puncta are colocalized with synaptobrevin-II (arrows). Magnification of the indicated areas from the neuron shown on the left. (D) Caβ subunits colocalize with the synaptic marker synapsin-I. Confocal images of the endogenous Caβ subunits in hippocampal neurons visualized with the pan-β antibody (left), synapsin-I visualized with an anti-synapsin-I antibody (middle), and overlay of the two pictures (right) reveal that endogenous Caβ subunits partially colocalize with the presynaptic marker synapsin-I. (E) Endogenous Caβ subunit mRNAs are expressed at different levels in cultured hippocampal neurons. The mRNA expression levels of Caβ1,2a, Caβ3, and Caβ4b were normalized to the mRNA level of the Caβ1b subunit. The bar graph shows that the Caβ3 mRNA level was approximately three times higher than Caβ1b, whereas the Caβ2a expression level was ~50% lower (n = 12; **, P < 0.01) in comparison with Caβ1b. The mRNA expression level for Caβ4b was not different from that of Caβ1b. Error bars represent SEM. Bars (A–C), 25 μm; (D) 5 μm.
We found that the Caβ1b and Caβ3 subunits reveal a more homogeneous distribution, whereas the Caβ2a and Caβ2b subunits are highly clustered (Fig. 2, A–C). When cells expressing these exogenous subunits were immunostained with the synaptic marker synaptobrevin-II or synapsin-1, we found that Caβ2a subunits revealed a higher degree of colocalization with synaptic markers than Caβ2b. Association of the Caβ subunits with the Caα1 subunits predicts that both proteins should be distributed in cytoplasmic as well as membrane regions, which we confirmed by Western blots from cytosolic and membrane fractions of whole rat brain using the pan-Caβ antibody (Fig. 3 C). Exogenous expression of the Caβ subunits revealed a similar distribution, with subtype-specific enrichment within either the cytoplasmic or the membrane fraction (Fig. 3 C). Caβ2a subunits are highly enriched in the membrane fraction, whereas Caβ1b was mostly concentrated in the cytoplasm (Fig. 3 C). Caβ2a and Caβ3 subunits were equally distributed in both fractions (Fig. 3 C).

Because Ca2+ channel Caβ2a and Caβ4b subunits reveal a mainly punctate distribution within the neurons, we wanted to know whether we can detect Caβ subunits in presynaptic terminals on vesicles or vesicular structures (Fig. 4). The high expression levels of the GFP-tagged subunits allowed us to study their localization by immunoelectron microscopy. As a negative control, we used the untagged GFP overexpressed in hippocampal neurons. As shown in Fig. 4, Caβ2a and Caβ4b subunits were detected on vesicular structures (Fig. 4, A and B) and close to presynaptic terminals (Fig. 4, C and D). We also observed that both Caβ2a and Caβ4b were attached to the plasma membrane (Fig. 4 D). In contrast, GFP was found only in the nucleus and outside of the nucleus but was not associated with vesicles or transported to the presynapse (unpublished data). The results suggest that both Caβ2a and Caβ4b subunits are transported to synaptic sites and to the plasma membrane, where they most likely associate with the Caα1 subunits to form channel complexes.

Figure 2. Exogenously expressed Caβ1–4 subunits distribute in different patterns in hippocampal neurons and colocalize to various degrees with presynaptic marker proteins. (A) Fluorescence pattern of neurons from low density hippocampal cultures infected with the indicated GFP-tagged Caβ subunit (i.e., Caβ1–4) reveal either a punctate (Caβ2a and Caβ4b) or a more diffuse, cytosolic staining (Caβ1b and Caβ3). (B) Increased magnification of hippocampal neurites reveal that Caβ2b and Caβ4b are clustered, whereas Caβ1b and Caβ3 are diffusely distributed. (C) The bar graph indicates that neurons overexpressing Caβ2a and Caβ4b mainly reveal punctate staining similar to the endogenous distribution of Caβ subunits, whereas the majority of neurons infected with Caβ1b and Caβ2a or GFP alone do not reveal punctate staining (n = 110–153 for each subunit; *, P < 0.01 compared with GFP). Quantification of the percentage of transfected neurons showing that punctate staining was performed by generating 11–13 randomly chosen fields within each group of neurons analyzed. In each field, the number of total infected neurons was counted, and the percentage of those showing punctate patterns was calculated. The percentages in each group were then averaged. n is equal to the total number of infected neurons counted. (D and E) Caβ2a and Caβ4b reveal differences in their percentages to colocalize with presynaptic marker proteins. (D) Cultured hippocampal neurons were infected with GFP-tagged Caβ2a and Caβ4b and costained with synaptobrevin-II or synapsin (red). Representative confocal images of hippocampal neurites reveal that the fluorescent puncta in Caβ2a- and Caβ4b (green)-expressing neurons reveal a different colocalization percentage with the presynaptic marker (red). (E) Quantification of the GFP puncta containing either Caβ2a or Caβ4b that colocalize with synaptobrevin-II. The percentage of synaptic colocalization is given as the number of Caβ GFP puncta, which colocalized with the number of synaptobrevin-II puncta relative to the amount of GFP puncta within the region of interest analyzed (n = 20–21; *, P < 0.01). Error bars represent SEM. Bars (A), 15 μm; (B and D) 2 μm.
Effect of Cav β subunits on Ca2+ channel currents in HEK293 cells and hippocampal neurons

Cav β subunits determine the biophysical properties of the Ca2+ channel. When expressed with the P/Q-type channel in Xenopus laevis oocytes or HEK293 cells, Cav β subunits determine the time course of inactivation in a subunit-specific manner. Cav β1b- and Cav β2a-assembled channels inactivate rapidly, whereas Cav β4b-assembled channels inactivate slowly (Stea et al., 1994). Cav β4b-assembled channels inactivate with a time course that lies between Cav β1b,3 and Cav β2a. The gating properties of the presynaptic Ca2+ channels determine Ca2+ influx into the presynaptic terminal and, therefore, determine transmitter release and synaptic plasticity, such as facilitation and depression.

