A Tripartite Interaction Among the Calcium Channel \(\alpha_1\)- and \(\beta\)-Subunits and F-Actin Increases the Readily Releasable Pool of Vesicles and Its Recovery After Depletion

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Neurotransmitter release is initiated by the influx of \(Ca^{2+}\) via voltage-gated calcium channels. The accessory \(\beta\)-subunit (Ca\(_V\beta\)) of these channels shapes synaptic transmission by associating with the pore-forming subunit (Ca\(_V\alpha_1\)) and up-regulating presynaptic calcium currents. Besides Ca\(_V\alpha_1\), Ca\(_V\beta\) interacts with several partners including actin filaments (F-actin). These filaments are known to associate with synaptic vesicles (SVs) at the presynaptic terminals and support their translocation within different pools, but the role of Ca\(_V\beta\)/F-actin association on synaptic transmission has not yet been explored. We here study how Ca\(_V\beta_4\), the major calcium channel \(\beta\) isoform in mamalian brain, modifies synaptic transmission in concert with F-actin in cultured hippocampal neurons. We analyzed the effect of exogenous Ca\(_V\beta_4\) before and after pharmacological disruption of the actin cytoskeleton and dissected calcium channel-dependent and -independent functions by comparing the effects of the wild-type subunit with the one bearing a double mutation that impairs binding to Ca\(_V\alpha_1\). We found that exogenously expressed wild-type Ca\(_V\beta_4\) enhances spontaneous and depolarization-evoked excitatory postsynaptic currents (EPSCs) without altering synaptogenesis. Ca\(_V\beta_4\) increases the size of the readily releasable pool (RRP) of SVs at resting conditions and accelerates their recovery after depletion. The enhanced neurotransmitter release induced by Ca\(_V\beta_4\) is abolished upon disruption of the actin cytoskeleton. The Ca\(_V\alpha_1\) association-deficient Ca\(_V\beta_4\) mutant associates with actin filaments, but neither alters postsynaptic responses nor the time course of the RRP recovery. Furthermore, this mutant protein preserves the ability to increase the RRP size. These results indicate that the interplay between Ca\(_V\beta_4\) and F-actin also support the recruitment of SVs to the RRP in a Ca\(_V\alpha_1\)-independent manner. Our studies show an emerging role of Ca\(_V\beta\) in determining SV maturation toward the priming state and its replenishment after release. We envision that this subunit plays a role in coupling exocytosis to endocytosis during the vesicle cycle.

Keywords: calcium channel, Ca\(_V\beta\) subunits, F-actin, RRP size, RRP refilling, EPSC
INTRODUCTION

Calcium entry through Ca\(_{\text{V}2.2}\)x high-voltage activated calcium channels is a pivotal step during action potential-evoked neurotransmitter release and synaptic plasticity (Wheeler et al., 1994; Cao and Tsien, 2010; Simms and Zamponi, 2014; Nanou and Catterall, 2018). The Ca\(_{\text{V}2.2}\)x calcium channel core complex in the mammalian brain is composed of one Ca\(_{\text{V}2.1}\) and one Ca\(_{\text{V}2.2}\) subunit (Müller et al., 2010).

The Ca\(_{\text{V}2.2}\) family belongs to the membrane-associated guanylate kinases (MAGUKs) class of scaffolding proteins encompassing two highly conserved domains, a Src 3 homology (SH3) domain and a guanylate kinase (GK) domain that are encompassing two highly conserved domains, a Src 3 homology (SH3) domain and a guanylate kinase (GK) domain that are flanked by variable regions (Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004). The four Ca\(_{\text{V}2.2}\) isoforms described until now, Ca\(_{\text{V}2.2}\)\_1 to Ca\(_{\text{V}2.2}\)\_4, associate with a highly conserved sequence among the high-voltage activated Ca\(_{\text{V}2.1}\) referred to as \(\alpha_1\) interaction domain (AID) that is located within the intracellular loop joining the transmembrane domains I and II (Pragnell et al., 1994). Association of Ca\(_{\text{V}2.2}\) with the AID site increases calcium current densities by altering the biophysical properties of the channel and its expression at the plasma membrane (Buraei and Yang, 2010). Accordingly, in mouse hippocampal neurons exogenously expressed Ca\(_{\text{V}2.4}\), the predominant Ca\(_{\text{V}2.2}\) isoform associated with the Ca\(_{\text{V}2.2}\) core complex (Müller et al., 2010), increases neurotransmitter release via slowing down voltage-dependent inactivation and promoting Ca\(_{\text{V}2.2}\)x channel cell surface expression (Wittemann et al., 2000; Xie et al., 2007; Etemad et al., 2014).

Several lines of evidence suggest that Ca\(_{\text{V}2.2}\)\_4 affects synaptic transmission by mechanisms that are independent of the upregulation of Ca\(_{\text{V}2.2}\)\_mediated currents. Dissociation from Ca\(_{\text{V}2.1}\) induced by membrane depolarization favors the association of Ca\(_{\text{V}2.2}\)\_4 with a phosphatase 2A regulatory subunit causing their translocation to the nucleus and regulation of transcriptional activity (Tadmouri et al., 2012; Ronjat et al., 2013). Ca\(_{\text{V}2.2}\)\_4 also associates with the Rab3 interacting molecule RIM1 and facilitates synaptic transmission by supporting the docking of synaptic vesicles (SVs; Kiyonaka et al., 2007).

We have previously shown that the Ca\(_{\text{V}2.2}\)\_4 isoform associates directly with F-actin and facilitates the trafficking of Ca\(_{\text{V}2.1}\)-containing transport vesicles toward the plasma membrane (Stöllting et al., 2015; Conrad et al., 2018). The actin-based cytoskeleton supports readily releasable pool (RRP) recruitment, docking step and recycling of SVs as well as of synaptic proteins after neurotransmitter release (Cingolani and Goda, 2008; Hallermann and Silver, 2013; Tanifuji et al., 2013; Hayashida et al., 2015; Rust and Maritzen, 2015; Miki et al., 2016). These two lines of evidence motivated us to investigate whether the interaction between Ca\(_{\text{V}2.2}\)\_4 and the actin cytoskeleton play a role in the recruitment of SVs to the active zone and replenishment after depletion. In order to dissect Ca\(_{\text{V}2.1}\)-dependent and independent functions (Hidalgo and Neely, 2007; Hofmann et al., 2015; Rima et al., 2016), we generated a wild-type (WT) Ca\(_{\text{V}2.2}\)\_4 and a mutant version with disrupted binding to Ca\(_{\text{V}2.1}\) (Opatowsky et al., 2004) and, expressed these constructs in primary mouse hippocampal neurons.

