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Abstract. High voltage gated calcium channels (VGCCs) are composed of at least three subunits, one pore forming α1-subunit, an intracellular β-subunit, and a mostly extracellular α2δ-subunit. Interactions between these subunits determine the kinetic properties of VGCCs. It is unclear whether these interactions are stable over time or rather transient. Here, we used single-molecule tracking to investigate the surface diffusion of α1- and α2δ-subunits at the cell surface. We found that α2δ-subunits show higher surface mobility than α1-subunits, and that they are only transiently confined together, suggesting a weak association between α1- and α2δ-subunits. Moreover, we observed that different α1-subunits engage in different degrees of association with the α2δ-subunit, revealing the tighter interaction of α2δ1 with Ca2+ 1.2 > Ca2+ 2.2 > Ca2+ 2.1 > Ca2+ 3.2. These data indicate a distinct regulation of the α1 /α2δ interaction in VGCC subtypes. We modeled their membrane dynamics in a Monte Carlo simulation using experimentally determined diffusion constants. Our modeling predicts that the ratio of associated α1- and α2δ-subunits mainly depends on their expression density and confinement in the membrane. Based on the different motilities of particular α1 /α2δ-subunit combinations, we propose that their dynamic assembly and disassembly represent an important mechanism to regulate the signaling properties of VGCC. © The Authors. Published by SPIE under a Creative Commons Attribution 3.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI. [DOI: 10.1117/1.NPh.3.4.041809]

Keywords: calcium channel; single-particle tracking; surface mobility.

1 Introduction

Transient activation of high voltage gated calcium channels (VGCCs) is critical and widespread cellular process. For example, VGCC activation is essential for vesicular transmitter release in neurons, excitation contraction coupling in muscle cells, and induction of intracellular signaling cascades via Ca2+ influx in many excitable cells.1 VGCCs are able to open and close so rapidly that, in conjunction with efficient Ca2+ -buffering and clearance, changes in Ca2+ -concentration can be confined to the nanometer range. Such a highly localized VGCC function plays an important role at neuronal synapses where the positioning of presynaptic VGCCs relative to synaptic vesicles (SV) is crucial for neurotransmitter release. At fast synapses, activation of a single VGCC may trigger release if it is in close proximity to a fusion-competent SV.7,8 Consistent with such a scenario, we reported recently that the number and mobility of VGCCs within active zones are relevant parameters for the synaptic function.9 It remained unclear, however, if the subunit-based molecular composition of VGCC affects these dynamic parameters.

VGCCs are composed of three principal subunits: the pore forming α1-subunit which determines the type of Ca2+ channel, and two auxiliary subunits, an intracellular β-subunit and a membrane-anchored α2δ-subunit with a large, highly glycosylated extracellular domain. It has been widely assumed that the three subunits within a VGCC display a stoichiometry of 1:1:1 and that the composition remains stable over time.6,7 Several distinct β- and α2δ-isofoms and splice variants are encoded in most vertebrate genomes, and changes in the combination of α1-, β- and α2δ-subunits within a particular VDCC have a substantial impact on kinetic properties and trafficking.7,8 For example, exchanging β-subunits altered the inactivation properties of presynaptic CaV2.1 and CaV2.2 and changed presynaptic transmitter release.10 While α2δ-subunits can also alter the voltage-dependent channel inactivation,11,12 their major function is their ability to promote channel trafficking and to tune the number of synaptic VGCCs.8,13 The latter property may account for pathological conditions characterized by an increase of surface expressed calcium channels.9,14 In addition, α2δ-subunits may assume VGCC-independent functions during synaptogenesis by their interaction with extracellular proteins like thrombospondins.15,16 Genomic aberrations of α2δ-subunits have been reported to cause epilepsy and intellectual disabilities as well as hyperinsulinism in humans caused by deletion of the CD36 gene.17

The extracellular von Willebrand A domain and cache domains of α2δ-subunits have been implicated in the physical interaction with the first three segments of the α1-subunit.18–20 Moreover, multiple glycosylation sites of the α2δ appear to