Figure 3. Cav β subunits are found in cytoplasmic and membrane fractions in hippocampal neurons. (A and B) Rat whole brains (postnatal day 0–3) were homogenized and fractionated in a discontinuous sucrose gradient. Primary membrane and cytosolic fractions were taken for Western blot analysis. (B) When immunoblotted with the pan-β antibody, the endogenous Cav β subunits were mainly located in the membrane fraction but also found in the cytosolic fraction. (C) Exogenously expressed Cav β subunits revealed a subunit-specific distribution between the cytosolic and membrane fraction. 13–16 h after infection with Cav β subunits, 14-d in vitro hippocampal neurons were harvested, and cell extracts were blotted with anti-GFP antibodies.

Figure 4. Immunoelectron microscopy reveals that Cav β2a and Cav β4b subunits are associated with membranes and vesicular structures and are targeted to presynaptic terminals in hippocampal neurons. (A and B) Immunoelectron microscopy pictures of 14-d in vitro autaptic neurons exogenously expressing GFP-tagged Cav β2a (A) or GFP-tagged Cav β4b (B) subunits. In neurons expressing Cav β2a and Cav β4b subunits, gold particles were found attached or close to vesicular structures (arrowheads). (C and D) In adult hippocampal slices, exogenously expressing GFP-tagged Cav β2a (C) and Cav β4b subunits (D) were found in presynaptic terminals close to synaptic vesicles and attached to the cell membranes (arrowheads). Bars, 50 nm.
We wanted to know how P/Q-type channels assembled with different Caβ subunits open and closed during action potential (AP) waveforms, which we obtained from cultured hippocampal neurons. We expressed Caα2.1 subunits together with the Caα2δ and the various Caβ subunits in HEK293 cells and applied 30 APs to analyze how many channels would be opened during AP trains. To determine the proportion of open channels, we used the following protocol. Based on the voltage dependence of the activation of P/Q-type channels, we applied a 10-ms depolarizing test pulse to a test potential in which ~100% of channels within the cells were open (Herlitze et al., 1996, 1997, 2001; Mark et al., 2000). This value is given by the amplitude of the tail current. We then compared the tail current elicited by the AP to the tail current elicited by the 10-ms depolarization to +100 mV. We were interested in three values. We wanted to know whether activation with the AP waveforms would reveal differences in the opening of the channels when assembled with different Caβ subunits. The results indicated that the AP opens between 55 and 65% of the channels. No considerable differences were observed between channels assembled with the different Caβ subunits (Fig. 5, A and B).

We next compared the ratio between the amount of channels opened by the first and the second AP (Fig. 5 A). By comparing this value, we gain information on differences on the influx of Ca2+ through Ca2+ channels into the presynaptic terminal, which may determine whether synapses facilitate or depress during paired pulses. No differences were detected between channels assembled with different Caβ subunits. We next analyzed whether a 20-Hz train of 30 APs leads to a decrease in channel opening, as would be expected from the inactivation of Ca2+ channels during long, constant depolarizations (Stea et al., 1994; Herlitze et al., 1997). When comparing the proportion of channels opened by the first AP relative to the amount of channels opened by the 30th AP, we found that currents mediated by

![Figure 5. Hippocampal AP waveform protocols detect differences in the amount of open P/Q-type channels assembled with the different Caβ subunits during long 20-Hz stimulations but not for paired pulses. (A) HEK293 cells expressing Caα2.1, Caα2δ, and one of the four Caβ1b, Caβ2a, Caβ3, and Caβ4b subunits were held at −60 mV, and Ca2+ currents were elicited by a 20-Hz AP train 1 s after a prepulse to 100 mV for 10 ms. This prepulse was given to open ~100% of the Ca2+ channels expressed in the cell (top). The tail current elicited by the prepulse was used to relate the tail current elicited by the AP to gain an understanding about the percentage of channels opened by the AP. The example whole cell currents (top) and the bar graph (bottom) indicate that approximately the same amount of channels were opened by the first AP and the second AP for the Caβ1–4-assembled P/Q-type channels analyzed. (B) Increased time resolution of the underlying current elicited by the AP. The deactivation time of the tail currents can be fitted with a single exponential. Only currents were included and analyzed in the experiments described in A–E, which reveal fast deactivation kinetics and no change in the deactivation kinetics between the first and the last tail current elicited. (C) Examples of P/Q-type channel currents assembled with Caβ1–4 subunits during a 20-Hz 30-pulse AP waveform train. (D) Relative ICa2+ ratio for the P/Q-type channel currents assembled with Caβ1–4 subunits assembled P/Q-type channels analyzed. (E) Increased time resolution of the underlying current elicited by the AP. The deactivation time of the tail currents can be fitted with a single exponential. Only currents were included and analyzed in the experiments described in A–E, which reveal fast deactivation kinetics and no change in the deactivation kinetics between the first and the last tail current elicited. (C) Examples of P/Q-type channel currents assembled with Caβ1–4 subunits during a 20-Hz 30-pulse AP waveform train. (D) Relative ICa2+ ratio for the P/Q-type channel currents assembled with Caβ1–4 subunits. The tail current amplitudes were related to the tail current elicited by the first AP. (E) Comparison of the relative amplitude of the tail currents elicited by the first and 30th AP during the 20-Hz AP train reveals that currents through P/Q-type channels assembled with Caβ1b and Caβ3 subunits are relatively smaller than currents through P/Q-type channels assembled with Caβ2a and Caβ4b. Error bars represent SEM, * P < 0.05; **, P < 0.01.
Ca_β_11_ and Ca_β_2- and Ca_β_3-assembled channels are reduced by 10–15% (Fig. 5, D and E). In contrast, currents mediated by Ca_β_2-assembled channels are reduced by 2% (Fig. 5, D and E), and currents mediated by Ca_β_2-assembled channels increased by 5% (Fig. 5, D and E). Thus, P/Q-type channels assembled with different β subunits reveal substantial differences in the amount of channel opening during long AP trains.

