RECI PROCAT INTERACTIONS REGULATE TARGETING OF CALCIUM CHANNEL β SUBUNITS AND MEMBRANE EXPRESSION OF α1 SUBUNITS IN CULTURED HIPPOCAMPAL NEURONS*

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Auxiliary β subunits modulate current properties and mediate the functional membrane expression of voltage-gated Ca2+ channels in heterologous cells. In brain all four β isoforms are widely expressed, yet little is known about their specific roles in neuronal functions. Here we investigated the expression and targeting properties of β subunits and their role in membrane expression of CaV1.2 α1 subunits in cultured hippocampal neurons. Quantitative RT-PCR showed equal expression and immunofluorescence a similar distribution of all endogenous β subunits throughout dendrites and axons. High-resolution microscopy of hippocampal neurons transfected with six different V5 epitope-tagged β subunits demonstrated that all β subunits were able to accumulate in synaptic terminals and to colocalize with postsynaptic CaV1.2, thus indicating a great promiscuity in α-β interactions. In contrast, restricted axonal targeting of β1 and weak colocalization of β4b with CaV1.2 indicated isoform specific differences in local channel complex formation. Membrane expression of external HA epitope-tagged CaV1.2 was strongly enhanced by all β subunits in an isoform specific manner. Conversely, mutating the alpha interaction domain of CaV1.2 (W440A) abolished membrane expression and targeting into dendritic spines. This demonstrates that in neurons the interaction of a β subunit with the AID is absolutely essential for membrane expression of α1 subunits, as well as for the subcellular localization of β subunits, which by themselves possess little or no targeting properties.

Voltage-gated Ca2+ channels (CaV) provide key pathways for Ca2+ entry into neurons and translate membrane depolarization into neurotransmitter secretion and gene regulation. CaVs are composed of a pore-forming α1 subunit and the auxiliary α2δ and β subunits (1). Whereas the α1 subunits are responsible for voltage sensing and ion conduction, the auxiliary subunits have been implicated in membrane targeting and modulation of channel properties (for review see 2). Presynaptic CaV8s regulate neurotransmitter release (3) and postsynaptic CaVs activate the transcriptional regulators CREB and NFAT (4,5) and thus modulate long term potentiation (6). These functions reflect both, the diversity of CaV isoforms expressed in brain (7-11), and their differential subcellular localization in neurons (12-15).

Four distinct β isoforms have been identified (16-19), all of which are expressed in brain (20-23). They contain an SH31 domain and a guanylate kinase domain (24-27). However, the GK fold is modified so that it can bind with high affinity to the so-called alpha interaction domain (AID) in the intracellular I-II linker of CaV α1 subunits (28,29). The SH3 and the GK-like domains are highly conserved among the four genes encoding β subunits (Cacnb1-b4, Fig. 1C), whereas the sequence connecting these domains as well as the N- and C-termini are subject to alternative splicing (30,31). When coexpressed with α1 subunits in heterologous expression systems, such as X. laevis oocytes or HEK cells, all four β isoforms modulate the current properties and cause a strong increase in the current density (17-19,32) by an enhanced functional membrane expression of the channel.
However, it is not clear whether association of a β subunit is also required for the membrane expression of CaVs in neurons. In skeletal muscle synaptic targeting of Ca\textsuperscript{2+} channels. On the other hand, as previously shown in heterologous cells, also in neurons the association of β subunits with the AID domain is essential for membrane expression of the postsynaptic CaV1.2.

**Experimental Procedures**

**Cell culture and transfection.** Low-density cultures of hippocampal neurons were prepared from 16.5-day-old embryonic BALB/c mice as described (15,37,69). Plasmids were introduced into neurons on day 6 using lipofectamine 2000 transfection reagent (Invitrogen GmbH) as previously described (15). For single transfection (pβA-β-V5 constructs) 0.5 – 2 µg of DNA, for co-transfection experiments (pβA-eGFP, pβA-CaV1.2-HA, pβA-β(V5) 1 – 2.5 µg total DNA at a molar ratio of 1:1 was used. Cells were immunostained and analyzed 6 – 19 days after transfection.

**Molecular biology.** All constructs were cloned into a eukaryotic expression plasmid containing a neuronal chicken β-actin promoter (pβA; 15,38). For details about sources and cloning strategies of all constructs used in this study see Supplementary Methods. Genbank\textsuperscript{TM} accession numbers: β1a, M25514 (16); β1b X61394 (39); β2a, M80545 (17); β2b, AF423193 (30); β3, NM_012828 (19); β4b, L02315 (18).

**Quantitative Taqman RT-PCR.** RNA isolation and reverse transcription: 2 weeks old BALB/c mice were euthanized by CO\textsubscript{2} exposure and brains were excised after decapitation. Brain regions were dissected in cold HBSS and total RNA was extracted from homogenized brain tissue using the RNeasy Protect Mini Kit (Qiagen, GmbH, Hilden, Germany). To isolate RNA from hippocampal neurons, cultures (24 DIV) were harvested by trypsin treatment, total RNA was extracted as described above and RNA concentrations were determined photometrically. Reverse transcription was performed with 1 µg (hippocampi) or 5 µl (cultured neurons) of RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, USA) and random primers (Promega, Madison, USA), the RT mix was incubated for 60 min at 37°C.

Quantitative Taqman RT-PCR (qRT-PCR): The relative abundance of different β transcripts was assessed by qRT-PCR using a standard curve method as described (40,41). Specific TaqMan Gene Expression Assays, designed to span exon-exon boundaries, were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA): β1, Mm00518940_m1; β2, Mm00659092_m1; β3, Mm00432233_m1; β4, Mm00521623_m1. The following primers (MWG Biotech, Ebersberg, Germany) were used for PCR-amplification of assay-specific fragments using whole brain cDNA as a template (F, forward; R, reverse): β1, 5'-gatcctctccatggtccagaa-3'; β2, 5'-gactatctggaggcatactggaag-3'; β2, 5'-ctgctcctctcatgaagttc-3'; β3, 5'-ctctcttgggtttcagagtcaaa-3'; β3, 5'-ctgcctccttccttaaggcttc-3'; β4, 5'-ctctcctcgactcgagcagtaagagc-3'; β4, 5'-ctctcctcgactcgagcagtaagagc-3'.
tgtctcatgtgactctgtaat-3’. The integrity of the obtained fragments was confirmed by sequencing (MWG Biotech). In order to calculate standard curves, fragment concentrations were determined in a TECAN Genios Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland) using the Quant-IT PicoGreen dsDNA Reagent (Molecular Probes/Invitrogen) according to the manufacturer’s instructions. Standard curves with 10-fold serial dilutions from 10⁷ to 10 molecules of the respective fragment were generated for each assay. qRT-PCR was performed in triplicate measurements using 20 ng total RNA equivalents of cDNA and the specific TaqMan Gene Expression Assay in a final volume of 20 µl in TaqMan Universal PCR Master Mix (Applied Biosystems). To compare the relative expression of CaV β subunits between hippocampus and the cultured neurons, data were normalized to HPRT1 expression (Mm00446968_m1). HPRT1 was determined to be the most stable control gene among 7 genes tested (not shown). Analysis was performed using the ABI PRISM 7500 Sequence Detector (Applied Biosystems).

**Immunocytochemistry.** Neurons were fixed in pF in PBS at room temperature. Fixed neurons were incubated in 5% NGS in PBS/BSA/Triton for 30 min. Primary antibodies were applied in PBS/BSA/Triton at 4°C overnight and detected by fluorochrome-conjugated secondary antibodies (15). For staining of surface-expressed HA-tagged CaV1.2 constructs living neurons were incubated with the rat anti-HA antibody for 30 min at 37°C (42,43). Then the cultures were rinsed in HBSS, pF fixed for 10 min, blocked with NGS, and incubated with the secondary antibody for 1 hour (15).

For colocalization analysis of surface-expressed CaV1.2-HA constructs and cytoplasmic β subunits, live cell-stained neurons were postfixed for 5 min in pF. Then neurons were rinsed in PBS, permeabilized, blocked again with 5% NGS in PBS/BSA/Triton and subsequently incubated with the second primary antibody overnight at 4°C. After washing, the Alexa 488-conjugated secondary antibody was applied for 1h at room temperature. Coverslips were then washed and mounted in p-phenylene-diamine-glycerol to retard photobleaching (44). Preparations were analyzed on an Axiohot or an Axio Imager microscope (Carl Zeiss, Inc) using 63 ×, 1.4 NA, 25 ×, 0.8 NA, and 16 ×, 0.5 NA objectives. Images were recorded with a cooled CCD camera (SPOT; Diagnostic Instruments, Stirling Heights, MI, USA) and Metavue image processing software (Universal Imaging, Corp., West Chester, PA, USA). Images were arranged in Adobe Photoshop 9 (Adobe Systems Inc.) and linear adjustments were performed to correct black level and contrast.