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contribute to the association with α1-subunits. The affinities between α1- and α2δ-subunits, however, appear rather weak as their association in the channel complex was below 10% compared to α1- or β-subunits. A direct interaction of α1- and α2δ-subunits via a transmembrane domain of the δ-domain was proposed, but identification of a glycosylphosphatidylinositol (GPI)-anchor present in all α2δ-subunits arguments against a transmembrane interaction. Here, we used single-particle tracking methods to investigate the surface dynamics and putative association between α1- and α2δ-subunits. We focused mostly on Cav2.2 and α2δ1-subunits because of their prominent role in the induction and expression of chronic pain in the peripheral nervous system.

2 Materials and Methods

2.1 Cell Culture

HEK293-T cells and tsA-201 cells (large SV40 T-antigen transformed HEK293) were grown in DMEM supplemented with 10% fetal calf serum (FCS), 1% antibiotic/antimycotic, and 1% L-glutamine. tsA-201 cells stably expressing rat Cav2.2 (in pcDNA6, basicidin resistance, GenBank No. AF055477), rat β3 (in pcDNA3.1, rocin resistance), and rat α2δ1 (in pcDNA3, hygroycin resistance, GenBank No. AF286488) were a gift from D. Lipscombe. All cells were cultured in 5% CO2 and a humidity of 95% at 37°C. All supplemented cell culture media were sterile filtrated (0.22 μm pore size) and kept at 4°C until use. Cells were transfected for 48 h before experiments using transfection reagents based on cationic lipids (FuGENE®HD Transfection Reagent, Roche).

Dissociated neuronal cultures were prepared from hippocampus as described before and transfected with calcium channel constructs at 3 to 5 days in vitro (DIV). For fluorescence recovery after photobleach (FRAP) and single-particle tracking (SPT) experiments, cultures of 14 to 21 DIV were mounted in an open chamber perfused with extracellular solution as specified below and imaged for up to 20 min at 36°C/RT.

2.2 Molecular Biology

Cav2.2::HA expression construct was modified from rat α1B-subunit (Q02294; kindly provided by Gerald Zamponi) by PCR to insert the HA-epitope (YPYDVPDYA) into the extracellular loop between the fifth and sixth transmembrane domain after F34, resulting in the duplication of C252/F254 after the epitope (HKACF254—HA-epitope—C254/FPNS). Two α1B-PCR fragments were generated using the following pairs of primers: (1) ratN-NotI-fw: cta ggc gcg cgctatatgggcgc ac/ ratN-5P(HA) RV-rev: P-gtc atg tga ata gaa ggc gct ctt atg gaa ttt g and (2) ratN-5P(HA)RV-fw: P-gtc ctt gat tgc gtc ttc ccc aac agc aca gat/ratN-syn_rev and ccc gta cgg cct cgg ctt gtt cgg. With the first and second halves of the epitope-sequence included in the forward and reverse ratN-5P(HA) primers and NotI or BsW1 sites included in the outer primers, respectively, the two fragments were tail-to-head ligated and thereafter used to replace the respective NotI-BsW1 fragment in the original rat Cav2.2 construct.

Similarly, Cav2.2::GFP was based on rat sequence (CAC1B_RA) and generated by inserting cGFP with short linkers coding for restriction sites into the last P-loop between amino acids 1672/1673 by site-directed mutagenesis. The resulting sequence was confirmed by sequencing as M QVF G N A L D D G T S I N R H N N F R T F L Q A L M L FR S-ATGEAWHEIMLSCLGNRACDP-Gly-Thr-eGFP-Thr-Glu-Gly-Thr>HANASEGDSFAYFY... N-terminal GFP-tagged Cav2.3.2 was provided by E. Bourinet (Montpellier, France), and generation of GFP-tagged Cav1.2 as well as of α2δ1-subunit with a double HA-epitope inserted after the predicted signal peptide into a rabbit cDNA (Genbank: M21948; expressed from neuronal β-actin promoter) were previously described. To allow the use of different labeling antibodies, we also exchanged the N-terminal HA-tag of the α2δ1-subunit to an FLAG epitope at the same position.