It has been shown that the biophysical properties of P/Q-type channels depend on the cellular environment in which the pore-forming Ca_α_1 subunit is expressed (Tottene et al., 2002). We found that the maximal current elicited by a 500-ms-long voltage ramp is shifted to more negative potentials (around 20 mV) in neurons expressing non-L-type channels in comparison with HEK293 cells expressing P/Q-type channels encoded by the Ca_α_2.1, Ca_α_δ, and Ca_β subunits (Fig. 6 A). Therefore, Ca_β subunit-mediated effects on presynaptic Ca^2+ channel (non-L-type) inactivation may be shielded in neurons by, for example, neuronal-specific channel-interacting proteins. To show that the Ca_β subunits (i.e., Ca_β_2a and Ca_β_3b) also change the biophysical properties of non-L-type channels in hippocampal neurons, we analyzed the Ca^2+ channel inactivation of somatic neuronal non-L-type channels. As shown in Fig. 6 B, the exogenous expression of Ca_β_2a and Ca_β_3b subunits reduce non-L-type channel inactivation in a subunit-specific manner. Ca_β_3b subunit expression leads to an increase in the non-L-type current during a 100-ms test pulse from −60 to 0 mV, whereas neuronal non-L-type currents in the presence of Ca_β_3b subunits do not change in size (Fig. 6 B).

Effects of Ca_β subunits on synaptic transmission

Our results on the recombinant P/Q-type channels and endogenous neuronal Ca^2+ channels suggest that Ca^2+ influx into the presynaptic terminal should be altered during long 20-Hz AP trains but not for paired-pulse responses. We analyzed the effect of the Ca_β subunits on paired-pulse facilitation (PFF) by comparing the first and second excitatory postsynaptic current (EPSC; defined as the paired-pulse ratio [PPR]) and analyzed the effect on synaptic depression by comparing the first and last EPSCs (averaged 27–30 EPSCs) within a 20-Hz stimulation protocol when 30 pulses were elicited in 4 mM of extracellular Ca^2+ (Fig. 7, A and C). Because we did not observe effects on synaptic transmission when Ca_β_1b and Ca_β_3 subunits were expressed in our initial studies (unpublished data), we only analyzed Ca_β_2a and Ca_β_3b subunit effects on synaptic transmission in the following experiments. According to our results regarding the effects of Ca_β subunits on the inactivation properties of Ca_2 channels, we found that Ca_β_2b subunits did not change the PPR as expected from the aforementioned biophysical analysis. However, Ca_β_3b subunits increased the PPR, leading to facilitation (Fig. 7, A and C).

We next analyzed whether the Ca_β_2a and Ca_β_3b subunits influence synaptic transmission during longer AP trains. The biophysical analysis predicts that in the presence of Ca_β_2a and Ca_β_3b subunits, Ca^2+ influx into the presynaptic terminal should be increased as a result of the noninactivating properties of the presynaptic Ca^2+ channels in comparison with Ca_β_1b and Ca_β_3 subunits. The increased Ca^2+ influx may cause more vesicle depletion (depression) and may influence asynchronous transmitter release, which has been shown to be proportional to the residual [Ca^2+]. (Atluri and Regehr, 1998). Analysis of the synaptic responses during 30 20-Hz AP trains revealed that Ca_β_2a- and Ca_β_3b-expressing neurons show larger depression in comparison with wild-type neurons (Fig. 7, A–C) Note that the amount of depression is related to the largest EPSC compared with the minimal EPSC at the end of the stimulus train. The largest EPSC in noninfected neurons and Ca_β_2a-expressing neurons is the EPSC elicited by the second pulse. Therefore, depression is significantly larger for Ca_β_3b (0.34 ± 0.01; n = 14) as well as for Ca_β_3b (0.49 ± 0.03; n = 15) in comparison with noninfected neurons (0.7 ± 0.01; n = 15). To determine whether

![Figure 6](image-url)

**Figure 6.** Ca_β subunits expressed in hippocampal neurons change the biophysical properties of the endogenous non-L-type channels. (A) The activation of non-L-type channels in hippocampal neurons is shifted to more negative potentials when compared with P/Q-type channels exogenously expressed in HEK293 cells. (top) Example current traces (IV curve) of non-L-type currents from hippocampal neurons in comparison with currents through P/Q-type channels (Ca_α_2.1, Ca_α_δ, and Ca_β subunits) expressed in HEK293 cells elicited by 500-ms voltage ramps from −60 to 90 mV. (bottom) Diagram of the voltage at which the peak current appears during the voltage ramp for P/Q-type channels expressed in HEK293 cells and non-L-type currents from hippocampal neurons in the presence or absence of Ca_β subunits. (B) The inactivation properties of non-L-type channels in hippocampal neurons are changed in the presence of Ca_β_2a and Ca_β_3b subunits. (left) Example traces of non-L-type currents elicited by a voltage pulse from −60 to 0 mV reveals that in the presence of Ca_β_2a and Ca_β_3b subunits, inactivation is slowed. (right) Diagram of the current change (percentage) within the 100-ms current trace. The current at the beginning of the test pulse (10 ms) is compared with the current at the end of the test pulse (95 ms). Error bars represent SEM. *, P < 0.05; **, P < 0.01.
Cavβ2a- and Cavβ4b-expressing neurons reveal more vesicle depletion during AP trains, we compared the readily releasable pool size before and after 20-Hz train stimulations. As shown in Fig. 7 (E and F), the pool size is substantially reduced in Cavβ2a-expressing neurons (12 ± 3.5%) and is slightly reduced in Cavβ4b-expressing neurons (9 ± 2.7%) in comparison with control neurons (3 ± 2.2%). However, the Cavβ4b effects were not substantial. To further verify that Cavβ2a- and Cavβ4b-expressing neurons may increase the Ca2+ influx into the presynaptic terminal, we analyzed the asynchronous release. We found that onset of the asynchronous release was much faster and the amount of asynchronous relative to the phasic release at the beginning of the AP train was increased in Cavβ2a- and Cavβ4b-expressing neurons in comparison with control neurons (Fig. 7 G). Although the total amount of phasic and asynchronous release (Fig. 7 H) as well as the mean EPSC amplitude (Fig. 7 D) were slightly increased in Cavβ2a-expressing neurons in comparison with control and Cavβ4b-expressing neurons, the differences were not substantial.