**Antibodies.** Primary antibodies: rat monoclonal anti-HA (clone 3F10, 1:1,000 and 1:100 for live cell labeling; Roche Diagnostics GmbH, Vienna, Austria), rabbit polyclonal anti-CaV1.2 (1:4,000; Sigma-Aldrich), rabbit polyclonal anti-CaV2.1 (1:2,000; Synaptic Systems), rabbit polyclonal anti-GFP (1:20,000; Molecular Probes, Eugene, OR, USA), mouse monoclonal anti-V5 (1:400; Invitrogen CA, USA); mouse monoclonal anti-synapsin 1 (clone 46.1, 1:2,000; Synaptic Systems), rabbit polyclonal anti-synapsin 1 and 2 (1:20,000 and 1:2,000 in combination with Alexa 350; Synaptic Systems), rabbit polyclonal anti-NaChpan (1:250; Sigma-Alrich), mouse monoclonal anti-β1 (1:10,000) and anti-β2 (1:250; both Neuromab, Davis, CA, USA), rabbit polyclonal anti-β2 (1:500; pab 425) and anti-β3 (1:500; pab MM 2; both generous gifts from Dr. Flockerzi). Secondary antibodies: goat anti mouse Alexa 488 (1:2,000) and Alexa 594 (1:4,000), goat anti-rabbit Alexa 350 (1:500), Alexa 488 (1:4,000), Alexa 594 (1:4,000), and goat anti-rat Alexa 594 (Invitrogen, 1:4,000).

**Analysis.** Quantification of the density and fluorescent intensity of CaV1.2-HA clusters: To analyze the effects of the coexpressed β subunits on the membrane expression of CaV1.2-HA, surface fluorescence intensity was measured in 17 DIV old cultured neurons as previously described (42). HA intensity values were expressed as % of control separately for each individual experiment (transfection and culture preparation). For each condition between minimally 4 and maximally 16 neurons were analyzed in each of 3 to 7 independent experiments (culture preparations and transfections; Fig. 5E).

Quantification of β-V5 fluorescent intensity: To analyze the subcellular distribution of the heterologously expressed V5-tagged β subunits we quantified the fluorescence intensity of the V5 stain in 13 DIV old cultured hippocampal neurons. To this end 14 bit gray-scale images of the red
(V5) and green (eGFP) channel of the neurons’ soma were acquired and the V5 image was corrected for uneven illumination and the camera dark current. For each cell a second image showing a segment of the axonal main branch at 1 mm distance from the soma was acquired and corrected accordingly. The corresponding eGFP image was used to distinguish the emerging axon from dendrites. A region of interest was manually traced around the soma and 30 µm long lines were placed along the proximal segments of one dendrite, the axon (representing the axon hillock), and along a distal segment of the axon 1 mm from the soma. Subsequently the region intensities were recorded and background was subtracted. V5 staining intensities in the dendrite, the axon hillock, and the distal axon were normalized to the soma intensity of each individual cell by calculating the dendrite/soma, distal axon/soma, and axon hillock/dendrite ratios. For each condition between minimally 2 and maximally 10 neurons were analyzed in each of 3 to 6 independent experiments (culture preparations and transfections; Tab. 1).

Colocalization analyses: To analyze the degree of colocalization of CaV1.2-HA and β-V5 clusters a Z-stack of 3 consecutive 14-bit gray scale images (interplane distance of 0.2 µm) of the corresponding red (live cell staining of CaV1.2-HA) and green (V5) color channels were acquired using Metavue software. Next the images were aligned and deconvolved using in ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2007) as previously described (45). Images were background subtracted and a region of interest was drawn around a dendritic segment. Colocalization was analyzed in ImageJ using two different methods; intensity correlation analysis (46) and distance based colocalization (JACoP plugin in ImageJ; 47). Results are expressed as mean ICQ ± 95% confidence intervals (c.i.) and mean percentage ± 95% c.i. of CaV1.2-HA or β-V5 objects colocalizing with β-V5 or CaV1.2-HA, respectively. For each condition the indicated number of 17 DIV old neurons was analyzed from two independent experiments (culture preparations and transfections; Fig. 5B). To determine the colocalization of endogenous β subunits with synapsin, 14-bit gray scale images of the corresponding red and green color channels were acquired using Metavue software. Subsequently images were 2D deconvolved (Metamorph) and analyzed in ImageJ using distance based colocalization (JACoP plugin in ImageJ; 47).

Quantification of dendritic CaV1.2-HA and CaV1.2-HA(W440A) expression: To analyze the distribution of the overall HA fluorescence along the dendrites of 18 DIV old hippocampal neurons transfected with CaV1.2-HA and CaV1.2-HA (W440A) 14 bit gray-scale images of the red (HA) and green (eGFP) channel were acquired using the 25 ×, 0.8 NA objective. For each neuron the HA fluorescence intensity of 2-5 dendrites was recorded along a single pixel line reaching from the soma to the distal tips of the dendrite. The corresponding eGFP image was used to distinguish dendrites from axons. The intensity values of one dendrite were normalized to the average intensity of the proximal 10 µm of the same dendrite and the moving average of 10 µm long bins was calculated for the entire length of the dendrite. Finally, the normalized intensity values were averaged between the dendrites of one neuron. N-numbers: 23 (normal) and 25 (W440A) neurons from 4 separate culture preparations.

Semi automated analysis of eGFP and HA fluorescent intensity in dendritic spines: Analysis of dendritic spine HA and eGFP intensities in 18 and 25 DIV old neurons was performed using a custom designed Metamorph Journal (macro). Briefly, 14-bit gray scale images of the corresponding red (permeabilized staining of CaV1.2-HA) and green (eGFP) color channels were acquired using Metavue software. Corresponding images were aligned and one dendritic segment of 20-50 µm length was selected for analysis. The HA and eGFP images were background-flattened and thresholded to trace fluorescent clusters (HA) and dendritic spines (eGFP) as accurately as possible. The thresholded eGFP images were binarized and a morphological filter (circular gradient) was applied to outline the edges of the dendritic spines. The binarized and gradient images were added and ROIs were drawn around the dendritic spines and the dendritic shaft. Using the integrated morphometric analysis option of Metamorph the spine size was measured in the binarized eGFP image and subsequently the ROIs were transferred onto the HA and eGFP images to record the
fluorescent intensities of each spine. Average spine intensities were background subtracted, normalized to the average fluorescent intensity of the dendritic shaft, and finally multiplied by the respective spine size.

Statistical Analysis. Results are expressed as mean ± standard error (SE) except where otherwise indicated. Data were organized and analyzed using MS Excel and SPSS statistical software (SPSS Inc, Chicago IL, USA) as indicated. Graphs and figures were generated using MS Excel, Origin 7, and Adobe Photoshop 8.0 software.

RESULTS

Cultured hippocampal neurons express mRNA and protein of all four Ca\textsuperscript{2+} channel \(\beta\) subunits. It has previously been shown that the hippocampus expresses mRNA and protein of the Ca\textsubscript{V} \(\alpha_1\) subunits (Ca\textsubscript{V}1.2, Ca\textsubscript{V}1.3, Ca\textsubscript{V}2.1, Ca\textsubscript{V}2.2, and Ca\textsubscript{V}2.3), \(\alpha_2\delta\) (\(\alpha_2\delta\)-1, \(\alpha_2\delta\)-2, \(\alpha_2\delta\)-3), and of all four \(\beta\) subunits (e.g. 12-15,21,23). Still little is known about the subcellular distribution of \(\beta\) subunits in neurons and about the specific subunit composition of Ca\textsubscript{V} complexes in pre- and postsynaptic compartments. Therefore we addressed the questions as to 1) which of the \(\beta\) subunits are expressed in a defined neuronal culture system – low density hippocampal neurons; 2) are these \(\beta\) subunits differentially distributed within hippocampal neurons; and 3) do their colocalization and functional interactions reveal evidence for preferred interaction partners of specific \(\alpha_1\) subunits?

Employing quantitative Taqman RT-PCR analysis we determined whether and how much mRNA of the four \(\beta\) subunits is expressed in hippocampus tissue of 2 weeks old BALB/c mice and in cultured hippocampal neurons (40,41). In hippocampus we detected similar expression levels of all \(\beta\) subunit isoforms. The mRNA levels of \(\beta_2\) and \(\beta_4\) were slightly but not significantly higher than those of \(\beta_1\) and \(\beta_3\) (Fig. 1A, left). Finding all four \(\beta\) subunits expressed in mouse hippocampus was not surprising considering the cellular heterogeneity of the hippocampal formation. Remarkably, however, mRNA of all four \(\beta\) subunits was also expressed in low-density cultured hippocampal neurons (Fig. 1A, right), which consist of ~90% glutamatergic pyramidal cells (37,48).