2.3 Electrophysiology

Whole-cell recordings from HEK cells were performed 48 to 72 h posttransfection for different combinations of Cav2.2-subunits, using an EPC 10 amplifier (HEKA, Germany) controlled by the PatchMaster software (HEKA). Patch pipettes were pulled from borosilicate glass capillaries with a resistance of 2 to 5 MΩ. The internal solution contained in mM: 130 CsCl, 3 MgCl2, 0.66 CaCl2, 11.7 EGTA, 10 HEPES (pH 7.3), and 305 mOsM. Prior to experiments, fresh ATP solution was added to a final concentration of 2 mM and the pH was adjusted to 7.3 with CsOH. The extracellular solution contained in mM: 140 NaCl, 10 BaCl2, 1 MgCl2, 10 HEPES, 10 D-(+)-glucose, pH 7.4 was adjusted with NaOH. Recordings were performed at room temperature (22 to 25°C) under constant perfusion with extracellular solution. Barium currents were recorded in whole-cell mode using the p/4 protocol to subtract leak currents. Data were analyzed using FitMaster (HEKA) and IGOR Pro (WaveMetrics) software. The stimulus protocols were designed within the PatchMaster Pulse Generator.

2.4 Immunocytochemistry

Primary antibodies were applied to live or fixed HEK293 or COS-7 cells to distinguish between surface and total populations of channel subunits. Live cell labeling was done in culture medium for 15 to 30 min at 37°C. Cells were fixed with 4% paraformaldehyde (PFA) in phosphate buffered salt solution (PBS) for 5 min, then washed and permeabilized for 2 min with 0.3% TritonX/PBS. Nonspecific immune reactivity was blocked by washing cells three times for 10 min with a washing buffer containing 10% FCS, 25 mM Glycin, and 2% bovine serum albumin (BSA) in PBS. Primary and secondary antibodies were applied consecutively for 1 h at RT. After additional washing steps, cells were mounted on glass slides with Mowiol (Sigma). The following primary antibodies were used: polyclonal rabbit anti-GFP antibody 1:200 (Invitrogen; A6455), monoclonal mouse anti-HA antibody (1:200; Covance; MMS-101P); secondary fluorescently labeled antibodies included: anti-mouse ATTO 647N 1:200 (Sigma-Aldrich, 50185-4ML-F) and anti-rabbit Abberior STAR 580 1:200 (Abberior; 2-0012-005-08) or Alexa 488 (Thermo-Fisher Scientific, A-11034).

2.5 Imaging and Colocalization Analysis

Fluorescence labeling of cells was examined by either using a conventional epifluorescence microscope coupled to a CCD camera (ImagerA2 microscope, Zeiss, coupled to a CoolSnap

Neurophotonics 041809-2 Oct-Dec 2016 • Vol. 3(4)
Myo CCD-camera, Roper Scientific) or by use of an stimulated emission depletion (STED) microscope (SP5, Leica, Germany). To determine the colocalization of extra- or intracellularly GFP-tagged CaV2.2 and α2δ1, HA-subunits, Z-stacks were recorded using a Leica TCS SP5 2-channel STED microscope equipped with an inverted microscope DMI 6000 and a 100x-STED objective (HCX PL APO 100x, 1.4 NA oil STED, Leica Microsystems). The fluorophores used, Abberior STAR580 and Atto647N, were sequentially excited with pulsed-diode lasers (PicoQuant) at 531 and 635 nm. The fluorescence signals were detected with avalanche photodiodes (Perkin Elmer Inc.) through BL HC 607/36 (Abberior STAR580) and ET BP 670/30 (Atto647N) emission filters separated by a dichroic beam splitter at 650 nm. Depletion was performed at 730 nm for Abberior STAR580 and at 750 nm for Atto647N with a titanium sapphire laser (Chameleon ultra II, Coherent). The stacks were acquired at a resolution of 12 bits and in 1024 x 1024 pixel format with a pixel size of 25.2 nm (due to 6 x zoom). The scan speed was set to 700 Hz by using 48x line averaging.