We found that Ca\(_{\text{V}2.2}\)\_4 also binds to actin filaments (F-actin) and, at excitatory synapses it enhances spontaneous and evoked postsynaptic currents as well as the size of the RRP of SVs and its recovery time after depletion. The enhanced synaptic transmission relies on the association of Ca\(_{\text{V}2.4}\)\_4 with Ca\(_{\text{V}2.1}\) and depends on an intact actin cytoskeleton. Ca\(_{\text{V}2.4}\) mutant retains the capability to interact with F-actin and to recruit SVs to the RRP, but it fails to increase neurotransmitter release. Our results add a new function of Ca\(_{\text{V}2.4}\) in shaping synaptic transmission and expand the already broad functional repertoire of this subunit.

MATERIALS AND METHODS
cDNA Constructs

For heterologous expression of Ca\(_{\text{V}2.2}\)/Ca\(_{\text{V}2.4}\) in HEK293T cells, the cDNA-encoding region of the human Ca\(_{\text{V}2.2}\)\_2\_1 pore-forming subunit of voltage-gated calcium channels (UniProtKB: Q00975-1) was subcloned into pEGFP-N1 to yield a C-terminal Ca\(_{\text{V}2.2}\)\_2\_1-GFP fusion protein. The human WT Ca\(_{\text{V}2.4}\) (UniProtKB: Q00305-2) was fused to mCherry to facilitate recognition of transfected cells. For recordings in hippocampal neurons, cDNA-encoding WT and Ca\(_{\text{V}2.1}\)-association deficient Ca\(_{\text{V}2.4}\) mutant where fused to eGFP and subcloned into FsY1.1 G.W lentiviral transfer vector (kindly provided by Dr. M Filippov, Nizhny Novgorod, Russia) to yield fusion constructs with the eGFP moiety fused at the C-terminus of Ca\(_{\text{V}2.4}\). To express a Ca\(_{\text{V}2.4}\)\_4 mutant with impaired Ca\(_{\text{V}2.1}\)-association M238A/L384A amino acid substitutions were introduced by PCR based techniques. For protein expression in E.coli, the coding region of the core regions of Ca\(_{\text{V}2.4}\) (residues 50–408) or Ca\(_{\text{V}2.1}\)\_4 was used. Briefly, the purified proteins were concentrated and stored at −80°C until use. The glutathione S-transferase (GST) protein alone or fused to the Ca\(_{\text{V}2.1}\)-anchoring domain (AID) have been previously described (Miranda-Laferte et al., 2014). The GST pull-down assay was done as previously (Hidalgo et al., 2006) and also described in Supplementary Material.

Recombinant Proteins

Histidine-tagged Ca\(_{\text{V}2.2}\) derivatives were purified from E.coli lysates as previously described (Hidalgo et al., 2006). In brief, proteins were purified from the soluble fraction of the crude lysate by metal affinity followed by size-exclusion chromatography. Fractions containing the purified proteins were concentrated and stored at −80°C until use. The glutathione S-transferase (GST) protein alone or fused to the Ca\(_{\text{V}2.1}\)-anchoring domain (AID) have been previously described (Miranda-Laferte et al., 2014). The GST pull-down assay was done as previously (Hidalgo et al., 2006) and also described in Supplementary Material.

F-Actin Cosedimentation Assay

Binding of the Ca\(_{\text{V}2.2}\) derivatives to F-actin was studied using the F-actin co-sedimentation assay according to the manufacturer’s instructions (Cytoskeleton, Inc, Denver, CO, USA) and as previously described (Stöllting et al., 2015). Each assay was performed in a volume of 50 µl. For each reaction, either protein alone (control) or together with F-actin, the same amount and stock of Ca\(_{\text{V}2.2}\) was used. Briefly, the purified proteins were
incubated with rabbit muscle actin in actin polymerization buffer containing (in mM): 10 Tris- HCl, 0.2 CaCl₂, 50 KCl, 2 MgCl₂, 1 ATP, pH 8.0, centrifuged for 1 h at 150,000 x g at 4°C in a Beckman TLA 100.1 rotor. After centrifugation, the whole supernatant was transferred to a new tube while the pellet was resuspended in a final volume of 50 µl containing 1× SDS-loading buffer. Supernatant and pellet fractions were then resolved by denaturing SDS-PAGE. Each lane was loaded with 25 fractions were then resolved by denaturing SDS-PAGE. Each lane was loaded with 25 µl to permit direct comparison of protein amounts bound or unbound to F-actin. Since Cavβ₄b core and actin exhibit overlapping migration in the mini gels (Bio-Rad, Hercules, CA, USA), the proteins were resolved using a Multiigel-Long chamber (Biometra, Göttingen, Germany). Proteins were visualized with Coomassie Blue and the prestained protein markers Dual color (Bio-Rad, Hercules, CA, USA) or PageRuler Plus (Thermo Fisher, Waltham, MA, USA) were used as molecular mass standards. All assays were repeated at least three times.

Cell Culture and Immunocytochemistry

Autaptic and mass cultures of hippocampal neurons were prepared from postnatal day 0 to day 3 C57/Bl6-N mice and maintained as previously described (Guzman et al., 2010). Briefly, hippocampi were isolated from brain and enzymatically treated with 15 units of papain (Worthington Biochemical Corp, Lakewood, NJ, USA) for 20 min at 37°C. After enzymatic digestion, neurons were mechanically dissociated. For autaptic cultures, neurons were diluted to a density of 1,000 cells/ml and plated onto micro-islands containing glial cells that were cultured 3–5 days prior to seeding neurons. For mass cultures, isolated neurons were diluted to a density of 300 cells/cm² on 25 mm glass coverslips previously treated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA). Neuronal cells were grown in NBA (Invitrogen, Carlsbad, CA, USA), supplemented with Glutamax at 1% (Thermo Fisher, Waltham, MA, USA) penicillin/streptomycin at 2% (Thermo Fisher, Waltham, MA, USA) and B-27 at 2% (Thermo Fisher, Waltham, MA, USA). Neurons were used after 10–14 days in vitro (DIV) for electrophysiological recordings and for fluorescence confocal microscopy imaging. HEK293T cells (Sigma-Aldrich, St. Louis, MO, USA) used for electrophysiological recordings of heterologously expressed Cavβ2.2 were cultivated in cell culture dishes using DMEM (Gibco) medium supplemented with 10% FBS and with 2% penicillin/streptomycin. All cells were cultivated at 37°C and in a humidified atmosphere with 5% CO₂.