Because quantitative RT-PCR analysis revealed the expression of all four \(\beta\) subunits in the cultured neurons, we next investigated their subcellular distribution using immunofluorescence labeling with antibodies specific for the individual \(\beta\) isoforms (Fig. 1B; Suppl. Fig. 1). All four \(\beta\) isoforms could be detected in the soma and in the dendrites. Higher magnification micrographs of dendritic segments (Fig. 1B, lower panel) revealed a delicate punctate staining pattern of all isoforms along the dendritic shaft (arrows) and adjacent to the shaft in positions typical for dendritic spines (open arrowheads). Double immunofluorescence labeling with an antibody against synapsin, marking the presynaptic vesicle compartment (50), showed that some of the \(\beta\) clusters overlapped or colocalized with synapsin (Fig. 1B, arrowheads). \(\beta_1\) clusters were primarily located adjacent to synapsin clusters, while a subset of \(\beta_2\) and \(\beta_4\) clusters was colocalized with synapsin (yellow in Fig. 1B). Partial overlap of all \(\beta\) subunits with synapsin was further supported by object-based colocalization analysis of 24 DIV old neurons [% of \(\beta\) clusters colocalized with synapsin ± 95% c.i. (n): \(\beta_1\), 46±4 (21); \(\beta_2\), 48±4 (22); \(\beta_3\), 42±4 (23); \(\beta_4\), 45±5 (22); ANOVA: F(3,84) = 1.64; p = 0.19]. Here the preferential colocalizations observed in the qualitative analysis were not detected, mainly because of the large abundance of extrasynaptic \(\beta\) clusters along the dendritic shaft.

The localization of \(\beta\) subunits along the dendritic shaft and at synaptic sites suggests their interaction with postsynaptic and presynaptic Ca\textsubscript{V} \(\alpha_1\) subunits, respectively. For example, the overall \(\beta\) subunit distribution along the dendrites was strikingly similar to the localization of endogenous L-type Ca\textsubscript{V}1.2 (15) and Ca\textsubscript{V}1.2-HA expressed in the membrane (15,42,49). Thus, we next investigated whether \(\beta_1\) and \(\beta_4\) subunits specifically colocalize with postsynaptic (Ca\textsubscript{V}1.2) or presynaptic (Ca\textsubscript{V}2.1) \(\alpha_1\) subunits. Double immunofluorescence demonstrated that a subset of Ca\textsubscript{V}1.2 clusters was precisely colocalized with \(\beta_1\) clusters, especially in positions typical for dendritic spines. In contrast, the overlap of Ca\textsubscript{V}1.2 and \(\beta_4\) clusters along the dendritic shaft seemed to be largely random (Fig. 1C, left). This observation was further supported by linescan analysis of
selected regions (Fig. 1C, lower panel). On the other hand, a subset of β4 clusters was precisely colocalized with Cav2.1 clusters, while β1 clusters were only loosely associated with this presynaptic α1 subunit (Fig. 1C, right). The inherent inability to distinguish membrane expressed channel clusters from intracellular pools and inevitable differences in the quality of the antibodies prevented the quantitative analysis of the colocalization. Together this emphasizes the need for a standardized method for investigating β subunit localization and the importance to specifically identify and analyze membrane expressed channels.

Six different V5-tagged β subunits show a similar distribution pattern in the somatodendritic compartment of cultured hippocampal neurons. Therefore we tagged β1a, β1b, β2a, β3, and β4 with a C-terminal V5 tag (Fig. 1D) and expressed them together with soluble eGFP or an extracellular HA-tagged Cav1.2 in the low-density cultured hippocampal neurons. This approach has the advantage of localizing all examined β subunits with the same antibody and of investigating their colocalization exclusively with the population of membrane expressed CaV channels. Furthermore it allowed us to extend the analysis to additional β splice variants for which no specific antibodies are available. The eGFP fluorescence enabled us to independently assess the quality of the transfected neurons and to unambiguously identify neuronal compartments such as axons with presynaptic boutons (e.g. Fig. 3 and 4) and dendrites with dendritic spines (e.g. Fig. 2B). To obtain similar expression levels the amounts of DNA of all transfected β constructs were titrated between 0.5 and 2 µg DNA per 60 mm culture dish. To exclude interference of elevated expression levels on the distribution pattern we analyzed exclusively medium to low expressing neurons.

As already suggested by the antibody labeling of the endogenous β subunits (Fig. 1B) also all recombinant β subunit constructs displayed very similar overall distribution patterns (Fig. 2A). All analyzed isoforms and splice variants were expressed in the somato-dendritic compartment as well as in the axons (arrows). In dendrites all β-V5 constructs were distributed in a punctate and discretely clustered pattern along the dendritic shaft and also in the dendritic spines (Fig. 2B, arrowheads). This distribution pattern and the apparent density of the β clusters was similar to the staining pattern of the endogenous β subunits (Fig. 1B). Moreover, analysis of the dendrite-over-soma ratio of the fluorescence intensity (Tab. 1) demonstrated that, with the exception of the β2a isoform, all β subunits had a similar expression density in the dendrites. The higher expression of the β2a isoform was the result of its accumulation at the membrane due to N-terminal palmitoylation (51,52); mutation of the palmitoylation site, the cysteins at positions 3 and 4 to serines, abolished this effect (Tab. 1).

Isoform specific localization of β-V5 subunits in the distal axon. Because all β-V5 subunits were able to enter the axonal compartment (Fig. 2A), we next sought to analyze their expression pattern in the distal axon. To this end we followed the main axonal branch of the transfected neuron for 1 mm (based on the eGFP stain; Fig. 3A) and analyzed the axon over soma ratio of V5 intensity separately for each individual neuron (Tab. 1). Axonal expression of all β-V5 constructs was evident as fine puncta along the axon (Fig. 3A). The expression levels of both β1 splice variants (β1a and β1b) were significantly lower compared to the other β subunits (Fig. 3A; Tab. 1). This reduced targeting of β1 subunits into the distal axon did evidently not depend on the expression levels of individual neurons. First, because this differential distribution pattern was evident in neurons displaying a wide range of expression levels, and secondly because β1 expressing neurons showed the same somatic β-V5 intensity as neurons expressing the other βs (ANOVA: * F (6,152) = 2.16; p = 0.018). The difference in axonal targeting was especially apparent when β1b-V5 was coexpressed together with a C-terminally GFP-tagged β4b subunit in the same hippocampal neuron (Fig. 3B). Both β subunits labeled the soma and the dendrites, including the most distal tips of the dendrites, to a similar degree. In contrast, the axonal localization of β1b-V5 was restricted to the proximal segments, whereas β4b-GFP label was intense throughout all the axonal branches (Fig. 3B, color overlay). Thus, β subunits display an isoform specific expression in the axonal compartment in that β2, β3, and β4 isoforms are expressed throughout the axon, whereas β1a and β1b are largely excluded from the distal axon and small axonal branches.
All \( \beta \) subunit isoforms can accumulate in the presynaptic compartment. As all \( \beta \) subunits were able to enter the axon, although to different degrees, it was important to examine whether they also accumulate in the presynaptic compartment. To this end presynaptic terminals were identified by the eGFP label and concomitant immunostaining with an antibody against synapsin (Fig. 4) or the vesicular glutamate transporter (vGlut1, not shown). Where axons of transfected neurons made contacts with non-transfected postsynaptic neurons, clusters of V5-labeled \( \beta \) subunits were unambiguously identified as presynaptic. In triple-labeling experiments we regularly observed clusters of all six examined \( \beta \) isoforms and splice variants colocalized with synapsin clusters in transfected, eGFP-positive axons (Fig. 4, anti V5 label and color overlay). This synaptic localization was repeatedly observed in both, \textit{en-passent} boutons (Fig. 4, \( \beta_{1a}-V5 \), \( \beta_{1b}-V5 \), \( \beta_{2a}-V5 \)) and in terminal synapses (\( \beta_{2b}-V5 \), \( \beta_{3a}-V5 \) and \( \beta_{6b}-V5 \)) for all the V5-tagged \( \beta \) subunits. Thus, even in those cases were only a limited amount of \( \beta \) was expressed in the distal axon (\( \beta_{1a} \) and \( \beta_{1b} \)), the \( \beta \) subunits could specifically accumulate in the synapse; presumably due to their association with presynaptic Cav5.