Maximal intensity projections of the STED stacks were generated and the background was subtracted with a rolling ball radius of 10 pixels using ImageJ (version 1.44). The colocalization was analyzed from 512 x 512 pixel image sections with the software OpenView (provided by Dr. N. Ziv, Tel Aviv, Israel). Here, fluorescent clusters positive for the GFP-tagged α2β1δ2 channel were determined by centering 10 x 10 pixel boxes around the local fluorescent maximum. The colocalization of fluorescent spots was defined by matching pixel boxes within a radius of 4 pixels. The matched area was set to overlap at least a 4 x 4 pixels region to be considered as colocalized.

2.6 Fluorescence Recovery After Photobleach Experiments

Hippocampal cultures were imaged on an inverted fluorescence microscope (Axio Observer D1, Zeiss) using a heated imaging chamber (TC-344B, Warner Instruments) and an EMCCD camera (Evolve 512, Photometrics) controlled by MetaMorph software. The FRAP laser (DL-473, Rapp Optoelectronics) was coupled to a point scanning device controlled by the software via a UGA40 control unit (Rapp Optoelectronics). The FRAP laser was pointed at up to 10 regions of interest (ROI) within one experiment with a dwell time per ROI of 10 ms. About 50 to 100 images were acquired before photobleach followed by an additional image acquisition for the next 5 min after photobleach. Image sequences were analyzed using MethaMorph and GraphPad Prism5 software. The recovery rate was determined after background subtraction and bleach correction. The relative recovery rate was calculated by the ratio of ROI fluorescent intensity for every time point versus the intensity before (set to 100%) and immediately after photobleach (set to 0%).

2.7 Single-Particle Tracking with Quantum Dots

GFP- or HA-tagged constructs were labeled with QDs precoated on monoclonal mouse anti-GFP (clone 7.1 and 13.1, 11814460001, Roche) or rat anti-HA antibody (clone 3F10, Roche), respectively. Precoating of QDs was performed as described before. In brief, 1 µl of 1 µM QD-655 conjugate against mouse or rat (goat F(ab’)2 anti-mouse or anti-rat IgG conjugate, Molecular Probes) was precoated with the corresponding antibody (0.5 µg, ∼0.5 µl) in 7.5 µl PBS for 15 min and blocked with 1 µl Casein solution (Vector Laborities) for an additional 15 min. Precoated QDs were used to label surface-expressed, epitope-tagged subunits of transfected cells at a final concentration of 0.1 to 0.01 nM for 1 to 5 min at 37°C. Labeled cells were washed three times for 1 min in extracellular solution containing 0.5% BSA before imaging.

HEK cells and primary hippocampal neurons were mounted in an open chamber (Ludin chamber, Basel, Swiss) and used for imaging experiments in an extracellular solution of the following composition in mM: 145 NaCl, 10 Glucose, 10 HEPES, 5 KCl, 2 CaCl2, 2 MgCl2. Imaging of QDs was conducted at 100x NA objective. Fluorescence of QD was excited by a Xenon lamp using excitation filter HC 531/40 (Semrock), and emitted fluorescence was acquired through a HC 655/15 bandpass filter (Semrock). Images were acquired at video rate (30Hz) using MetaMorph stream acquisition software.

Tracking of QDs was performed by the use of custom-made software. Trajectories of single QDs were reconstructed by allowing reconnection of positions within two pixels distance to the previous image. The mean square displacement (MSD) was calculated and plotted over time for reconnected trajectories of at least 100 frames. Diffusion coefficients were calculated by linear fit of the first four points of the MSD plots versus time. The diffusion coefficient and confinement index were calculated using custom software as described in Ref. 31.