These aforementioned results support the idea that during AP trains, the Ca2+ influx into the presynaptic terminal is larger in the presence of Cavβ2a and Cavβ4b subunits. A larger Ca2+ influx into the presynaptic terminal during AP trains in Cavβ2a and Cavβ4b subunit–expressing neurons should also result in faster
vesicle recycling (Stevens and Wesseling, 1998). To test this hypothesis, we repeated the experiments described in Stevens and Wesseling (1998). We first analyzed the recovery of the readily releasable vesicle pool (RRP) after RRP depletion without 20-Hz stimulation trains applied during depletion. No differences were found for the recovery of the RRP regardless of whether Caβ2a or Caβ2b subunits were expressed in the neurons (Fig. 8, A and B). Also, recovery of the EPSC after RRP depletion was not different between neurons expressing or not expressing Caβ2a and Caβ2b subunits (Fig. 8, C and D), suggesting that exogenously expressed Caβ2a and Caβ2b subunits most likely do not interfere with the vesicle recycling. We next analyzed the RRP recovery after 20-Hz stimulation trains were applied during the initial sucrose application (Fig. 8 E). We confirmed the observation described by Stevens and Wesseling (1998) that the RRP recovery for all neurons analyzed (regardless of whether Caβ subunits were expressed or not) was accelerated by the 20-Hz stimulus train (Fig. 8, E and F). Interestingly, RRP recovery was faster in Caβ2a and Caβ2b subunit–expressing neurons in comparison with control neurons (τrec without 20-Hz train stimulation: control = 11.4 s, Caβ2a = 9.1 s, and Caβ2b = 12.6 s; τrec after 20-Hz train stimulation: control = 4.1 s, Caβ2a = 1.8 s, and Caβ2b = 1.6 s), suggesting again that Ca2+ influx into the presynaptic terminal is increased during 20-Hz stimulation trains in Caβ2a and Caβ2b subunit–expressing neurons.

Although the exogenous expression of Caβ subunits determines the synaptic responses during long AP trains according to the biophysical properties of the assembled presynaptic Ca2+ channels, the induced facilitation by Caβ2a during paired pulses cannot be explained by the biophysical properties of presynaptic Ca2+ channel assembled with the Caβ2a subunit. However, this may suggest that in the presence of Caβ2a subunit,
the Ca\textsuperscript{2+} dependence of the vesicle release is altered, which may result in a reduced release probability (Thomson, 2000). Therefore, we compared the release probability of noninfected and Ca\textsubscript{\beta2a} as well as Ca\textsubscript{\beta2b}-expressing neurons. The release probability can be examined by comparing the RRP with the number of vesicles elicited by a single AP. The RRP size is determined by application of a hypertonic sucrose solution (Rosenmund and Stevens, 1996). As shown in Fig. 9, we found that the release probability in the presence of Ca\textsubscript{\beta2b} was reduced in comparison with noninfected and Ca\textsubscript{\beta2a}-expressing neurons. No differences in the mean RRP and EPSC size were detected between the neurons expressing different Ca\textsubscript{\beta} subunits, probably because only a small number of cells were analyzed.

The relationship between the Ca\textsuperscript{2+} influx into the presynaptic terminal and the vesicle release is approximately given by the following equation: vesicle release \( \propto \left[ \text{Ca}^{2+}\right]^{H} \). The Hill coefficient is defined as the Ca\textsuperscript{2+} cooperativity. The Ca\textsuperscript{2+} cooperativity in many synapses is high (three to four), indicating that a small change in Ca\textsuperscript{2+} channel domains necessary for efficient vesicle release. Thus, in our experiments, the Hill coefficient gives an indirect measure of the Ca\textsuperscript{2+} influx through presynaptic Ca\textsuperscript{2+} channels relative to the transmitter release. This means that a change in the number, localization, or organization of the presynaptic Ca\textsuperscript{2+} channels most likely results in a change in Ca\textsuperscript{2+} dependence of the transmitter release. Interestingly, in the presence of the Ca\textsubscript{\beta2b} subunits, the Ca\textsuperscript{2+}-dependent transmitter release dose-response curve became more shallow, with a small change in the half maximal \([\text{Ca}^{2+}]_o\) concentration (EC\textsubscript{50}) when compared with wild-type neurons or neurons exogenously expressing Ca\textsubscript{\beta2a} subunits (Fig. 9). Because the Ca\textsubscript{\beta2b} subunit particularly changed the cooperativity of the transmitter release, this result may suggest that Ca\textsubscript{\beta2b} is involved in organization of the Ca\textsuperscript{2+} channel domains necessary for efficient vesicle release. For example, Ca\textsubscript{\beta2b}-assembled channels may be further apart from the release machinery. If this is the case, synaptic transmission in Ca\textsubscript{\beta2b}-expressing neurons should be more sensitive to the slow Ca\textsuperscript{2+} buffer EGTA. Indeed, we found that when 10 mM EGTA was applied intracellularly or 50 \textmu M EGTA-AM was applied extracellularly, the EPSC amplitude was substantially more reduced in Ca\textsubscript{\beta2b}-expressing neurons to 60 and 93\%, whereas in Ca\textsubscript{\beta2a}-expressing neurons and control neurons, the EPSC amplitude was reduced only by 50 and 87–89\% (Fig. 9, E and F).