**All \( \beta \) subunit isoforms can interact with the postsynaptic L-type channel Cav1.2.** A colocalization of \( \beta \) subunits with presynaptic marker proteins is indicative of their association with presynaptic Cav complexes. Likewise the punctate distribution of the \( \beta \) in the somato-dendritic compartment (cf. Fig. 1B and Fig. 2) is suggestive for their association with postsynaptic CavVs. Cav1.2 is the only postsynaptic L-type channel whose precise subcellular localization on the soma, dendrites and dendritic spines of cultured hippocampal neurons is known (15). Therefore we coexpressed the V5-tagged \( \beta \) subunits together with the external epitope-tagged Cav1.2-HA to analyze \( \beta \) subunit interaction with postsynaptic CavVs. As previously described, live cell staining using an anti HA antibody revealed the localization of the membrane-expressed Cav1.2-HA in small clusters on the shafts of the dendrites and in the dendritic spines (Fig. 5A) at a density of 1-2 clusters per \( \mu \)m (15,42). Subsequent fixation and permeabilization of the live-cell labeled neurons allows to immunostain the intracellular \( \beta \) subunits in addition. Such double staining clearly showed that clusters of membrane incorporated Cav1.2-HA are colocalized with each one of the co-expressed \( \beta \) subunits (Fig. 5A), indicating the association of all of the examined \( \beta \) isoforms with Cav1.2-HA in the postsynaptic compartment.

In order to reveal potential differences in the degree of \( \alpha_{1}\)-\( \beta \) colocalization, we further analyzed the images by two independent quantification methods; intensity correlation analysis (ICA; 46) and object based colocalization (47). ICA (Fig. 5B) showed similar degrees of colocalization for all \( \beta \) subunits except for \( \beta_{4b} \), for which the intensity correlation quotient (ICQ) was slightly but significantly reduced. Object based colocalization supported the initial, visual, observation by demonstrating that the vast majority of the membrane expressed Cav1.2-HA clusters (~80%) was colocalized with each of the coexpressed \( \beta \) subunit isoforms (Fig. 5C). Conversely, between ~50 to 80% of the \( \beta \) subunit clusters colocalized with membrane expressed Cav1.2-HA. Interestingly, also in this analysis the \( \beta_{3b} \) showed the lowest degree of colocalization (Fig. 5D). Together these analyses demonstrate that in cultured hippocampal neurons clusters of postsynaptic Cav1.2 channels are almost fully occupied by each of the \( \beta \) isoforms and splice variants. However clusters of \( \beta \) subunits also exist independently of the Cav1.2-HA clusters and this is most evident in the case of the \( \beta_{4b} \) isoform.

In contrast to other Cav targeting studies we routinely express the \( \alpha_{1} \) subunit without auxiliary \( \alpha_{2}\delta \) and \( \beta \) subunits in cultured hippocampal neurons (15,42,49). As a consequence subcellular localization, targeting, and membrane expression of these expressed subunits entirely depend on the interaction of the heterologous channel with endogenous \( \beta \) and \( \alpha_{2}\delta \) subunits. This minimizes possible effects of overexpression on the subcellular localization. To test whether the amount of \( \beta \) subunits is limiting for membrane expression of Cav1.2, we analyzed the intensity of Cav1.2-HA surface expression upon co-expression of the different \( \beta \) subunits. As control we coexpressed a plasmid bearing the same promoter but no coding sequence together with Cav1.2-HA (Fig. 5E, mock). Coexpression of all \( \beta \)-V5 subunits resulted in a substantial increase of the membrane expression of Cav1.2-HA up to 300% (\( \beta_{1a} \) of
controls without a β (Fig. 5E and F). This suggests that also in neurons the amount of membrane expressed CaV α₁ subunits is limited by the amount of available β subunits. Interestingly, with the β₁a, β₁b and β₂a isoforms the increase in membrane expression of Cav1.2-HA was larger and statistically significant compared to that with β₃, β₄b and the non-palmitoylated β₂a mutant. This observation indicates that β subunits enhance membrane expression in an isoform-specific manner.

**Intact α₁-β subunit interaction at the AID is essential for neuronal membrane expression of CaV1.2-HA.** Studies in heterologous expression systems have demonstrated the requirement of α₁-β interactions for functional membrane expression of the CaV complex (reviewed in 31). Recently, the mutation of a key tryptophane in the AID (53) of the intracellular I-II linker of CaV2.2 has been shown to strongly reduce surface expression of the channel in HEK cells (54). To determine the role of α₁-β interactions for membrane expression of the α₁ subunit and for the targeting of the β subunit in a native neuronal cell type, we mutated the analogous tryptophane in the AID of the Cav1.2-HA to alanine (W440A). Live cell staining of normal CaV1.2-HA showed the typical clustered membrane expression pattern (Fig. 6A, anti HA, left; n=56 neurons from 7 independent culture preparations and transfections). In contrast, the mutated channel CaV1.2-HA(W440A) was not detectable on the surface of cultured hippocampal neurons in live cell staining experiments (Fig. 6A, anti HA, right) in all neurons analyzed (n=54 neurons from 7 independent culture preparations and transfections). This was not due to an overall failure of protein expression, since subsequent permeabilization and immunolabeling with an antibody against CaV1.2 revealed the presence of similar amounts of normal and W440A mutant channel protein in the transfected cells (Fig. 6A, anti CaV1.2).

To test whether an excess of β subunits, which should enable β subunits to occupy even low affinity interaction sites (55), might at least partially rescue the loss of membrane expression in the W440A mutant, we co-expressed the different β isoforms with CaV1.2-HA and CaV1.2-HA(W440) (Suppl. Fig. 2). Whereas increased membrane expression of the CaV1.2-HA was observed with all the β subunits, none of the β subunits induced a detectable membrane expression of CaV1.2-HA(W440A), suggesting an exclusive and essential role of the α₁-β interaction at the AID for neuronal membrane expression.

In principle the failure in membrane expression of CaV1.2-HA(W440A) could also be explained by a compromised trafficking of the mutated channel into the distal parts of the dendritic tree. Thus, we analyzed the distribution of the overall fluorescence signal in the dendrites of permeabilized neurons transfected with normal and mutated CaV1.2-HA. The HA labeling intensity in the proximal dendrite was indistinguishable between normal and the W440A mutant channels (t(45) = -0.04; p = 0.96; n-numbers: normal, 22; W440A, 25). Analysis of the relative decrease in fluorescence (Fig. 6B) showed a very similar rate of decrease of CaV1.2-HA and CaV1.2-HA(W440A) expression along the dendrites; and even in the most distal regions of the dendrites (~250 μm) HA labeling of the W440A mutant was robust. The small reduction in the total HA fluorescence in CaV1.2-HA(W440A) compared to the normal channel along the entire length of the dendrite is likely the effect of the missing membrane-fraction of the HA staining (Fig. 6B). Thus, mutation of AID-β interactions did neither reduce expression levels, nor the overall targeting of the CaV1.2(W440A) throughout the dendritic tree, but specifically disrupted the membrane expression of the channel.

**Intact α₁-β subunit interaction at the AID is essential for dendritic spine targeting of CaV1.2-HA.** A hallmark of the subcellular distribution of CaV1.2 is its clustered localization in the heads and necks of dendritic spines close to, but not necessarily within the postsynaptic density (15,42). Therefore we next investigated the importance of the functional α₁-β interaction for the characteristic localization of CaV1.2 channel complexes in dendritic spines. The localization of CaV1.2-HA in the membrane of dendritic spines is most strikingly revealed by live cell labeling of the extracellular HA epitope (15,42). Nevertheless, also in fixed and permeabilized neurons the clusters in dendritic spines are discernable from the HA stain in the dendritic shaft, which resembles both, the membrane fraction and the intracellular pool of CaV1.2-HA (Fig. 6C, 18 and 25 DIV, normal). At
18 DIV the majority of spines are long and filopodia-like, and accordingly CaV1.2-HA clusters are spread out over a fair distance from the shaft. At 25 DIV many spines assumed a mature mushroom-like shape, leading to a more regular alignment of CaV1.2-HA clusters at both sides of the dendritic shaft. In contrast, in permeabilized neurons transfected with CaV1.2-HA(W440A) dendritic spines were devoid of HA-immunolabel, both at 18 and 25 DIV (Fig. 6C, W440A). The immunolabel of the W440A mutant channel was restricted to the dendritic shafts, where it also lacked the clustered appearance of the membrane-incorporated channels. Thus, the lack of membrane incorporation of the W440A mutant Ca2+ channel revealed that cytoplasmic membrane organelles containing the α1 subunits are absent from dendritic spines.

The lack of W440A label from the spines was observed in both, immature filopodia-like spines at 18 DIV and in mature mushroom-like spines in 25 DIV old cultured neurons (magnified micrographs in Fig. 6C). Semi-automated analysis (see Materials and Methods) of the dendritic spine size based on the eGFP intensity revealed a significant increase of the average spine volume between 18 DIV and 25 DIV old neurons [eGFP intensity (arbitrary units ± SEM) 18 DIV, 0.30±0.01; 25 DIV, 0.34±0.01; t(2376) = 2.53, p = 0.011]. However, at both time points the mean spine volumes (Tab. 2) and spine volume distributions (Fig. 6D) were indistinguishable between neurons transfected with the normal and the W440A mutant CaV1.2-HA. Moreover, the overall shape and density of spines were similar in both conditions (Fig. 6C; quantitation not shown). This indicates that the absence of CaV1.2-HA(W440A) label from spines did not result from potential deficits in the maturation of spines when the neurons were transfected with the mutant channel.