2.8 Single-Particle Tracking Photoactivated Localization Microscopy (Sptpalm) Imaging

A TIRF set-up was based on an inverted microscope (IX71 Olympus, Germany) and equipped with a manual TIRF illuminator arm and TIRF objective (100x, NA1.49). Generation and use of the mEos2-tagged CaV2.2 construct was described in a previous work. The tag was inserted on the same position as GFP in the N-terminus. Briefly, we used laser diodes to photocexcite and excite the fluorophore by continuous illumination of the probe with a 405-nm laser (2% to 5% of 100 mW) and a 561-nm laser (25% to 40% of 100 mW). Images were acquired by an EMCCD camera (Andor, EMCCD, IXon Ultra). The green fluorescence of mEos2 (exitation at 488 nm) was used to identify transfected cells. Images were recorded at 30 Hz for up to 4000 frames. We used a 1.6 magnification lens to reduce the pixel size to 100 x 100 nm2.

2.9 Image Analysis

Localization and trajectory reconstruction of mEos2 signals was performed by the use of a wavelet based algorithm, implicated in the super resolution software application from MetaMorph. Trajectories of mEos2-tagged molecules were reconstructed by a simulated annealing algorithm, taking into account molecule localization and total intensity. It has been described that mEos2 molecules can show blinking-like behavior. To avoid false reconnections between trajectories, all subtrajectories of mEos2 were analyzed as individual trajectories. The dynamic behavior of single molecule was computed from the MSD curves for all trajectories of at least 8 frames. Diffusion coefficients were calculated by linear fit of the first four points of the MSD plots. MSD plots of immobilized molecules (on fixed samples) revealed that under our imaging conditions D ≥ 0.001 µm2/s can be considered to be mobile. This threshold
was used for QD analysis as well. Trajectories from QD-labeled subunits were used to calculate the confinement and explored membrane surface area as described.23

### 3 Monte Carlo Simulation of Subunit Interactions

The simulation has been implemented in MATLAB R2015b. The random number generation was carried out using different pseudorandom number algorithms (RNGs) such as “mersenne twister,” “combined multiple recursive,” and “multiplicative lagged fibonacci.” Within the statistical averages from 20 independent runs, all results were similar from the different RNGs in the framework of calculated mean values and error variances. Typical run times of simulations were on the order of a few minutes.

#### 3.1 Statistics

Analysis of differences in data distributions or mean values was done by the use of GraphPad Prism software, using statistical tests as indicated in the legends. Data shown either as median and an interquartil range, or as mean ± SEM. Significant levels are given as *, p < 0.05; **, p < 0.005; and ***, p < 0.0005; n.s., nonsignificant.

### 4 Results

#### 4.1 Functional Characterization of Extracellularly Tagged Ca\(^{2+}\) Channel Subunits

Live imaging of endogenous VGCCs ideally requires surface labeling with antibodies against an extracellular domain of \(\alpha_1\)- or \(\alpha_2\beta\)-subunits. Despite numerous attempts by several groups, this strategy has not yet been successful, presumably because of the small size of the extracellular domains and blocking of normal channel function of \(\alpha_1\)-subunits, and/or limited antigenicity due to extensive glycosylation of \(\alpha_2\beta\)-subunits. Using an alternative strategy, surface labeling of VGCC has been achieved by insertion of small epitope tags in an outer loop of the pore forming \(\alpha_1\)-subunit of CaV \(1.2\) and CaV \(2.2\) channels without major impact on channel traffic and function.15 However, a similar approach was not successful for the CaV \(2.1\) channel,16 a serious drawback as CaV \(2.1\) is the predominant variant in most excitatory synapses. In this study, we, therefore, tested additional positions in the outer loops of CaV \(2.1\) and CaV \(2.2\) for the insertion of HA- and GFP-epitopes. The successful positions for both tags in the pore-forming subunit of CaV \(2.2\) are indicated in Fig. 1(a). Subunits carrying an epitope were expressed on the surface of heterologous HEK293 cells and reliably detected by live labeling using anti-HA or anti-GFP antibodies [Fig. 1(b)]. We then tested the expression of the tagged CaV \(2.2\) constructs in cultured hippocampal or DRG neurons, but obtained very heterogeneous results with respect to a detectable surface population which could not be improved by coexpression of \(\alpha_2\delta\)-subunit (data not shown). Problems with reliable neuronal surface expression of extracellularly tagged VGCCs are in contrast with the successful targeting of pore-forming subunits where the epitope is placed on its cytoplasmic N-terminus.3 For our investigation of the VGCC subunit interaction and surface dynamics, we, therefore, chose the heterologous cell expression system.