Virus Production, Transduction of Hippocampal Neurons and HEK Cells Transfection

To deliver the cDNA encoding for the Cavβ₄b constructs hippocampal cultures were infected with lentivirus. The helper plasmids pRSVREV, pMDLg/pRRE, and vesicular stomatitis virus G protein expressing plasmid were kindly provided by Dr. Thomas Südhof (Howard Hughes Medical Institute, Stanford University, Stanford, CA, USA). Lentivirus was produced (Barde et al., 2010) and transduced as previously described (Stößing et al., 2015). Calcium phosphate transfection was used to co-transfect the lentiviral transfer vector and the three helper plasmids into HEK293FT cells. After 14 h transfection, cell culture medium containing (DMEM, 10% FBS, 100 mM sodium pyruvate, 100 mM non-essential amino acids, and 100 mM Glutamax) was renewed. Solutions were purchased from Thermo Fisher (Waltham, MA, USA). Cell culture medium containing lentiviral particles was withdrawn from the cell surface and ultracentrifuged for 2 h. Lentiviral particles were immediately resuspended in culture medium, frozen in liquid nitrogen and stored at −80°C. Hippocampal neurons were infected using 30–50 µl of viral suspension at 1–3 DIV.

HEK cells were transiently co-transfected with Cav2.2-GFP and either with WT or mutant Cavβ₄b-mCherry using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA, USA). Cells were split 12 h after transfection, and the electrophysiological experiments were performed 24 h later.

Immunostaining and Confocal Microscopy

Mass hippocampal neurons infected with lentiviral particles encompassing the Cavβ₄b-eGFP constructs were fixed with 4% paraformaldehyde (PFA) in PBS during 10 min at room temperature (RT). PFA was removed and 0.1% Triton X-100 in PBS was added to neurons and incubated at RT for 10 min. Primary antibodies incubation using anti-MAP-2 (Synaptic Systems, Göttingen, Germany), and anti-VGLUT1 (Synaptic Systems, Göttingen, Germany) was performed overnight at 4°C. Transduced neurons were incubated with anti-GFP (Abcam, Cambridge, UK) to enhance eGFP fluorescence. Samples were then rinsed five times with PBS and incubated with secondary antibodies conjugated either with 647-Alexa (Thermo Fisher, Waltham, MA, USA), Dylight 549 (Jackson ImmunoResearch, UK) or 488-Alexa (Thermo Fisher, Waltham, MA, USA) for 60 min at RT. After washing in PBS three times, samples were fixed in mounting medium and confocal images were acquired with a Leica TCS SP5 II inverted microscope (Leica Microsystems, Wetzlar, Germany) equipped with a ×63 oil immersion objective. Alexa fluorophores, 488 and 647, were excited with a 488-nm argon laser and with a 633-nm helium-neon laser, respectively. Emission signals were acquired after filtering with 500–550 nm and 640–700 nm bandpass filters, respectively. Dylight 549 was excited with a 543-nm helium-neon laser and emission signal was acquired after filtering with 570–610 nm bandpass filter. Changes in synapse formation were evaluated by analyzing the synaptic contact density per 50 µm length of neuronal structures. Overlapping positive puncta structures were identified between immunolabeled signals generated by presynaptic and postsynaptic markers. Confocal images were analyzed using Fiji (Schindelin et al., 2012).

Electrophysiology

Electrophysiological studies were conducted using the whole-cell voltage-clamp configuration with an EPC-10 amplifier equipped with the PatchMaster software (HEKA, Elektronik). Excitatory postsynaptic currents (EPSCs) were obtained from autaptic neuronal cultures containing a layer of glial cells and a single neuron forming autapses as described (Guzman et al., 2010). The composition of the external recording solution was (in
mM): 130 NaCl, 10 NaHCO₃, 2.4 KCl, 1.25 CaCl₂, 1.3 MgCl₂, 10 HEPES, 10 D-glucose, pH 7.3 with NaOH, osmolarity, 310 mOsm. The extracellular solution was supplemented with 20 μM bicuculline (Tocris Bioscience, Bristol, UK) to ensure the recording of EPSCs. Borosilicate patch pipettes with resistances of 3.5–6 MΩ were pulled on a Sutter P-1000 puller (Sutter, Novato, CA, USA) and filled with the intracellular solution containing (in mM): 137.5 K-gluconate, 11 NaCl, 4 MgATP, 0.4 Na₂GTP, 1.1 EGTA, 11 HEPES, 11 D-glucose, pH 7.3 with CsOH, osmolarity, 310 mOsm. EPSCs were triggered by a brief somatic depolarization from a holding potential of −70 mV to +10 mV during 0.7 ms. Data were registered at 10 or 50 kHz and Bessel filtered at 3.0 kHz. The resulting series resistance was usually less than 12 MΩ, and only neurons with series resistance below 15 MΩ and 70% to 85% resistance compensation were used for analysis. The amplitude of the evoked current was calculated from the baseline amplitude subtracted prior to stimulation artifact. The amount of the RRP of vesicles was measured by application of a hypertonc sucrose solution (500 mM) for 4 s with aid of a fast-flow perfusion system (AutoMate Scientific, Berkeley, CA, USA). The amplitude and charge of evoked EPSCs and the charge of RRP sucrose responses were evaluated by means of the following software: Clampfit (Molecular Devices, San Jose, CA, USA) and OriginPro (OriginLab Corporation, Northampton, MA, USA). The RRP size has been estimated as previously described (Stevens and Sullivan, 1998). The replenishment of RRP was measured after RRP depletion by applying paired-pulses of hypertonc sucrose solution during 3 s at 1 s, 3 s, 7 s, 15 s, 30 s and 60 s. Then, the time course of RRP refilling was analyzed by calculating firstly the percentage of RRP recovery at several interpulse time intervals relative to the last application of hypertonc sucrose (60 s). Second, the percentage of RRP recovery was fitted to a single exponential function to simplify the analysis of data as proposed (Stevens and Wesseling, 1998). To evaluate postsynaptic receptor saturation, 1 mM γ-D-Glutamylglycine (Tocris Bioscience, Bristol, UK) was added to the sucrose solution as described in Supplementary Figure S6. Miniature excitatory spontaneous postsynaptic currents (mEPSCs) were recorded in mass hippocampal neurons at holding potential of −70 mV during 1 min in the presence of 20 μM bicucullin and 1 μM tetrodotoxin (TTX; Biotrend, Köln, Germany) which was added to the standard extracellular solution to avoid spontaneous depolarization of the neurons. Spontaneous events with peak amplitudes higher than 15 pA (−5 times the standard deviation of the background noise) and with charges higher than 25 fC were evaluated using the software MiniAnalysis by Synaptosoft. Data were sampled at 10 kHz and Bessel filtered at 3.0 kHz. Recordings of Cav2.2/Cavβ₄b-mediated currents in HEK293T cells are described in Supplementary Material.