Analysis of the dendritic spine HA intensity revealed a dramatic difference between neurons transfected with normal CaV1.2-HA or with W440A (Tab. 2). This difference is clearly reflected in the cumulative frequency distribution diagram of the spine HA intensities (Fig. 6E). Plotting the relative frequencies of the individual recorded spine HA intensities against their intensity values indicated that the reduced overall mean fluorescence values in spines of neurons transfected with CaV1.2-HA(W440A) were due to the greatly increased population of spines containing no HA staining at all. Whereas with CaV1.2-HA only 29% (18 DIV) and 17% (25 DIV) of spines contained no HA staining, with the W440A mutant spines without HA staining made up 72% (18 DIV) and 58% (25 DIV). In contrast, the fractions of the spines with high HA intensity values were not different between normal and W440A CaV1.2-HA (Fig. 6E). This population appears to correspond to the similarly large population of spines with high eGFP intensity (compare Fig. 6D and E), and it is likely that these large “spines” actually represent sprouts of dendritic branches, which cannot be distinguished from large spines. Together with the altered staining pattern (Fig. 6C) this quantitative analysis of wildtype and W440A mutant channels indicates that most, if not all spines lack the cytoplasmic organelles containing Ca2+ channels en route to the plasma membrane.

Correct subcellular localization of β subunits depends on their interaction with an α1 subunit. The observation that all V5-tagged β subunits could accumulate in presynaptic terminals and colocalize with the postsynaptic CaV1.2-HA clusters suggested that the subcellular localization of the β subunits may exclusively depend on their interaction with an α1 subunit (see above). Alternatively, specific interactions of these MAGUK proteins with pre- and postsynaptic anchoring proteins might determine their localization and in turn contribute to the specific localizations of the α1 subunits. Coexpression of β subunits with normal and W440A mutant CaV1.2-HA now provided an experimental paradigm to test these hypotheses. We reasoned that in the absence of interactions with CaV1.2-HA(W440A) excess co-expressed β subunits will be mistargeted if their subcellular localization depends on their association with the CaV complex by binding the AID. This was indeed observed. Coexpression of the palmitoylated β2a-V5 with CaV1.2-HA showed colocalized clusters in the dendrites (Fig. 7A, left, arrowhead) and in the axon initial segment (Fig. 7A, left, arrow). Upon co-expression with CaV1.2-HA(W440A) the clustered distribution pattern of β2a-V5 was much less pronounced. Remaining weakly labeled clusters likely represent V5-tagged β subunits colocalized with clusters of endogenous
channels (Fig. 7A, right). Quantitative analysis showed that the relative staining intensity was similar in neurons expressing CaV1.2-HA or CaV1.2-HA(W440A) (Fig. 7A, graph). Thus, the altered labeling pattern on co-expression of CaV1.2-HA(W440A) did not arise from reduced β expression levels, but from the redistribution of the palmitoylated β2a subunits in the membrane when it could not bind the AID motif of the channel.

Mutation of the N-terminal double cysteins at positions 3 and 4 to serines removed the membrane anchor of β2a-SS. When co-expressed with CaV1.2-HA both subunits colocalized in clusters at the membrane of dendrites and the initial segment of the axon (Fig. 7B, arrowhead and arrow, respectively). Again, co-expression with CaV1.2-HA(W440A) changed the overall distribution of β2a-SS-V5 to a less intense and more uniform staining pattern. Quantitative analysis confirmed that this altered staining was due to redistribution and not reduced overall expression of β2a-SS-V5 (Fig. 7B, micrographs and graph).

Interestingly, when co-expressed with CaV1.2-HA(W440A), a distinct accumulation of β2a-SS-V5 in the most proximal part of the axon was regularly observed (Fig. 7B; right, arrow). This labeling pattern was neither observed with β2a-V5 nor when β2a-SS-V5 was co-expressed with the normal CaV1.2-HA. Thus, when β2a-SS-V5 cannot interact with an α1 subunit because its AID had been mutated, it is mistargeted to other neuronal structures. Together these findings suggest that the correct subcellular localization of β subunits depends on their interaction with an α1 subunit.

Specific accumulation of heterologously expressed β2b and β4b in the axon hillock. The accumulation in the most proximal part of the axon was not unique to β2a-SS-V5. Apart from their localization in pre- and postsynaptic compartments, we regularly observed a similar accumulation of β2b and β4b in the most proximal part of the axon when expressed without additional α1 subunits (Fig. 7C and D; Tab. 1). This staining pattern was especially pronounced in neurons expressing the β subunits at very low levels close to the limit of detection. In double staining experiments the accumulations of β2b and β4b overlapped with immunolabel of voltage-gated Na+ channels, identifying the β2b- and β4b-containing structure as the axon hillock (Fig. 7D). However, in variance with the membrane labeling pattern of the Na+ channel, β2b and β4b were located in an intracellular and fibrous structure (Fig. 7D, color overlay). This is consistent with the observation that the normal palmitoylated β2b did not show this staining pattern (Fig. 7A; see above). The fact that the β2a-SS-V5 staining was primarily cytoplasmic and that it was more pronounced in combination with the CaV1.2-HA(W440A) mutant, further suggests that this accumulation of β subunits in the axon hillock is not related to their association in a Ca2+ channel complex. Whether this phenomenon occurs with endogenous β subunits and what binding partners might be involved remains to be shown in future studies.

**DISCUSSION**

Specificity of α/β pairing is not determined by β subunit expression and localization. Many cell types achieve exclusive α/β combinations by the selective expression of specific isoforms. For example, skeletal muscle expresses CaV1.1 and β1a, cardiac myocytes CaV1.2 and β2, and retina photoreceptor cells CaV1.4 and β2 (2,56). In cerebellum CaV2.1 and β3 are the predominant CaV isoforms (9,18,57), however, not to the exclusion of others. Consistent with previous reports (17-19,58-60) our quantitative RT-PCR analysis detected mRNA of all four β isoforms in hippocampus. This uniform abundance does not preclude a more selective expression of β isoforms in distinct types of neurons within the hippocampal formation. Unexpectedly however, we found that cultured hippocampal neurons, consisting of >90% glutamatergic pyramidal cells (37,48), also expressed all four β isoforms at similar levels. Furthermore, immunofluorescence revealed similar staining patterns of the four endogenous β proteins. Together this indicates that in cultured hippocampal neurons α/β subunit specificity is not the result of a selective β subunit expression pattern.

Another possible mechanism for achieving subunit specificity would be the differential targeting of β subunits into distinct compartments. Immunofluorescence analysis of the endogenous βs did not indicate such distinct localizations, however, showed some preference in their...
association with pre- and postsynaptic Cav α1 subunits. Yet, due to different antibodies and the inability to distinguish between splice variants, a direct comparison of labeling patterns is difficult. Expression of epitope-tagged β subunits and subsequent immunofluorescence with a single antibody presents a powerful approach to circumvent these limitations and also demonstrated a more divergent targeting behavior. On the one hand all six examined β subunits were found in the somato-dendritic and in the axonal compartment. On the other hand β1a and β1b showed clearly reduced targeting into the distal axon, indicating a preferential role of β1 in the postsynaptic compartment. The overall pattern of all β subunits was clustered and not diffuse (cf. Fig. 2 and 3) consistent with their localization in complexes with endogenous somato-dendritic and axonal Cav-s, the latter possibly in vesicles or in preassembled transport packages (61).

Previous analysis of Cav2.2 splices lacking the SYNPRINT domain suggested the existence of separate check-points for axonal targeting and the incorporation of channels into the synapse (49). Also axonal β subunits might differ in their ability to be incorporated into the presynaptic compartment. However, this was not the case. Not only were all β2, β3, and β4 constructs found colocalized with the presynaptic marker synapsin, but also synapses were identified in which β1a and β1b were accumulated. Even though the β1 variants were poorly targeted into distal axons, they could be incorporated into the nerve terminal like any other β subunit.

The fact that all β subunits can accumulate in presynaptic terminals suggests that they all can form complexes with presynaptic channels in situ. This observation is consistent with the great permissiveness of α1–β interactions observed upon heterologous coexpression and indicates that in neurons the affinities of specific β-AID pairs (28) by themselves do not determine the specificity of α1/β assemblies. Interestingly, low neuronal α1/β selectivity was also suggested by immunoprecipitation experiments showing similar β subunit compositions of neuronal L-type, P/Q-type, and N-type channels (20-22).