To characterize the extracellularly tagged CaV \(2.2\) variants electrophysiologically, we performed whole-cell recordings of

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**Fig. 1** Functional characterization of extracellular tagged constructs of the CaV \(2.2\) channel. (a) The position of the two tagging positions is indicated in the sketch of the \(\alpha_1\)-subunit of the channel, the inset position is given for both the HA-tag and GFP-tag plus the flanking linkers (in gray). (b) Immune fluorescent labeling of the constructs expressed in HEK cells for surface expressed channel population (live labeling, extra) and total channel population after fixation of the cells (total). (c) Representative barium currents evoked by depolarizing potential steps from −90 mV holding potential to −20; −10; 0; 10 mV for 200 ms and stepping back to 90 mV. (d) Current voltage relationship for the WT, CaV \(2.2\) : : HA and the CaV \(2.2\) : : GFP construct, note the clear differences in the current density as well as minor changes in the maximal activation voltage. (e) Quantification of the current density for WT CaV \(2.2\) / CaV \(2.2\) : : HA/CaV \(2.2\) : : GFP and WT CaV \(2.2\) expressed with the \(\beta_3\)-subunit only. (f) Illustrating example for the voltage-dependent inactivation for a test potential to 10 mV for the WT CaV \(2.2\) (black) and the WT CaV \(2.2\) expressed with the \(\beta_3\)-subunit only (blue). (g) Quantification of the time constant for voltage-dependent inactivation at different test potentials, the different combinations are given in the legend. The data are from 5 to 20 cells for each combination of channel subunits. Statistical differences are determined by one-way ANOVA test followed by a posthoc Bonferroni-test. Data given as mean ± SEM, *, p < 0.05.
transfected HEK293 cells, coexpressing subunits α1B with and without epitope tags, β3, and α2δ1 [Figs. 1(c)–1(g)]. Representative traces [Fig. 1(e)], I/V-curves [Fig. 1(d)], and maximal current densities [Fig. 1(e)] were not different between the WT and HA-tagged channels. Expression of the GFP-tagged CaV2.2 :: α2δ1 channel complex containing such a large epitope still showed similar current densities to WT CaV2.2/β1/VGCCs without coexpressed α2δ1-subunits.

We next probed activation and steady-state-inactivation properties of our tagged CaV2.2::α2δ1 and compared these parameters to barium currents recorded from a stable CaV2.2 cell line with no tags on α1- or α2δ1-subunits (Table 1). Since the association of α2δ1 with α1-subunit not only promotes surface expression, but also influences the time course of voltage-dependent inactivation,4 we tested this parameter for all three channel constructs at different potentials. Similar to their current densities, the HA-tagged (CaV2.2::HA) channel was not significantly different from the WT channel. The extracellular GFP-tagged CaV2.2 (CaV2.2::GFPextra) showed a slower inactivation, comparable to WT-CaV2.2 channel expressed without α2δ1 [Figs. 1(c) and 1(e)]. Coexpression of the α1-subunit with the β3 alone led to similar current kinetics as seen with the CaV2.2::GFPextra [Fig. 1(d)]. Thus, both current density and inactivation time course indicate that the large insert of an extracellular GFP molecules when expressed alone [Fig. 2(a)], revealing a diffusion coefficient almost identical to GPI-anchored GIF [Fig. 2(e), items 1 and 2]. These results provide support for the proposed GPI-anchorage of α2δ-subunits.54 In contrast, coexpression of α2δ1 with either extra- or intracellularly tagged CaV2.2 channels (including the β3-subunit as in all experiments) triggered the formation of surface clusters and partial confinement of α2δ1-subunits [Figs. 2(b)–2(d)]. While all CaV2.2 variants were able to cluster α2δ1, the restrictive effect of CaV2.2 on the mobility of α2δ1-subunit was stronger for the intracellularly tagged CaV2.2 channels compared to the extracellular variants [Fig. 2(e), items 3 versus 4]. Moreover, the extracellularly tagged CaV2.2 channels themselves displayed significantly different surface mobility than intracellularly tagged CaV2.2 channels [Fig. 2(e), items 5, 6]. Further tests with other channels (CaV2.1, CaV1.2, and CaV3.2) showed an isomorph specific effect on the diffusion properties of α2δ1-subunits with different pore-forming α1-subunits [Fig. 2(f)]. The low-voltage activated CaV3.2 channel did not induce a reduction of α2δ1-subunit surface dynamics [Fig. 2(f), item 6], whereas the surface trafficking of CaV3.2 channels is reported to be supported by the expression of α2δ1-subunits.55