Figure 9. Ca\textsubscript{\beta2b} subunits expressed in hippocampal neurons reduce the synaptic release probability, change the Ca\textsuperscript{2+}-dependent transmitter release, and are more sensitive to EGTA. (A, left) Representative EPSC traces evoked by 2-ms depolarizing pulses from \(-60\) to \(10\) mV are shown for noninfected and Ca\textsubscript{\beta2a}- and Ca\textsubscript{\beta2b}-infected neurons. (right) Representative traces of the hypertonically mediated release of quanta from the same neuron shown on the left upon the application of 500 mM sucrose for 4 s. (B) Probability of synaptic vesicle release was evaluated by calculating the ratio of release evoked by the AP to that evoked by hypertonic sucrose. In autaptic neurons infected with Ca\textsubscript{\beta2b}, the vesicular release probability is significantly reduced compared with noninfected or Ca\textsubscript{\beta2a} subunit–infected neurons. (C) Representative EPSC traces elicited by the application of increasing extracellular Ca\textsuperscript{2+} concentrations of autaptic hippocampal neurons expressing Ca\textsubscript{\beta2b} subunits. (D) Dose-response curve of the EPSC amplitude by increasing extracellular Ca\textsuperscript{2+} concentrations. The Ca\textsuperscript{2+}-dependent EPSC responses of noninfected, Ca\textsubscript{\beta2a}, or Ca\textsubscript{\beta2b}-infected neurons were free fitted according to the Hill equation \([\text{EPSC} = \text{EPSC}_{\text{max}}/\{1 + \text{EC}_{50}/([\text{Ca}^{2+}]_o)^H\}]\). The EPSCs were then normalized to the maximal EPSC given by each fit. The mean normalized EPSCs for the given Ca\textsuperscript{2+} concentrations are shown. The curves again were fitted according to the Hill equation. The Hill coefficients are 2.7 ± 0.4 for control and Ca\textsubscript{\beta2a}-infected neurons and 1.9 ± 0.4 for Ca\textsubscript{\beta2b}-infected neurons. (E) Representative EPSC traces before and after a 50-\textmu M EGTA-AM application evoked by two 2-ms depolarizing pulses from \(-60\) to \(10\) mV within 50 ms are shown for noninfected and Ca\textsubscript{\beta2a}- and Ca\textsubscript{\beta2b}-infected neurons. (F) Bar graph of the remaining EPSC amplitude after 10 mM EGTA was applied intracellularly for 20 min (top) and after 50 \textmu M EGTA-AM was applied extracellularly for 15 min (bottom). Error bars represent SEM. * \(P < 0.05\).
Discussion

In this study, we investigated the targeting and function of Ca,β subunits in hippocampal neurons. We found that Ca,β2a and Ca,β2b are sufficiently targeted to synaptic sites, where they influence synaptic transmission during long AP trains according to the biophysical properties that these subunits induce in the presynaptic Ca2+ channel. During paired pulses, Ca,β2a subunits also altered the Ca2+ dependence of transmitter release. The physiological consequences and implications of the findings are discussed below.

Targeting of Ca,β subunits to the plasma membrane and synaptic terminals

We show that Ca,β2a and Ca,β2b are targeted to synaptic sites and colocalize with synaptic markers. All Ca,β subunits (exogenously and endogenously expressed) are found to various degrees in cytoplasmic and membrane fractions, as suggested by an overexpression study of Ca,β subunits in HEK293 cells (Chien et al., 1998). In particular, Ca,β2a subunits are associated with the membrane fraction, as predicted from their N-terminal located palmitoylation site (Dolphin, 2003; Herlitze et al., 2003). This is in agreement with previous studies performed in HEK293 cells in which palmitoylated Ca,β2a subunits reach the plasma membrane independently of the Ca,α1 subunit (Chien et al., 1998; Bogdanov et al., 2000). Ca,β2a subunits could also be found on vesicular structures, supporting the view that they most likely are associated with Ca,α1 subunits, where they are transported as preassembled channel complexes to synaptic sites (Ahmar et al., 2000; Shapira et al., 2003). Our studies for Ca,β1a and Ca,β1 reveal that these subunits, when expressed alone, distribute more homogenously in neurons and do not substantially influence the synaptic parameters analyzed. The reason for this could be that Ca,β1a and Ca,β1 are not sufficiently transported to the presynaptic terminals as suggested by Maximov and Bezprozvanny (2002). On the other hand, because Ca,β1 is the main mRNA detected in hippocampal neurons, most synaptic Ca2+ channels could be assembled with Ca,β1 subunits. Therefore, the biophysical properties of the presynaptic Ca2+ channels would not be affected by either Ca,β1a and Ca,β1, because the biophysical differences of channels assembled with these subunits are small.

Ca,β subunits may determine synaptic plasticity during longer AP trains as a result of the effects on the inactivation properties of the presynaptic Ca2+ channel complexes