In theory the presynaptic accumulation of all β subunits could arise from anchoring mechanisms other than the AID of the α1 subunits (62). The colocalization of β subunits with membrane-expressed Cav1.2-HA clusters, however, provides compelling evidence that their subcellular localization in neurons is essentially determined by this α1-β interaction. Interestingly, upon coexpression of the W440A mutant, in which the α1-β interaction was abolished, the localization of β subunits was altered. This result unambiguously demonstrates that specific targeting of β subunits in the postsynaptic compartment requires the interaction with the Cav1.2 α1 subunit, and that the AID is essential for this interaction. A similar conclusion was earlier reached in skeletal muscle cells, in which a Y366S mutation in the AID of Cav1.1 abolished colocalization of the β subunit, but not its ability to modulate the current density (63). Together these data indicate that an intact AID is essential for the specific localization of β subunits in nerve and muscle cells, but that low affinity interactions are sufficient for current modulation by β subunits (54,55,64).

**β subunit interaction at the AID is absolutely required for Cav membrane expression in neurons.** Functional membrane expression of Cav-s in heterologous cell systems requires the presence of a β subunit (31). In contrast, only little information is available on the role of β subunits for membrane expression in the native environment of nerve cells (65). Here we demonstrate for the first time that membrane expression of an α1 subunit in differentiated neurons absolutely depends on its interaction with a β subunit. Membrane incorporated Cav1.2 channels were never observed when the AID was mutated (W440A), even though the channel was expressed in cytoplasmic membrane compartments. Apparently, without a β-AID interaction the channel is retained in the endoplasmic reticulum, as previously suggested (33). That the β requirement for membrane expression of α1 subunits observed in heterologous cells also applies to native channels in differentiated neurons was not necessarily to be expected. In skeletal muscle of the immotile zebra fish mutant relaxed, which lacks the β subunit, this is not the case. In the absence of the β subunit Cav1.1 channels were not only incorporated into the membrane, but even correctly targeted to the triadic junctions (34). Evidently, the requirement of β-AID interactions for Cav membrane targeting

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differs between nerve and muscle cells. Remarkably, membrane expression of the W440A mutant could not be rescued by coexpression of any one of the β subunits; even though coexpression with the wildtype CaV1.2 resulted in a substantial increase of membrane expression. Therefore we can conclude that putative additional binding sites, which may be specific for certain β isoforms (66), are not sufficient to induce detectable membrane expression of the channel, even if β subunits are available in excess.

Do specific α/β pairs exist in neurons? Both, the observation that all tested β subunits can colocalize with CaV1.2-HA in membrane clusters, and the finding that β coexpression enhances membrane insertion of CaV1.2-HA underscore the general permissiveness of α-β interactions. Nonetheless, we also observed some remarkable β isoform-specific differences in their localization and interactions. First, β2 showed a lower degree of colocalization with CaV1.2-HA than all the other β isoforms. Secondly, β1 and β2 isoforms enhanced CaV1.2-HA membrane expression more than β3 and β4 isoforms; and finally, β1 was poorly transported into distal axons. Although the evidence is indirect, these subtle differences in neuronal targeting properties and in α-β interactions suggest that β1 and β2 are better partners of the somato-dendritic CaV1.2 channel than β3 and β4; and conversely, β4 is a more likely partner for presynaptic Ca^{2+} channels than for example β1. The latter is consistent with previous findings reporting presynaptic functions of the β4 subunit (35,36). Yet, neither expression patterns, nor differential targeting or isoform-specific α-β interactions indicated the existence of explicit α/β pairs in hippocampal neurons. Strictly speaking, exclusive α/β pairs may not exist in neurons expressing multiple isoforms. However, the observed subtle differences in targeting properties and in promoting membrane expression of CaV1.2-HA, together with previously reported differences in β-AID affinities (28) may lead to the formation of preferential α/β pairs. These may be in a dynamic steady-state with free β subunits and change depending on the relative local concentrations of the β isoforms (67). A modest surplus of one β subunit may shift the balance towards this isoform, thus emphasizing the non-specific, promiscuous nature of α-β interactions.

In vivo, changes in relative expression levels or an activity-dependent export of free β subunit from the nucleus, as suggested by our recent work (70), might alter the subunit composition in one or the other neuronal compartment, and thus contribute to the dynamic modulation of particular neuronal Ca^{2+} currents.

CaV1.2 channels enter dendritic spines via lateral diffusion. Finally, this study revealed the absence of cytoplasmic CaV1.2 channels from dendritic spines. Although live cell staining demonstrated that CaV1.2-HA(W440A) failed to reach the plasma membrane, staining in fixed/permeabilized neurons showed that it was distributed throughout the dendritic arbor like the wildtype CaV1.2-HA. This clearly indicated that the overall CaV1.2 targeting properties are independent of an interaction with a β subunit and that CaVs are inserted into the plasma membrane locally in the periphery of the neuron. However, spines of permeabilized neurons only contained CaV1.2 clusters similar to the bona fide membrane clusters observed in live-stained neurons but not cytoplasmic organelles containing CaV1.2. Their absence from spines suggests that CaV1.2 channels must be inserted into the membrane in the dendritic shaft and subsequently enter the spine via lateral diffusion (see model in Fig. 6). A similar pathway has recently been suggested for the AMPA receptor GluR2 subunit by elegant FRAP studies (68). However, as opposed to the AMPA receptor, whose dynamic recycling in the postsynaptic membrane underlies synaptic plasticity, the size and density of CaV1.2 clusters in spines are stable during NMDA-induced synaptic remodeling (42).

In conclusion our data demonstrate that all four β isoforms are expressed in cultured hippocampal pyramidal neurons, are able to assemble with pre- and postsynaptic CaVs, and regulate membrane expression of CaVs by high-affinity binding to the AID in the I-II loop of the α1 subunit. Whereas β subunits depend on this non-specific interaction for their own subcellular localization, additional interactions with the α1 subunit and other binding proteins may determine β isoform-specific differences in axonal targeting and the promotion of membrane expression of neuronal Ca^{2+} channels.
REFERENCES


**FOOTNOTES**

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1 The abbreviations used are: AID, alpha interaction domain; BSA, bovine serum albumin; eGFP, enhanced green fluorescent protein; GK, guanylate kinase; HA, hemagglutinin; ICA, intensity correlation analysis; ICQ, intensity correlation quotient; NGS, normal goat serum; PBS/BSA/Triton, PBS containing 0.2% BSA and 0.2% Triton X-100; pF, 4% paraformaldehyde, 4% sucrose; RT, reverse transcription; SH3, src homology 3

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**FIGURE LEGENDS**

**Fig. 1.** mRNA expression and immunocytochemical localization of all four Ca²⁺ channel β subunit isoforms in cultured mouse hippocampal neurons. **A**, Taqman RT-PCR expression profile of the four Ca²⁺ channel β subunits in hippocampi from 2 weeks old mice (left) and cultured mouse hippocampal neurons differentiated 24 days in vitro (DIV). In hippocampus β2 and β4 isoforms are expressed at slightly but not significantly higher levels than β1 and β3 (ANOVA: F(3,8) = 2.42; p = 0.14). Cultured hippocampal neurons express all β isoforms at similar levels (ANOVA: F(3,16) = 1.20; p = 0.34).

N-numbers: 3 tissue and 5 culture preparations; data are presented as mean number of transcripts per 20 ng RNA ± SEM. **B**, Representative cultured hippocampal neurons (20 DIV) labeled with monoclonal (β₁, β₄) or polyclonal (β₂, β₃) antibodies show a similar expression and distribution pattern of all four endogenous β subunits in the soma and dendrites. Contrast in micrographs is optimized to visualize the weak labeling on the dendrites, therefore the staining in the somata appears saturated. Dendrite segments of all βs shown at higher magnifications (lower panel) reveal a similar punctate staining pattern along the dendritic shafts (red, arrows) and adjacent to the shafts in dendritic spines (open arrowheads). β₂, β₃, and β₄ puncta partially overlap or colocalize with the presynaptic marker synapsin (yellow, arrowheads). **C**, Double immunofluorescence labeling and linescan analyses of monoclonal β₁ and β₄ antibodies together with polyclonal anti CaV1.2 (left) and anti CaV2.1 (right) antibodies in 24 DIV cultured neurons. A subset of CaV1.2 clusters colocalizes with β₁ along the dendritic shaft and in dendritic spines (linescan analysis,
lower panel), while association with $\beta_4$ is less pronounced. In contrast, presynaptic CaV2.1 clusters colocalize with $\beta_4$ but rarely with $\beta_1$ clusters. D. Comparative exon structures of all carboxyterminally V5-tagged $\beta$ subunit isoforms and splice variants used in this study. Exon similarities of the $\beta_1$, $\beta_3$, and $\beta_4$ genes were identified in the mouse genome (NCBI m37 assembly) in relation to the overall structure of the $\beta_2$ gene (30,31). Color codes: orange, conserved exons in the SH3 and GK-like domain; blue, high sequence similarity; light blue, lower sequence similarity. Bars, 25 $\mu$m and 5 $\mu$m (B) and 5 $\mu$m (C).