**Statistical Analysis**

The data are presented as column scatter dot plots with the mean value ± standard error of the mean (SEM) of each distribution shown by a line. Experiments were conducted in at least three different cell culture preparations with at least three different batches of lentivirus. Statistical significance was analyzed by a comparison between the data sets using one-way analysis of variance (ANOVA). p values: *p < 0.05, **p < 0.01, ***p < 0.001 were considered statistically significant. The variability between tested groups was conducted with SigmaPlot version 12.3 (Systat Software, San Jose, CA, USA). The data obtained to calculate the time course of replenishment of RRP were fitted with OriginPro.

**RESULTS**

**Cavβ Associates With Actin Filaments and the Cavα₁-Anchorin Domain in a Non-exclusive Manner**

We used an in vitro co-sedimentation assay to test the association of Cavβ with actin filaments. For this assay, actin is dissolved in a low salt polymerization buffer to promote the formation of F-actin. After centrifugation, proteins that associate with F-actin are recovered from the pellet fraction. Since recombinant full-length Cavβ, are not stable under low salt conditions (Stölting et al., 2015), we purified the core of the human neuronal Cavβ₄b containing the two highly conserved SH3 and GK domains. We verified that Cavβ₄b core associates with F-actin as previously described for the core domain derived from Cavβ₂ (Stölting et al., 2015; Figures 1A, B and Supplementary Figure S1).

We next examined if the interaction of Cavβ with F-actin and Cavα₁ are mutually exclusive or not. To assess Cavβ-Cavα₁ association, the highly conserved AID site (Pragnell et al., 1994) was fused to GST (GST-AID) as previously described (Hidalgo et al., 2006). The Cavβ₄b protein construct bearing a double mutation at two residues critical for association with the AID domain (M238A/L384A, Chen et al., 2004; Opatowsky et al., 2004) resulted in low yield and poor stability in low salt buffers. Thus, we used the core of Cavβ₂ as background and introduced the two point mutations at the analogous residues. This Cavα₁ association-deficient Cavβ₂ core mutant is more stable in the F-actin polymerization buffer and preserves the ability to associate with F-actin (Figure 1C). GST-AID co-sedimented together with F-actin in the presence of WT Cavβ₂, but not of the mutant version with impaired AID association (Figure 1D).

These results demonstrate that GST-AID is mobilized to the pellet fraction, together with F-actin, through its association with Cavβ. We conclude that Cavβ can simultaneously associate with Cavα₁ and F-actin.

**Cavβ₄b Increases the Frequency of Miniature Excitatory Postsynaptic Currents in the Presence of an Intact Cytoskeleton**

To study the functional role of the Cavβ₄b/F-actin association in synaptic transmission, we generated two lentiviral gene delivery expression vectors encoding either full-length Cavβ₄b (hereafter denoted as β₄WT) or the Cavβ₄b M238A/L384A mutant with
impaired Ca\textsubscript{\(\text{v}\)}\textsubscript{1}-binding (hereafter denoted as \(\beta\textsubscript{4-Mut}\)). Both Ca\textsubscript{\(\text{v}\)}\textsubscript{\(\beta\)}\textsubscript{b} constructs were linked to either eGFP or mCherry at their carboxy-terminal ends for visualization by fluorescence microscopy. \(\beta\textsubscript{4-WT}\), but not \(\beta\textsubscript{4-Mut}\), expressed in HEK293T associated in vitro with GST-AID (Supplementary Figure S2A) and when expressed together with Ca\textsubscript{\(\text{v}\)}2.2 supported robust ionic currents (Supplementary Figures S2B,C). \(\beta\textsubscript{4-Mut}\) failed to yield Ca\textsubscript{\(\text{v}\)}2.2-mediated currents.

In mass cultures of dissociated hippocampal neurons exogenous \(\beta\textsubscript{4-WT}\) and \(\beta\textsubscript{4-Mut}\) accumulate in synapses as judged by the co-localization with the presynaptic and postsynaptic markers VGLUT-1 and the MAP2, respectively (Figures 2A,B). We analyzed the number of excitatory synaptic contacts (VGLUT-1 positive puncta) in non-transfected hippocampal neurons and neurons expressing either \(\beta\textsubscript{4-WT}\) or \(\beta\textsubscript{4-Mut}\) by immunostaining (Figures 2A–C). Neither \(\beta\textsubscript{4-WT}\) nor \(\beta\textsubscript{4-Mut}\) alters synapse density at excitatory synapses (Figure 2D). Thus, exogenous Ca\textsubscript{\(\text{v}\)}\textsubscript{\(\beta\)}\textsubscript{b} does not modify synaptogenesis.