Ca,β in particular determines the time course of inactivation of high voltage–activated Ca2+ channels. How P/Q-type channels assembled with different Ca,β subunits behave when AP waveforms derived from hippocampal neurons are used as command potentials has not been studied before. Interestingly, we did not detect substantial differences in the opening of the channels for the first two APs, which would determine the Ca2+ influx into the presynaptic terminal during paired pulses underlying short-term synaptic plasticity, but found that Ca,β1a- and Ca,β3-assembled channels exhibited substantial differences in the proportion of channels open after 30 APs or longer trains when compared with the Ca,β2a- and Ca,βα2,-assembled channels (20 Hz; Fig. 5 D). We have to point out that the determination of the biophysical properties of the P/Q-type channel in HEK293 cells cannot directly be compared with the effects these subunits have on the native presynaptic Ca2+ channels. For example, Tottene et al. (2002) showed that the maximal current amplitude (when the peak current was analyzed with voltage step protocols) of the pore-forming human Ca,α2.1 subunit expressed in neurons from Ca,α2.1 knockout mice was shifted by ~20 mV when compared with the same channel subunit coexpressed with Ca,α2.3β3 and Ca,β2a in HEK293 cells. A similar shift in the maximal current amplitude was seen in our experiments when we compared the voltage ramps of rat Ca,α1.2, Ca,α2.3, and Ca,β2a-assembled channels in HEK293 cells with the non-L-type currents elicited by voltage ramps in noninfected or Ca,β subunit–infected neurons. This indicates that non-L-type currents in neurons differ in their biophysical properties probably because of cell type–specific interacting proteins and variations as well as combinations of splice variants contributing to the non-L-type current. The differences in channel opening and, therefore, Ca2+ influx correlate well with the observed effects Ca,β subunits have on synaptic depression, asynchronous release, and activity-dependent RRP recovery.

Synaptic depression can be achieved via various cellular mechanisms. Therefore, an increase in Ca2+ influx leading to faster vesicle depletion is only one possibility (Zucker and Regehr, 2002). Synaptic depression can also be independent of vesicle depletion. For example, a decrease in presynaptic Ca2+ influx into the calyx of Held is the major cause of synaptic depression at this synapse type (Xu and Wu, 2005). In addition, a reduction in the AP amplitude during high repetitive firing (>20 Hz) has been correlated with a reduction in the transmitter release (Brody and Yue, 2000). Because we did not observe any change in the AP amplitude when we elicited and measured 20-Hz AP trains in the presence or absence of Ca,β2a and Ca,βα2 subunits, a decline in AP amplitude is most likely not involved in the depression effects observed (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200702072/DC1). Because our synaptic terminals are too small to directly record the Ca2+ influx, we cannot exclude the possibility that the presynaptic Ca2+ influx into the terminal is reduced. However, the decrease in channel inactivation, particularly for Ca,β2a subunit–assembled channels, correlated with the faster RRP recovery and faster onset of asynchronous release does not agree with this mechanism but rather suggests a larger Ca2+ influx into the presynaptic terminal.

Ca,βα2 subunits change the cooperativity of transmitter release

Exogenous expression of Ca,βα2 subunits induced PPF. PPF occurs at low release probability synapses during high frequency stimulation and is associated with a restricted Ca2+ influx during the first AP accompanied by a build up in presynaptic Ca2+ concentration and, thus, an increase in the synaptic release probability once the second AP reaches the presynaptic terminal (Thomson, 2000; Zucker and Regehr, 2002). To analyze whether the increase in PPF in the presence of Ca,βα2 subunits could

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account for a reduction in channel opening caused by Ca,β2αβ, we examined the possibility of detecting differences in the amount of channels opened by a hippocampal AP. We could not detect substantial differences between the Ca,β-assembled channels during the paired-pulse protocol used. To provide an explanation for the facilitation behavior of Ca,β3-expressing synapses, we analyzed several parameters, including Ca2+ dependence of the transmitter release, the effect of the expression of Ca,β subunits on the contribution of N- and P/Q-type channels to synaptic transmission, and somatic non-L-type currents. We found that the expression of Ca,β4 changes the shape of the Ca2+ response curve, which is most likely not correlated with a change in the ratio between the P/Q- or N-type channel or a Ca,β4 channel-specific effect on the terminal (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200702072/DC1). This result suggests that in the presence of Ca,β4 subunits at the presynaptic terminal, the cooperativity of the Ca2+-dependent transmitter release is changed. Recently, the cooperativity of the transmitter release was determined using the same rat hippocampal autapse system. The cooperativity was estimated to around 3 (Reid et al., 1998), a value which we determined and confirmed in our study of wild-type and Ca,β2-expressing neurons. The authors did not find a difference in the contribution between N- or P/Q-type channels. This is important to note because Ca,β4 could preferentially assemble with the other channel type. For example, the preferential assembly with N- or P/Q-type channels would have important implications for the synaptic transmission at the calyx of Held, where N-type channels are suggested to be further apart from the release site than P/Q-type channels (Wu et al., 1999). The change in cooperativity in the presence of Ca,β4 subunits may suggest that the coupling between the Ca2+ channels and the release machinery is affected or that the Ca2+ channels are more distant from the release site. The idea is supported by our finding that Ca,β2α-expressing neurons are more sensitive to the slow Ca2+ chelator EGTA. This is an important finding given the recent observation that the N terminus of the Ca,β4 subunit can bind synaptotagmin and the microtubule-associated protein 1A (Vendel et al., 2006). This raises the possibility that the Ca,β4 subunit is creating a Ca,β subunit–specific anchor between the Ca2+ channel and the synaptic release machinery (Weiss, 2006), whereas the Ca,β4 subunit would not. Therefore, Ca,β4 subunit–assembled channels might be further apart from the release machinery or may change the placement of the readily releasing vesicle next to the Ca2+ channels, which may cause the change in the Ca2+ response curve. In fact, it has been suggested recently that recruitment and placement of the synaptic vesicles to sites where Ca2+ channels cluster are important for rapid neurotransmitter release (Wadel et al., 2007).