Fig. 2. Somatodendritic distribution pattern of six V5-tagged $\beta$ subunit isoforms and splice variants in cultured hippocampal neurons. Cultured hippocampal neurons (2 weeks old) transfected with different $\beta$-V5 constructs together with eGFP and labeled with an antibody against the C-terminal V5 epitope. A. Immunostaining reveals a similar expression of all V5-tagged $\beta$ subunits in the soma, the dendrites, and in the proximal regions of the axon (arrow). The axon was identified based on its characteristic appearance in the eGFP image (lower panel). In order to visualize the weak staining in the smaller dendrites and axons the contrast of the images was enhanced and thus the staining of the cell soma appears saturated. B. Details of dendritic segments: all $\beta$-V5 subunits display a punctate, clustered distribution pattern along the dendritic shaft. In addition all $\beta$ subunits are localized in small clusters in the dendritic spines (arrowheads) identified in the eGFP image. Note that the palmitoylated $\beta_{2a}$ isoform also shows a diffuse staining of the membrane. Bars, 25 $\mu$m (A) and 10 $\mu$m (B).

Fig. 3. Isoform specific localization of V5-tagged $\beta$ subunits in the distal axon of cultured hippocampal neurons. A. All examined $\beta$-V5 subunit isoforms display a clustered staining pattern along the axonal main branch of 2 weeks old cultured hippocampal neurons at $\sim$ 1 mm distance from the soma. The intensity and frequency of axonal clusters is much lower for $\beta_{1a}$ and $\beta_{1b}$ compared to the other $\beta$ subunits (for quantification see Tab. 1). B. Representative cultured hippocampal neuron (17 DIV) co-transfected with $\beta_{1b}$-V5 and $\beta_{4b}$-eGFP, and double labeled with anti-V5 and anti-GFP antibodies. The distribution of $\beta_{1b}$-V5 is confined to the somatodendritic compartment and the proximal regions of the axon (left, yellow in the color overlay), whereas $\beta_{4b}$-eGFP expression is similarly high throughout the axon and the axonal branches (middle, green in the color overlay). Bars, 10 $\mu$m (A) and 25 $\mu$m (B).

Fig. 4. Localization of $\beta$-V5 subunits in the presynaptic compartment of cultured hippocampal neurons. Representative axonal segments of triple-labeled 17-20 DIV old hippocampal neurons, transfected with different $\beta$-V5 constructs and eGFP (postsynaptic non-transfected neurons are not stained). eGFP fluorescence allows to morphologically identify axons with their varicosities typical for en-passent synapses (examples in $\beta_{1a}$-V5, $\beta_{1b}$-V5, and $\beta_3$-V5) and short axonal branches with presynaptic terminals (examples in $\beta_{2a}$-V5, $\beta_{2b}$-V5, and $\beta_{4b}$-V5). Double immunostaining with an antibody against synapsin (anti-Syn) identifies these axonal varicosities and terminals as presynaptic compartments. All six V5-tagged $\beta$ subunit isoforms (anti-V5) were found to accumulate in presynaptic terminals as based on their colocalization with synapsin (anti-Syn) and eGFP (examples indicated by arrowheads). Presynaptic accumulation was observed repeatedly for all isoforms in 3 to 12 analyzed cells of at least two independent experiments. Bar, 10 $\mu$m.

Fig. 5. Colocalization of $\beta$-V5 subunits with membrane expressed CaV1.2-HA in dendrites of cultured hippocampal neurons. A. Dendritic segments of hippocampal neurons (18 DIV), transfected with CaV1.2-HA and a V5-tagged $\beta$ subunit, labeled with an antibody against the extracellular HA epitope prior to fixation (anti-HA, live) and with anti-V5 after subsequent permeabilization. Clusters of all $\beta$ subunits were colocalized with the membrane-expressed CaV1.2-HA (examples indicated by arrowheads; yellow in color overlay). B. Intensity correlation analysis (ICA) of $\alpha_2$-$\beta$ colocalization reveals a similar intensity correlation coefficient (ICQ) for all co-expressed $\beta$ subunits with the exception of $\beta_{4b}$, for which it was significantly reduced. ANOVA: $F_{(4,54)} = 5.88$; $p = 0.001$. C. Object-based colocalization shows that $\sim$80% of CaV1.2-HA clusters are colocalized with the co-transfected $\beta$ subunit independent of the
respective isotype. ANOVA: $F_{(4,54)} = 0.76; p = 0.56$. Conversely, ~60% to 75% of $\beta$-V5 clusters are colocalized with CaV1.2-HA clusters. Interestingly, again the colocalization with $\beta_{4b}$ was reduced when compared to $\beta_{2a}$ and $\beta_{2b}$ (p = 0.002 and 0.001, respectively). ANOVA: $F_{(4,54)} = 5.81; p = 0.001$ and Tukey post hoc analysis. 

E. Coexpression of CaV1.2-HA with different $\beta$ subunit isoforms and splice variants (example images of $\beta_{2b}$ and $\beta_{3}$) results in a strong increase in CaV1.2-HA membrane expression compared to control cells (mock transfected). F. Quantification of surface HA staining intensity reveals a significant effect of the $\beta_{1}$ and $\beta_{2}$ isoforms on surface expression of CaV1.2-HA, compared to mock transfected control (c) neurons. ANOVA: $F_{(7,271)} = 12.78; p < 0.0001$; Tukey post hoc analysis: $p < 0.001$ (\(\beta_{1a}\)), $p = 0.014$ (\(\beta_{1b}\)), $p = 0.005$ (\(\beta_{2a}\)), $p = 0.0623$ (\(\beta_{3}\)), $p = 0.134$ (\(\beta_{4b}\)). Bars, 10 $\mu$m (A) and 25 $\mu$m (E), error bars indicate 95% confidence intervals.

Fig. 6. Mutation of an essential residue of the AID (W440A) of CaV1.2 prevents neuronal membrane expression and dendritic spine targeting of CaV1.2-HA. A. Representative cultured hippocampal neurons (17 DIV) transfected with CaV1.2-HA (normal) or the W440A mutant (W440A) labeled with an antibody against the extracellular HA epitope prior to fixation (anti-HA, live) and with anti-CaV1.2 after permeabilization. Surface expression (anti-HA, live) of CaV1.2-HA(W440A) is completely missing, although expression of total CaV1.2 protein (anti-CaV1.2) was similar for CaV1.2-HA and W440A. Comparable neurons were selected based on the expression of co-transfected eGFP (eGFP). B. Quantification of total fluorescence intensity (anti-HA in permeabilized cells) shows a similar expression of CaV1.2-HA and CaV1.2-HA(W440A) throughout the entire length of the dendrites of 18 DIV old hippocampal neurons. Error bars represent ± SEM. N-numbers: 23 (normal) and 25 (W440A) neurons from 4 separate culture preparations. C. Dendritic segments of 18 and 25 DIV old neurons transfected with normal or W440A CaV1.2-HA plus eGFP. Neurons were immunolabeled with anti-HA after pF fixation and permeabilization. Similar to live-stained neurons, CaV1.2-HA is localized in small clusters in the dendritic shaft and spines. In contrast CaV1.2-HA(W440A) label is restricted to the shaft and clusters are missing. eGFP reveals filopodia-like spines at 18 DIV and mushroom-shaped spines at 25 DIV. Magnified color overlays of anti-HA (green/yellow) and eGFP (green) demonstrate the presence of CaV1.2-HA clusters and the absence of CaV1.2-HA(W440A) staining in both types of spines D. Plotting the cumulative frequency of total eGFP intensity per dendritic spine (arbitrary units) shows that dendritic spine sizes are not different in neurons transfected with CaV1.2-HA or W440A, although many spines are larger in 25 DIV (red and green lines) compared to 18 DIV old neurons (light blue and blue lines). E. The cumulative frequency distribution of total HA intensity per dendritic spine demonstrates that in neurons expressing CaV1.2-HA(W440A) ~60 to 70 % of the spines are devoid of HA staining (red and light blue lines), whereas the vast majority of spines express CaV1.2-HA (only ~20 to 30 % without HA stain, green and blue lines). F. Model of CaV1.2 targeting into dendritic spines: Cytoplasmic membrane systems containing CaV1.2 are confined to the dendritic shaft. Association of a $\beta$ subunit promotes the insertion of the channel into the dendritic membrane. Channel complexes enter the spine by lateral diffusion in the membrane.