We recorded mEPSCs in mass cultures of hippocampal neurons transduced with the Ca\textsubscript{\(\text{v}\)}\textsubscript{\(\beta\)}\textsubscript{b}-encoding plasmids (Figure 3). We found that \(\beta\textsubscript{4-WT}\) significantly increased the average frequency of the mEPSCs, whereas \(\beta\textsubscript{4-Mut}\) produced no changes (Figures 3A,B). No alterations in the mean decay time constant of the averaged mEPSC responses were observed in neurons expressing \(\beta\textsubscript{4-WT}\) or \(\beta\textsubscript{4-Mut}\) by immunostaining (Figures 3A–C). Neither \(\beta\textsubscript{4-WT}\) nor \(\beta\textsubscript{4-Mut}\) alters synapse density at excitatory synapses (Figure 3D). The increased mEPSC frequency combined with the absence of alterations in mEPSCs decay time and in synapse density suggests a presynaptic function of Ca\textsubscript{\(\text{v}\)}\textsubscript{\(\beta\)}\textsubscript{b} in modulating neurotransmitter release as proposed (Wittermann et al., 2000; Xie et al., 2007). Both Ca\textsubscript{\(\text{v}\)}\textsubscript{\(\beta\)}\textsubscript{b} constructs induced a reduction in the average amplitude and charge transfer of the mEPSCs (Figures 3D,E), resulting in a slight leftward shift of the corresponding cumulative probability curve of the mEPSCs with respect to the distribution obtained from non-transduced neurons (Supplementary Figures S3A,B). These changes may arise from a decreased vesicular loading or from a reduction in the density of functional postsynaptic receptors triggered by Ca\textsubscript{\(\text{v}\)}4. Up-to-date there is no evidence associating Ca\textsubscript{\(\text{v}\)}4 with SV glutamate loading (Blondeau et al., 2004; Morciano et al., 2005; Takamori et al., 2006). Furthermore, since Ca\textsubscript{\(\text{v}\)}\textsubscript{\(\beta\)}\textsubscript{4} can be also targeted postsynaptically (Wittermann et al., 2000; Xie et al., 2007), we favor the idea that postsynaptic mechanisms account for these rather minor differences.
To investigate whether the Cavβ4b-mediated increase in mEPSC frequency depends on the integrity of the actin cytoskeleton, hippocampal neurons were exposed to the actin filament disruptor cytochalasin D prior to the electrophysiological recordings (Figure 4). Disruption of the actin cytoskeleton fully abolished the increase in the mEPSC frequency induced by β4-WT (Figures 4A,B). Our biochemical analysis indicates that the lack of effect observed for β4-Mut is not due to an impaired F-actin association (Figure 1C). Cytochalasin D treatment did not alter the mEPSC frequency in non-transduced neurons (untreated 1.4 ± 0.12 Hz; cytochalasin D-treated 1.17 ± 0.17 Hz, Figures 4A,B) suggesting that the actin cytoskeleton does not act as a barrier for spontaneous SV fusion (Sankaranarayanan et al., 2003).

The average mEPSCs decay time in non-transduced neurons was significantly increased after exposure to cytochalasin D (from 0.38 ± 0.03 ms to 0.61 ± 0.06 ms in untreated and cytochalasin D-treated neurons, respectively; Figure 4C).

**FIGURE 2 |** Exogenous WT Cavβ4b and the Cavα1 association-deficient mutant are targeted to synaptic contacts and do not alter synapse density in hippocampal neurons. Laser scanning confocal images of hippocampal neurons either transduced with Cavβ4b WT (β4-WT) fused to eGFP (A) or with Cavβ4b mutant with impaired Cavα1 binding (β4-Mut) fused to eGFP (B) and non-transduced neurons (ctrl; C). Neurons were immunolabeled with the glutamatergic presynaptic terminal marker VGLUT-1 (red) and the dendrite-selective marker MAP2 (blue). Cavβ4b fluorescence signal is shown in green. (D) Scatter dot plot of the number of synaptic contacts per 50 µm dendritic length for the indicated conditions. The number of synapses was quantified by counting the number of VGLUT-1 positive puncta overlapping with Cavβ4-eGFP signal along MAP2-positive neuronal processes per 50 µm length. Lines represent the average value ± standard error of the mean (SEM). n.s, not significant. Scale bar: 10 µm.
This is consistent with the notion that actin filaments participate in the spatial organization of the postsynaptic receptors at synapses (Okamoto et al., 2004). Minor changes in the amplitude and charge transfer of the spontaneous response were observed when comparing untreated neurons with their corresponding cytochalasin D-treated counterparts (Figures 4D,E and Supplementary Figures S3C,D). Nevertheless, a more pronounced effect was found in neurons expressing β4-Mut, where the charge transfer decreased from 116 ± 7 fC to 102 ± 6 fC after disruption of the actin cytoskeleton.

Our results show that spontaneous neurotransmitter release is regulated by Cavβ4b and that this regulation relies on an intact actin cytoskeleton and competent binding to Cavα1.

A Tripartite Interaction Among Cavβ, Cavα and F-Actin Increases Excitatory Postsynaptic Currents Induced by Depolarization in Hippocampal Neurons

We next recorded evoked EPSCs from autaptic cultures of dissociated hippocampal neurons expressing either β4-WT or...
The enhancement of the mEPSC frequency induced by exogenous Ca\textsubscript{v}β\textsubscript{4b} relies on a competent actin cytoskeleton. (A) Representative traces of mEPSC from neurons exposed to the cytochalasin D actin filament disruptor for 1 h (20 µM). For clarity, traces and plots from non-transduced neurons (ctrl) and transduced with either β\textsubscript{4-WT} or β\textsubscript{4-Mut} exposed to cytochalasin D are shown in blue in all figures. (B) Statistical analysis of mEPSC frequency for neurons exposed to cytochalasin D as shown in (A). (C) Scatter dot plot of the mean weighted decay time (mean τ decay, left panel) and ensemble averages of the mEPSCs (right panel) from the neurons exposed to cytochalasin D for the indicated conditions. (D) Scatter dot plot of the mEPSC amplitude recorded from the indicated neurons treated with cytochalasin D. (E) Scatter dot plot of the mEPSC charge transfer estimated for the indicated conditions. Lines represent the average value ± SEM. n.s, not significant; *p < 0.05, **p < 0.01, ***p < 0.001 one-way ANOVA.