Table 1 Activation and steady-state inactivation voltage of tagged and antibody labeled CaV2.2 channels expressed in HEK cells together with β3- and α2δ1-subunits. Data are means ± SEM (N = 5 to 10 cells for each condition), significant changes are indicated as (*) in respect to the stable cell line.

<table>
<thead>
<tr>
<th>Plasmid combination</th>
<th>V_{1/2active} (mV)</th>
<th>V_{1/2inactive} (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaV2.2 + β3 + α2δ1 (stable cell line)</td>
<td>-10.7 ± 3.5</td>
<td>-70.4 ± 0.5</td>
</tr>
<tr>
<td>CaV2.2 :: HA\text{extra} + β3 + α2δ1</td>
<td>-4.8 ± 2.9 (*)</td>
<td>-71.5 ± 0.2</td>
</tr>
<tr>
<td>CaV2.2 :: HA\text{extra} + β3 + α2δ1 + anti-HA antibody</td>
<td>-3.2 ± 4.1 (*)</td>
<td>-67.7 ± 0.4</td>
</tr>
<tr>
<td>CaV2.2 :: GFP\text{extra} + β3 + α2δ1</td>
<td>-5.8 ± 3.7 (*)</td>
<td>-65.7 ± 0.4 (*)</td>
</tr>
<tr>
<td>CaV2.2 :: GFP\text{extra} + β3 + α2δ1 + anti-GFP antibody</td>
<td>-2.8 ± 3.1 (*)</td>
<td>-59.9 ± 0.2 (*)</td>
</tr>
</tbody>
</table>

4.2 Single-Particle Tracking of Calcium Channel Subunits

To evaluate whether the differences in Ca2+ current densities and kinetics recorded from untagged and various tagged VGCCs reflect differences in the α1/α2δ1 interaction on the cell surface, we used SPT (Fig. 2). When we monitored the position and surface dynamics of VGCC by conjugating QDs with antibodies to tagged CaV2.2 pore-forming and α2δ1-subunits in this analysis (tagging schemes at left in Fig. 2), we observed that labeling densities varied between the different combinations of tagged channel subunits. Localization density maps and trajectories recorded from CaV2.2 channels and α2δ1-subunits expressed in different combinations show their distinct distributions in the membrane [Figs. 2(a)–2(d)]. We found that the surface expression of the extracellularly tagged CaV2.2 :: GFP\text{extra} channels strictly depends on the coexpression of an α2δ1-subunit. Moreover, HA-tagged α2δ1-subunits showed a different diffusion behavior with only a small population of clustered molecules when expressed alone [Fig. 2(a)], revealing a diffusion coefficient almost identical to GPI-anchored GIF [Fig. 2(e), items 1 and 2]. These results provide support for the proposed GPI-anchorage of α2δ-subunits.54 In contrast, coexpression of α2δ1 with either extra- or intracellularly tagged CaV2.2 channels (including the β3-subunit as in all experiments) triggered the formation of surface clusters and partial confinement of α2δ1-subunits [Figs. 2(b)–2(d)]. While all CaV2.2 variants were able to cluster α2δ1, the restrictive effect of CaV2.2 on the mobility of α2δ1-subunit was stronger for the intracellularly tagged CaV2.2 channels compared to the extracellular variants [Fig. 2(e), items 3 versus 4]. Moreover, the extracellularly tagged CaV2.2 channels themselves displayed significantly different surface mobility than intracellularly tagged CaV2.2 channels [Fig. 2(e), items 5, 6]. Further tests with other channels (CaV2.1, CaV1.2, and CaV3.2) showed an isoform specific effect on the diffusion properties of α2δ1-subunits with different pore-forming α1-subunits [Fig. 2(f)]. The low-voltage activated CaV3.2 channel did not induce a reduction of α2δ1-subunit surface dynamics [Fig. 2(f), item 6], whereas the surface trafficking of CaV3.2 channels is reported to be supported by the expression of α2δ1-subunits.55