Fig. 7. The subcellular localization of $\beta$ subunits depends on their interaction with an $\alpha_{1}$ subunit, and specific accumulation of heterologously expressed $\beta_{2a}$ and $\beta_{4b}$ in the axon hillock. A, B. Cultured hippocampal neurons transfected with CaV1.2-HA (normal) or the CaV1.2-HA(W440A) mutant (W440A) together with $\beta_{2a}$-V5 (A) or $\beta_{2a}$-SS-V5 (B) live cell stained with the anti HA antibody (anti HA, live) and labeled with the V5 antibody after fixation and permeabilization (anti V5). A, $\beta_{2a}$ colocalizes with membrane incorporated CaV1.2-HA in clusters along the dendrites (arrowhead) and proximal axon (arrow). In contrast, the clustered distribution pattern of $\beta_{2a}$-V5 is less pronounced when co-expressed with CaV1.2-HA(W440A). The remaining and weakly labeled clusters likely represent V5-tagged $\beta$ subunits colocalized with clusters of endogenous channels (right). Quantitative analysis showed that the relative staining intensity is similar in neurons expressing CaV1.2-HA or CaV1.2-HA(W440A) (Graph: light gray, normal; dark gray, W440A). B, Coexpression of CaV1.2-HA with the non-palmitoylated mutant,
β_{2a}-SS results in similar colocalization on dendrites (arrowhead) and axons (arrow). When co-expressed with Ca\textsubscript{v}1.2-HA(W440A), β_{2a}-SS-V5 labeling is enriched in the proximal part of the axon, presumably the axon hillock (right, arrow). This is most obvious by comparing the relative V5 intensity in the axon hillock of normal and W440A (t test: \( t_{(23)} = -5.25; p < 0.001 \)). 

C. Cultured hippocampal neuron (12 DIV) expressing β_{2b}-V5 and eGFP reveal a strong staining of β_{2b} (anti V5) in the axon initial segment, identified based on the distinct morphology in the eGFP image (arrow). 

D. Double labeling of cultured hippocampal neurons (19 DIV) expressing the non-palmitoylated β_{2a}-SS-V5 mutant or β_{4b}-V5 with anti V5 and a pan anti Na\textsuperscript{+} channel antibody (anti NaCh) identifies the strong V5 staining in the axon hillock (see also Tab. 1). However, whereas the immunolabel of the membrane expressed Na\textsuperscript{+} channel seems to envelop the axon hillock (anti NaCh, blue), the V5 staining (anti V5, red) appears to be restricted to an intracellular, filamentous structure.
### TABLES

**Tab. 1.** Intensity analyses of $\beta_x$-V5 subunit distribution in the dendrite (dendrite/soma ratio), the distal axon (axon/soma ratio), and the axon hillock (axon hillock/dendrite ratio).

<table>
<thead>
<tr>
<th></th>
<th>$\beta_{1a}$</th>
<th>$\beta_{1b}$</th>
<th>$\beta_{2a}$</th>
<th>$\beta_{2a-SS}$</th>
<th>$\beta_{2b}$</th>
<th>$\beta_3$</th>
<th>$\beta_{4b}$</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mean sem (n)</td>
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<tr>
<td></td>
<td>0.20</td>
<td>0.21</td>
<td>0.43 1)</td>
<td>0.17</td>
<td>0.22</td>
<td>0.28</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>(24)</td>
<td>(24)</td>
<td>(20)</td>
<td>(21)</td>
<td>(21)</td>
<td>(26)</td>
<td>(23)</td>
<td></td>
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<tr>
<td><strong>Axon</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mean sem (n)</td>
</tr>
<tr>
<td></td>
<td>0.015 2)</td>
<td>0.005 3)</td>
<td>0.030</td>
<td>0.036</td>
<td>0.049</td>
<td>0.038</td>
<td>0.051</td>
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<td>0.001</td>
<td>0.003</td>
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<td>(24)</td>
<td>(14)</td>
<td>(15)</td>
<td>(20)</td>
<td>(25)</td>
<td>(20)</td>
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<td><strong>Axon hillock</strong></td>
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<td></td>
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<td></td>
<td>mean sem (n)</td>
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<tr>
<td></td>
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<td>1.06</td>
<td>2.84 4)</td>
<td>2.27 4)</td>
<td>0.90</td>
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<td>(20)</td>
<td>(21)</td>
<td>(21)</td>
<td>(26)</td>
<td>(23)</td>
<td></td>
</tr>
</tbody>
</table>

Numbers of neurons analyzed are given in parentheses. For each condition 2-10 neurons were analyzed in 3-6 independent culture preparations and transfections.

ANOVA: * $F_{(6,152)} = 13.3; \text{p} < 0.001$; ** $F_{(6,130)} = 7.0; \text{p} < 0.001$; *** $F_{(6,152)} = 34.1; \text{p} < 0.001$

Post hoc (Tukey):  
1) other $\beta$s: $\text{p} < 0.001$  
2) $\beta_{2b}$: $\text{p} = 0.008$; $\beta_{4b}$: $\text{p} = 0.005$  
3) $\beta_{2a-SS}$: $\text{p} = 0.029$; $\beta_{2b}$, $\beta_{4b}$: $\text{p} < 0.001$; $\beta_3$: $\text{p} = 0.003$  
4) $\beta_{1a}$, $\beta_{1b}$, $\beta_{2a}$, $\beta_3$: $\text{p} < 0.001$
**Tab. 2.** Analysis of channel expression (HA intensity) in anti-HA immunolabeled fixed/permeabilized neurons transfected with Ca\(_v\)1.2-HA or Ca\(_v\)1.2-HA(W440A) compared to dendritic spine volume (eGFP intensity).

<table>
<thead>
<tr>
<th>Intensity</th>
<th>Age</th>
<th>Ca(_v)1.2-HA</th>
<th>Ca(_v)1.2-HA(W440A)</th>
<th>Statistic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>18 DIV</td>
<td>0.39±0.02 [0.17]</td>
<td>0.27±0.03 [0.00]</td>
<td>Z = -13.80 (^2)</td>
<td>p &lt;&lt; 0.001</td>
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<tr>
<td></td>
<td>25 DIV</td>
<td>0.39±0.02 [0.29]</td>
<td>0.32±0.04 [0.00]</td>
<td>Z = -8.12 (^2)</td>
<td>p &lt;&lt; 0.001</td>
</tr>
<tr>
<td>eGFP</td>
<td>18 DIV</td>
<td>0.31±0.01 [0.18]</td>
<td>0.29±0.01 [0.15]</td>
<td>(t_{(1618)} = 1.05) (^3)</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>25 DIV</td>
<td>0.33±0.01 [0.26]</td>
<td>0.35±0.02 [0.24]</td>
<td>(t_{(756)} = 0.83) (^3)</td>
<td>0.41</td>
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</tbody>
</table>

Number of spines (neurons) analyzed:
18 DIV: Ca\(_v\)1.2-HA, 852 (24); Ca\(_v\)1.2-HA(W440A), 768 (24) from 4 independent culture preparations
25 DIV: Ca\(_v\)1.2-HA, 359 (9); Ca\(_v\)1.2-HA(W440A), 399 (9) from 2 independent culture preparations

1) Intensity in arbitrary units (total spine grayscale intensity)
2) Mann-Whitney U test
3) independent samples t test
Figure 2

A

\[
\begin{array}{cccccc}
\beta_{1a-V5} & \beta_{1b-V5} & \beta_{2a-V5} & \beta_{2b-V5} & \beta_{3-V5} & \beta_{4b-V5} \\
\text{anti-\textbeta-V5} & & & & & \\
\text{eGFP} & & & & & \\
\end{array}
\]

B

\[
\begin{array}{cccccc}
\beta_{1a-V5} & \beta_{1b-V5} & \beta_{2a-V5} & \beta_{2b-V5} & \beta_{3-V5} & \beta_{4b-V5} \\
\text{\textbeta-V5} & & & & & \\
\text{V5/eGFP} & \text{eGFP} & & & & \\
\end{array}
\]
Figure 3
Figure 4
Figure 7

A

\( \beta_{2a} + \)

<table>
<thead>
<tr>
<th>anti V5 (perm)</th>
<th>anti HA (live)</th>
<th>anti HA/anti V5</th>
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<tr>
<td>normal</td>
<td>normal</td>
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</tr>
<tr>
<td>W440A</td>
<td>W440A</td>
<td>W440A</td>
</tr>
</tbody>
</table>

\( \beta_{2a} \)-V5 distribution

B

\( \beta_{2a} - SS + \)

<table>
<thead>
<tr>
<th>anti V5 (perm)</th>
<th>anti HA (live)</th>
<th>anti HA/anti V5</th>
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<tbody>
<tr>
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</tr>
<tr>
<td>W440A</td>
<td>W440A</td>
<td>W440A</td>
</tr>
</tbody>
</table>

\( \beta_{2a} - SS \)-V5 distribution

C

\( \beta_{2b} \)-V5

anti V5

eGFP

D

\( \beta_{2a} - SS \)-V5

anti V5

anti NaCh (pan)

anti V5/anti NaCh

\( \beta_{4b} \)-V5

anti V5

anti NaCh
Reciprocal interactions regulate targeting of calcium channel β subunits and membrane expression of α1 subunits in cultured hippocampal neurons

Gerald J. Obermair, Bettina Schlick, Valentina Di Biase, Prakash Subramanyam, Mathias Gebhart, Sabine Baumgartner and Bernhard E. Flucher

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