β\textsubscript{4-Mut} (Figure 5). Consistent with previous work (Wittemann et al., 2000; Xie et al., 2007), we observed that exogenous β\textsubscript{4-WT} resulted in significantly larger amplitudes of the evoked postsynaptic response and increased charge transfer (Figures 5A,B,D). The half-width of the averaged EPSCs were unaffected (Supplementary Figure S4A). Expression of β\textsubscript{4-Mut}
caused no changes in the amplitude, charge transfer and kinetics of the EPSCs (Figures 5A,B,D and Supplementary Figure S4A). Comparison of the EPSCs amplitudes from day 10–14 DIV showed no significant changes suggesting that during this time window, exogenous $\beta_4$ has reached equilibrium with its endogenous counterparts (data not shown). Moreover, during the same time window, it does affect the RRP size, as shown below. Thus, we do not attribute the lack of effect of $\beta_4$, to a failure in its ability to actively displace endogenous Cav,$\beta_4$.

To assess the impact of the actin cytoskeleton, we exposed hippocampal autaptic cultures expressing either $\beta_4$-WT or $\beta_4$-Mut to cytochalasin D before recording the postsynaptic currents. Cytochalasin D treatment did not change the amplitude, charge transfer or kinetics of the evoked EPSC response in non-transduced neurons, but blunted the effect of $\beta_4$-WT on the EPSC amplitude (Figure 5A, blue traces and C-E and Supplementary Figure S4B). In order to investigate if the effects of cytochalasin D can be mimicked by other actin cytoskeleton disruptor, we treated non-transfected and $\beta_4$,-transfected neurons with latrunculin A. We found that this drug also suppressed the increase in EPSC amplitude and charge mediated by $\beta_4$-WT (Supplementary Figures S5A,C,D).

Altogether, our findings demonstrate that a tripartite interaction among Cav,$\beta$, Cav,$\alpha_1$ and F-actin upregulates spontaneous and depolarization-evoked neurotransmitter release via activation of presynaptic mechanisms. The requirement of an intact actin cytoskeleton for the increase in synaptic strength regulated by Cav,$\beta_4$ and F-actin in mobilizing SVs at different steps of the SV cycle (Cingolani and Goda, 2008).

**Cav,$\beta_4$ Increases the Mobilization of Synaptic Vesicles to the Readily Releasable Pool in an F-Actin Dependent Manner**

To test whether Cav,$\beta$/F-actin association is involved in SV translocation, we estimated the size of the RRP. To compare the impact of WT and mutant Cav,$\beta_4b$ per se, and not due to calcium-dependent effects, we use hypertonic sucrose stimulation that is well-established strategy to measure the RRP size in a calcium-independent manner (Rosenmund and Stevens, 1996). The RRP size was measured 5 s after recording the depolarization-evoked response in the same autaptic neuron (Figure 6). Neurons transfected with $\beta_4$-WT and $\beta_4$-Mut displayed an augmented overall RRP size (Figures 6A,B). To assess whether or not our measurements of the RRP size in Cav,$\beta_4$-expressing neurons were affected by postsynaptic receptor saturation, we estimated the size of the RRP in neurons expressing $\beta_4$-WT in the presence and in the absence of 1 mM γ-D-Glutamylglycine, a fast dissociating AMPA receptor antagonist (Liu et al., 1999). The result demonstrated that in our study, postsynaptic receptor saturation does not contribute to the RRP size measurements (Supplementary Figure S6). This is consistent with the previous report using another AMPA receptor antagonist (Schottenham et al., 2015). Postsynaptic receptor desensitization contributing to RRP estimations using 500 mM sucrose solution in hippocampal neurons has been negligible (Pyott and Rosenmund, 2002).

In non-transduced neurons, the pharmacological disruption of the actin cytoskeleton led to a reduction in the average of the RRP size (Figure 6A, blue traces, and C). Thus, cytochalasin D sensitive actin filaments appear to facilitate, rather than hamper the mobilization of SVs to the RRP under resting conditions.

In neurons transfused with either $\beta_4$-WT or $\beta_4$-Mut, pharmacological disruption of actin filaments by either cytochalasin D or latrunculin A blunted the increase in the RRP size triggered by this subunit (Figures 6A,C and Supplementary Figures S5B,E). Cytochalasin D treatment was less efficient in inhibiting the increased RRP size mediated by $\beta_4$-Mut (Figure 6C). The remaining cytochalasin D-insensitive pool of mobilized SVs may reflect a cluster of remote vesicles that escape the actin network surrounding the active zone.

**The Recovery of the RRP Size After Depletion Is Facilitated by Cav,$\beta_4$ Through a Cav,$\alpha_1$-Dependent Binding and Is Tightly Controlled by F-Actin**

Finally, we investigated if the interaction between Cav,$\beta_4$ and F-actin also effects the time course of the RRP recovery after depletion (Figure 7). In control autaptic neurons and neurons expressing the Cav,$\alpha_1$ association-deficient mutant subunit, the RRP is replenished with a time constant of about 12 s (Figures 7A,C,E), which is consistent with the literature (Stevens and Tsujimoto, 1995). However, $\beta_4$-WT greatly reduces the time constant for the vesicular replenishment of the RRP ($\tau_{rec} = 5.7 \pm 0.9$ s as compared to $\tau_{rec} = 12.3 \pm 2.0$ s from non-transduced and $\tau_{rec} = 12.3 \pm 2.6$ s for neurons transfused with $\beta_4$-Mut). Pharmacological treatment of the neurons with cytochalasin D reverted the effect of the WT subunit and yielded comparable rates of the RRP recovery time among the three groups of neurons, non-transduced and transfused with either $\beta_4$-WT or $\beta_4$-Mut (Figures 7B,D,F). These results indicate that the association of Cav,$\beta_4b$ with Cav,$\alpha_1$ and cytochalasin D-sensitive F-actin accelerates the recovery of the RRP after depletion.

**DISCUSSION**

We here show that the changes in synaptic transmission elicited by Cav,$\beta_4$ in hippocampal neurons at excitatory synapses are mediated by actin filaments and involve presynaptic mobilization and maturation of SVs toward a fusion-competent state. Direct comparison of the effects of the WT Cav,$\beta_4b$ and the Cav,$\alpha_1$ association-deficient mutant on neurotransmission allowed us to define a novel channel-independent function of this subunit in recruiting vesicles to the RRP.