### Physiological consequences of neuronal Ca2+ channels assembled with different Ca,β subunits

Ca,β2αβ-subunit–specific effects on synaptic transmitter release (i.e., facilitation and/or depression) will arise if a certain subunit is abundant in a neuronal circuit or synapse. For example, in the thalamus, a brain region that is critical for seizure activity, high expression levels of Ca,β4 subunits are found, whereas Ca,β1,3 subunits seem to be absent or at a lower abundance (Tanaka et al., 1995; Burgess and Noebels, 1999). Loss of Ca,β4 subunit function results in absence seizure epilepsy correlated with a reduced excitatory synaptic transmission in the thalamus (Caddick et al., 1999). Ca,β2 subunits have been suggested to play a crucial role for Ca,1.4 function at the ribbon synapse of the outer plexiform layer of the retina, where these channels mediate glutamate release, whereas the role of Ca,β2 within the brain is poorly understood. Because Ca,β subunits are targets of protein phosphorylation and regulate the trafficking of the Ca2+ channels (Dolphin, 2003; Herlitze et al., 2003), it can be expected that activity-dependent trafficking of specific Ca,β subtypes in and out of synaptic terminals may occur as an important mechanism for the regulation of synaptic plasticity within a presynaptic terminal.

### Materials and methods

#### Cell culture

Microisland and cortical cultures of hippocampal neurons were prepared according to a modified version of published procedures (Bekkers and Stevens, 1991). In brief, hippocampal CA1-CA3 neurons from newborn rats (postnatal day 0–3) were enzymatically dissociated in 2 U/ml DME plus papain (Worthington) for 60 min at 37°C. Dissociated neurons were either plated onto astrocyte-covered poly-L-lysine/collagen (Sigma-Aldrich)-treated microislands that were prepared 3–5 d before plating (autaptic cultures) or were plated onto poly-L-lysine/collagen-treated coverslips that were placed inverted over astrocyte feeder cells (cortical cultures). Neuronal cultures were grown in Neurobasal-A media (Invitrogen) supplemented with 4% B-27 (Invitrogen) and 2 mM Glutamax (Invitrogen) for 12–15 d.

#### Immunocytochemistry and imaging

Contingent hippocampal cultures were prepared as described in the previous section and were infected with GFP-tagged Ca,β subunits. 12–18 h after infection, neurons were fixed with 4% PFA and permeabilized with 0.2% Triton X-100 in PBS. Anti–synaptobrevin-II (SY5) and antisyntapsin (Invitrogen) antibodies were used to label the synaptic markers. Neurons were incubated with the primary antibody overnight at 4°C, washed, and incubated with AlexaFluor568-conjugated secondary antibody (Invitrogen) for 30 min at room temperature. Cells were embedded in Prolong Gold Antifade (Invitrogen). Images were acquired with a confocal microscope (LSM 510; Carl Zeiss Microlmaging, Inc.) mounted on an inverted microscope (Axiowert 200M; Carl Zeiss Microlmaging, Inc.). Images were acquired with a 63× oil plan Apo NA 1.4 objective at room temperature, processed with the built-in LSM 510 software (version 3.5; Carl Zeiss Micromaging, Inc.), and analyzed by using VOLUCITY software (Improvement).

#### Pan-β antibody

Polyclonal anti–pan-β antibody was raised by Harlan Bioproduct for Science according to a published procedure (Vance et al., 1998). In short, a highly conserved peptide sequence presented in all β subunits (CE5YTSRFSDQDSSELEEDRE) was synthesized and a standard 112-d protocol was used for polyclonal antibody production (Harlan Bioproduct for Science). The specificity of the product was documented with Western blots using rat brain homogenate as well as homogenates of HEK293 cells expressing Ca,β3α, Ca,β3β, Ca,β3γ, or Ca,β4 subunit and resulted only in bands with desired molecular weights.

#### Electrophysiology and analysis

For HEK293 cell recordings, HEK293 cells (tS2A21 cells) were transfected with the Ca2+ channel subunits Ca,α2,1.1 and Ca,α2,δ with Ca,β3α, Ca,β3β, and Ca,β4 and with GFP to identify positively transfected cells (molar ratio of 2:1:1:0:25). Whole cell recordings were performed as described previously (Li et al., 2005). For EPSC recordings, only dots containing a single neuron forming excitatory synapses (autapses) were used using an EPC-9 amplifier (HEKA). Recordings were performed at room temperature.
For EPSC measurements as well as for recordings of Ca2+ currents in HEK293 cells, the extracellular recording solution contained 172 mM NaCl, 2.4 mM KCl, 10 mM Hapes, 10 mM glucose, 4 mM CaCl2, and 4 mM MgCl2, pH 7.3; the internal solution contained 145 mM potassium gluconate, 15 mM Hapes, 1 mM potassium EGTA, 4 mM Na-ATP, and 0.4 mM Na-GTP, pH 7.3. For EGTA experiments (Fig. 9, E and F), the internal solution contained 10 mM potassium EGTA (Sigma-Aldrich), or 50 μM EGTA-AM (Invitrogen) was applied 15 min before recording to the extracellular recording solution. Currents were elicited by a 2-ms-long test pulse to 10 mV and recorded and analyzed as described previously (Wittmann et al., 2000). For recordings using various extracellular Ca2+ concentrations (extracellular [Ca2+]o), solutions containing different extracellular [Ca2+]o were applied directly onto the recorded neurons by using a fast-flow perfusion system (AlaScientific). Non–L-type channel recordings in cultured hippocampal neurons were performed as previously described (Li et al., 2005; Han et al., 2006). The internal recording solution contained 120 mM N-methyl-D-glucamine, 20 mM tetraethylammonium-Cl, 10 mM Hapes, 1 mM CaCl2, 14 mM phosphocreatine (Tris), 4 mM Mg-ATP, 0.3 mM Na2GTP, and 11 mM EGTA, pH 7.2, with methanesulfonic acid. The external solution contained 145 mM tetraethylammonium, 10 mM Hapes, 10 mM CaCl2, and 15 mM glucose, pH 7.4, with methanesulfonic acid. In addition, 1 μM tetrodotoxin (Sigma-Aldrich) and 5 μM nimodipine (Sigma-Aldrich) were added to the external solution to block voltage-dependent Na+ channels and L-type Ca2+ channels. Non–L-type currents were elicited by 500-ms voltage clamp ramps from −60 to 90 mV with 1-min intervals and 50-ms current pulses from −20 to +20 mV (Fig. 6 B). Here, capacitative and tail currents were subtracted after the experiment. The sizes of RRs were measured according to published procedures (Rosenmund and Stevens, 1996; Han et al., 2006). In short, 500 mM sucrose was applied directly onto the recorded autaptic neurons for 4 s by using a fast-flow perfusion system (AlaScientific). The EPSC and RRP charge was calculated by integrating the currents elicited by the single AP or the successive current injections.