**The Functional Interplay Among Cav,$\alpha$, Cav,$\beta$ and F-Actin Regulates Synaptic Transmission**

Several roles of the actin cytoskeleton at the presynaptic terminal have been considered (Morales et al., 2000;...
FIGURE 5 | The tripartite interaction among \( \text{C}_{\alpha_2}\beta, \text{C}_{\alpha_2}\alpha \), and F-actin is required for increasing depolarization-evoked EPSCs in individual hippocampal neurons. (A) Averaged EPSC response from autaptic hippocampal neurons evoked with the voltage protocol shown at the top. Overlapped average EPSC responses from neurons non-exposed (untreated, black traces) and exposed to 20 \( \mu \)M cytochalasin D for 1 h (blue traces) and expressing either \( \beta_4\text{-WT} \) or \( \beta_4\text{-Mut} \) or non-transduced (ctrl) are shown. (B,C) Scatter dot plots of the EPSCs amplitude recorded from untreated neurons and cytochalasin D-treated neurons, respectively. (D,E) Scatter dot plots of the EPSCs charge transfer estimated from untreated and cytochalasin D-treated neurons, respectively. Lines represent the average value ± SEM. n.s, not significant; ***p < 0.001 one-way ANOVA.

Cingolani and Goda, 2008; Choquet and Triller, 2013; Nelson et al., 2013; Rust and Maritzen, 2015). Actin filaments may serve as tracks for the active translocation of SVs in synaptic terminals or as a scaffold to recruit and promote the interaction of regulatory proteins required for neurotransmitter release. They may also act as a barrier for SV mobilization toward the release site (Halpain, 2003; Sankaranarayanan et al., 2003; Miki et al., 2016).
CaV and Actin Modulate Neurotransmission

The increased mean decay time of the miniature response following cytochalasin D treatment (Figure 4C) is most likely due to the involvement of the actin cytoskeleton on the spatial arrangement of the postsynaptic receptors (Okamoto et al., 2004). Disruption of the actin cytoskeleton with cytochalasin D per se did not alter the frequency of the miniature response or the amplitude of the depolarization-evoked EPSC response in non-transfected neurons but prevented their potentiation triggered by exogenous CaVβ4. Comparable results on evoked response were obtained when actin polymerization was inhibited with latrunculin A (Supplementary Figure S5).

The lack of effect of F-actin disruption on non-transfected hippocampal neurons has been suggested to reflect a counteracting effect between the two opposing presynaptic roles attributed to the actin cytoskeleton; as a barrier as well as a facilitator for the mobilization of SVs (Cingolani and Goda, 2008). Within this framework, our observation that cytochalasin D treatment decreased the RRP size (Figure 6) implies that under our conditions, F-actin acts predominantly as an entryway to repopulate the RRP (Wu et al., 2016).

The tripartite interaction would acquire physiological relevance during high synaptic activity whereby efficient coupling between SVs and CaVα1 (and upregulation of the RRP size and their replenishment rate) is required. Enhanced neurotransmission mediated by CaVβ/F-actin association would rely on higher availability of this subunit to be rerouted toward this function. In other words, the accessibility of this subunit may be a key determinant for regulating synaptic strength via F-actin cytoskeleton and may constitute a rate-limiting step for neurotransmission adaptation in response to the increased activity via F-actin association.

Whether spontaneous vesicle release depends on CaV2.x-mediated calcium influx (Kaeser and Regehr, 2014; Williams and Smith, 2018) and whether the same pool of SVs (Groemer and Klingauf, 2007; Ikeda and Bekkers, 2009) or a distinct subset (Fredj and Burrone, 2009) is dedicated to miniature and evoked neurotransmitter release remains under discussion (for review, Truckenbrodt and Rizzoli, 2014). In spite of the controversy, our results demonstrate that the increased frequency of spontaneous release depends on a competent F-actin and intact CaVα1 binding site suggesting that close proximity of SVs to CaVα1 is required. This requirement may reflect a dependence of the spontaneous release on calcium permeation through CaV2.x channels. In such a case,
**FIGURE 7** | Ca$_V$$_{α_1}$-binding confers Ca$_V$β$_4$ the ability to accelerate the time course of recovery of the RRP of SVs after depletion. (A) Representative recordings of sucrose responses at increasing time points after depletion from autaptic hippocampal neurons expressing or not the indicated Ca$_V$β constructs. (B) Representative recordings of sucrose responses as (A) but for neurons exposed to cytochalasin D. (C,D) Average recovery time course of the RRP replenishment after depletion from data as shown in (A,B), respectively. Continuous lines depict monoexponential fits, and the corresponding time constants ($\tau_{rec}$) are reported in the graph. (E,F) Scatter dot plot of $\tau_{rec}$ from the individual untreated neurons and treated with cytochalasin D, respectively. Lines represent the average value ± SEM. n.s, not significant; **$p < 0.01$** one-way ANOVA.
Ca\textsubscript{2}\textbeta/F-actin association tethers SV to the channel complex and promotes spontaneous release upon stochastic channel opening (Ermolyuk et al., 2013). The finding that the same tripartite interaction, Ca\textsubscript{2}\textbeta, F-actin and Ca\textsubscript{2}\textalpha\textsubscript{1}, is required for the increased evoked response supports the concept of an overlapping pool of vesicles and release sites, which mediates these two modes of neurotransmission.

We propose that the tripartite interaction is mandatory for generating a fusion-competent SV by allowing the coupling of primed SVs with the calcium channel at the release site. Ca\textsubscript{2}\textbeta\textsubscript{4} mutant with no capability to associate with Ca\textsubscript{2}\textalpha\textsubscript{1} (Supplementary Figure S2) fails in tethering the SV to the calcium channel and explains the lack of effect of the mutant Ca\textsubscript{2}\textbeta\textsubscript{4} on the frequency of the spontaneous neurotransmitter release and evoked response.