The asynchronous and phasic release was calculated as described in Otsu et al. (2004). In brief, we estimated the phasic release by integrating the EPSC after each pulse within the 20-Hz stimulation protocol after subtraction of a baseline value measured 1 s before each test pulse. The asynchronous release was calculated by subtracting the phasic release from the total integrated current for each EPSC. The holding current was subtracted before integration in every experiment. Statistical significance throughout the experiments was evaluated with analysis of variance using Igor Pro software (WaveMetrics). Standard errors are mean ± SEM.

Quantitative real-time PCR

107 cells of acutely dissociated hippocampal neurons were plated on 10 mM Hapes, 1 mM CaCl2, 14 mM phosphocreatine (Tris), 4 mM Mg-ATP, 0.3 mM Na2GTP, and 11 mM EGTA, pH 7.2, with methanesulfonic acid. The external solution contained 145 mM tetraethylammonium, 10 mM Hapes, 10 mM CaCl2, and 15 mM glucose, pH 7.4, with methanesulfonic acid. In addition, 1 μM tetrodotoxin (Sigma-Aldrich) and 5 μM nimodipine (Sigma-Aldrich) were added to the external solution to block voltage-dependent Na+ channels and L-type Ca2+ channels. Non–L-type currents were elicited by 500-ms voltage clamp ramps from −60 to 90 mV with 1-min intervals and 50-ms current pulses from −20 to +20 mV (Fig. 6 B). Here, capacitative and tail currents were subtracted after the experiment. The sizes of RRs were measured according to published procedures (Rosenmund and Stevens, 1996; Han et al., 2006). In short, 500 mM sucrose was applied directly onto the recorded autaptic neurons for 4 s by using a fast-flow perfusion system (AlaScientific). The EPSC and RRP charge was calculated by integrating the currents elicited by the single AP or the successive current injections.

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Quantitative real-time PCR

107 cells of acutely dissociated hippocampal neurons were plated on poly-L-lysine–collagen–coated plates for continental culture as described in the Cell culture section. The total RNA was extracted from 14-d-old primary cultured neurons with the RNAeasy Mini kit (Qiagenet) and purified with on-column DNase digestion using the RNase-Free DNase Set (Qiagenet). For RTPCR, 1 μg RNA was used for reverse transcription with the Advantage RT-for-PCR kit (BD Biosciences) to generate 100 μl cDNA, and 3 μl of the final RT product was used for real-time PCR of each β subunit. Real-time PCR quantification was performed on the iCycler iQ Detection System (Bio-Rad Laboratories) with Cybr green dye (Bio-Rad Laboratories). The DNA fragments of Caβ1β, Caβ2a, Caβ3, and Caβ4 were amplified from cDNA with the following primer pairs: Caβ1β forward (GGCTGTAGGAGTTGTTTCCAT) and Caβ1β backward (TGTCAGCTGATCGTTGG); Caβ2a forward (CTACGCGACGGTGGTGG) and Caβ2a backward (TTCAGGAGTGGTCA); Caβ3 forward (CAGGAGTGGATGATGACA) and Caβ3 backward (CGTTCACGCATCCCCAGGG); Caβ4 forward (TGGGTGACTGGTCAAGA) and Caβ4 backward (TCCAGCGGCTCTTCACG). Procedures and conditions for reverse transcription, PCR amplification, and labeling, etc., were the same as for cultured neurons.

cDNAs and virus production

Rat Caβ1β, Caβ2a, Caβ3, and Caβ4 were gifts from T. Snutch (University of British Columbia, Vancouver, Canada) and E. Perez-Reyes (University of Virginia, Charlottesville, VA). They were cloned in frame into pGFP-C3 vectors (Clontech Laboratories, Inc.) and then into the Semliki forest virus vector pSFV1 (Life Technologies) for virus production. Thus, the GFP tag is located on the N terminus of the Caβ subunits.

Membrane fractionation

About 8 × 107 hippocampal neurons were cultured on four collagen-poly-L-lysine–coated 100-mm culture dishes for 14 d and infected with GFP-tagged Caβ1β, Caβ2a, Caβ3, and Caβ4, carrying virus for 1–16 h. Infected or noninfected cells were scraped in 0.32 M sucrose-TBS (0.15 M NaCl and 0.05 Tris, pH 7.4) containing 1 M Complete Mini protease inhibitor (Roche) and were homogenized for 50 strokes with Dounce tissue grinder (Wheaton Millville) before promptly being loaded on top of freshly prepared 0.8 M/1.2 M sucrose-TBS gradient for centrifugation. Centrifugation was performed in a J-221 M/E ultracentrifuge (Beckman Coulter) at 3 × 105 rpm with a SW25.1 rotor for 45 min at 4°C. Equal volumes of the cytosol and membrane fractions were used for Western blots, which were performed according to standard procedures (Mark et al., 1995).

Online supplemental material

Fig. S1 shows that the AP amplitude during 20-Hz stimulations is not reduced in noninfected or Caβ1β, Caβ2a, and Caβ4 subunits expressing hippocampal neurons. Fig. S2 shows that Caβ3 subunits expressed in hippocampal neurons do not change the relative contribution of N- and P/Q-type channels to non–L-type currents and EPSCs. Fig. S3 shows that the N terminus of the Caβ3 subunits interferes with synaptic transmitter release in hippocampal neurons. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200702072/DC1.

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