**Ca\textsubscript{2}\textbeta\textsubscript{4}/F-Actin Interaction Recruits Synaptic Vesicles to the Readily Releasable Pool and Is Involved in Its Replenishment**

We found that both tested Ca\textsubscript{2}\textbeta s—WT and mutant without the ability to associate with Ca\textsubscript{2}\textalpha\textsubscript{1}—support the recruitment of SVs to the RRP in concert with F-actin (Figure 6). Thus, the Ca\textsubscript{2}\textbeta-regulated increase in the RRP size operates independently of Ca\textsubscript{2}\textalpha\textsubscript{1} function. This is in line with the idea that neurotransmitter release evoked by hypertonc sucrose solution in hippocampal neurons is independent of calcium (Rosenmund and Stevens, 1996). Our results differ from a previous study, which reported unaltered RRP size in hippocampal neurons overexpressing Ca\textsubscript{2}\textbeta\textsubscript{4} and accelerated RRP recovery only after train stimulation. These studies suggested that the latter arose from an increased calcium influx (Xie et al., 2007). We believe that these differences are due to the use of two distinct Ca\textsubscript{2}\textbeta fusion constructs; whereas we linked eGFP to the C-terminus of the \textbeta subunit, the authors of the other study attached the GFP to the N-terminus. The SH3 domain located at the N-terminal moiety of the protein is a main determinant of F-actin binding (Stöltling et al., 2015), and N-terminal fusion might have masked functionally relevant protein-protein interactions.

The capability of Ca\textsubscript{2}\textbeta\textsubscript{4} to increase the RRP size hints to a direct role of this subunit in the translocation of vesicles along F-actin (Evans et al., 1998; Cingolani and Goda, 2008; Rust and Maritzen, 2015). This subunit may accumulate in the actin network surrounding SVs at the synaptic terminal and aid their passive recruitment to the RRP or their active trafficking along actin filaments by yet to be established protein-protein interactions. In analogy, Ca\textsubscript{2}\textbeta/F-actin association appears to recruit Ca\textsubscript{2}\textalpha\textsubscript{1} containing transport vesicles nearby the plasma membrane for recycling in cardiac cells (Stöltling et al., 2015; Conrad et al., 2018).

In excitatory hippocampal neurons, SVs within the RRP differ in their readiness to release their content upon a depolarizing stimulus (Hanse and Gustafsson, 2001; Moulder and Mennerick, 2005, 2006; Alabi and Tsien, 2012; Taschenberger et al., 2016; Kaeser and Regehr, 2017). Moreover, reluctant SVs can be converted into fast-releasing ones in an actin-dependent manner by bringing SVs closer to Ca\textsubscript{2} channels, supporting the so-called positional priming hypothesis (Lee et al., 2012). Within this context, our results are consistent with the notion that Ca\textsubscript{2}\textbeta\textsubscript{4} mutant mobilizes a subset of reluctant vesicles to the RRP whereas the WT protein translocate vesicles to the RRP and through its ability to associate with Ca\textsubscript{2}\textalpha\textsubscript{1} renders SV fusion competent.

Diverse protein-protein interactions are involved in positioning SVs and Ca\textsubscript{2}V within nanometer distance at the release site (Eggermann et al., 2011; Davydova et al., 2014; Gundelfinger et al., 2015; Nakamura et al., 2015; Korber and Kuner, 2016; Stanley, 2016; Wang et al., 2016; Kusch et al., 2018; de Jong et al., 2018). Among those, the multi-domain RIM associates directly with Ca\textsubscript{2}V\textsubscript{2.x} channels (Hibino et al., 2002). It has been reported that Ca\textsubscript{2}\textbeta also interacts with RIM (Kiyonaka et al., 2007) as well as with synaptotagmin I (Vendel et al., 2006). We here show that Ca\textsubscript{2}\textbeta\textsubscript{4} can simultaneously associate with F-actin and Ca\textsubscript{2}\textalpha\textsubscript{1}. This interplay may provide a molecular scaffold to hold in place the plethora of proteins involved in SV docking and priming. Moreover, the dimerization of Ca\textsubscript{2}\textbeta (Miranda-Laferne et al., 2011) may also enlarge the number of potential interacting partners regulating synaptic activity. The idea that Ca\textsubscript{2}\textbeta may operate as a scaffold between the calcium channel and the release machinery has been anticipated (Vendel et al., 2006; Weiss, 2006; Xie et al., 2007).
the clearance of the release sites in different model systems, including hippocampal neurons (Kawasaki et al., 2000; Hosoi et al., 2009; Wu et al., 2009; Soykan et al., 2017). It is recruited to endocytic sites via interaction with several proteins containing SH-3 domains. We have previously shown that Cavβ interacts with dynamin via its SH3 domain and promotes endocytosis (Gonzalez-Gutierrez et al., 2007; Miranda-Laferte et al., 2011). It is tempting to propose that Cavββ3 facilitates RRP replenishment by promoting dynamin/F-actin-dependent endocytosis that speeds up the removal of SV components and excess of membrane for a new cycle of release at the release site after exocytosis. Since undersupply of SVs and cleared release sites can cause transient synaptic depression during high synaptic activity, upregulation of the RRP size and their replenishment mediated by exogenous Cavββ3/F-actin association would support stable neurotransmission during sustained synaptic activity.

In conclusion, a physical association between Cavββ3, Cavβ3-3 and F-actin appears to be a sine-qua-non condition for bringing the SV within the permissive range of the Cavβ calcium nanodomain for release (Naraghi and Neher, 1997; Neher and Sakaba, 2008; Park et al., 2012; Nakamura et al., 2015; Stanley, 2015, 2016). This scenario places Cavββ3 as essential for the maturation of the SVs toward a fusion-competent state acting as a tether for the functional priming of the SV during spontaneous and depolarization-evoked synaptic transmission.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the German Law for the Protection of Animals. The protocol was approved by the Forschungszentrum Jülich GmbH and LANUV (State Agency for Nature, Environment and Consumer Protection) of North Rhine-Westphalia.

AUTHOR CONTRIBUTIONS

GG designed and performed the experiments, analyzed the data and edited the manuscript. RG analyzed data and edited manuscript. NJ produced all recombinant proteins and performed the biochemical assays and immunostainings. PH designed and supervised the research, and wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2019.00125/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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