We next used the extracellularly tagged CaV2.2 channel to characterize the surface affinity between the two subunits. The labeling efficiency between extracellular HA-tagged and GFP-tagged CaV2.2 channels was substantially different. First, only coexpression of CaV2.2, β3-, and α2δ1-subunits was sufficient to bring a population of extracellular GFP-tagged CaV2.2 channels to the surface. Second, labeling efficiency was weaker for extracellular HA-tagged CaV2.2 channels than GFP-tagged CaV2.2 channels if all subunits are expressed. Third, the surface diffusion was significantly different between the two extracellular tagged CaV2.2 channels [Fig. 2(e), items 5, 6]. Probing the surface dynamics of α2δ1-subunits coexpressed with the extracellular tagged CaV2.2 channel is complementary to the dynamics of tagged CaV2.2 channels. Expression of α2δ1-subunits with extracellular GFP-tagged CaV2.2 channels slightly...
reduced the mobility of α2δ1-subunits. Whereas in combination with the intracellular GFP-tagged CaV2.2 channel, the reduction of α2δ1-subunit surface dynamics was more prominent [Fig. 2(e), items 3, 4]. This indicates that CaV2.2 channels are less mobile and more confined in the cell membrane than their associated α2δ1-subunits. Second, the association of the α2δ1-subunits is influenced by the extracellular tag of the CaV2.2 channel, which might influence the voltage-dependent inactivation [Figs. 1(f) and 1(g)].

To assess whether the HA-tag within the CaV2.2 channel affects its association with α2δ1, we expressed CaV2.2:HAextra channels together with an FLAG-tagged α2δ1-subunit. The diffusion properties of the latter were similar to the HA-tagged α2δ1-subunit in combination with the intracellularly tagged channel CaV2.2::GFPintra (data not shown), implying that the HA-tag in the outer loops of CaV2.2 channels has little if any impact on its association with α2δ1.

Thus, the size of the extracellular label has a significant impact on the association of CaV2.2 channels and α2δ1-subunits expressed in different combinations. Note that only a very small fraction of channels and subunits is indeed immobile (D < 0.001 μm²/s).
Within long, reconnected single-particle trajectories periods of transient confinement are frequent. These observations indicate a dynamic association of channel subunits in the cell membrane [Fig. 3(a)]. The different diffusion behavior is visible in the distribution of the diffusion coefficient for $\alpha_2\delta_1$-subunits and $\text{CaV}_{2.2}$ channels [Fig. 2(g)]. Plotting the MSD versus time interval [Fig. 3(b)] confirmed the idea of general stronger confinement of $\text{CaV}_{2.2}$ channels versus $\alpha_2\delta_1$-subunits. Fitting the MSD of transient confined periods of diffusion [Fig. 3(a)], was used to determine the averaged surface of the explored area. The area of these transient confinements are different between the tested combinations as, $\alpha_2\delta_1$-subunit alone, $\alpha_2\delta_1$-subunits expressed together with intracellular tagged $\text{CaV}_{2.2}$ channel and extracellular tagged $\text{CaV}_{2.2}$ channels [Fig. 3(b)]. The differences in transient confinement strongly indicate a dynamic association of extracellular subunits with the pore forming subunits. Using dual color STED microscopy, we confirmed that there are changes in the colocalization of $\text{CaV}_{2.2}$ channel with intracellular tagged $\text{CaV}_{2.2}$ channel and $\alpha_2\delta_1$-subunits, dependent on the position of the GFP-tag in the $\text{CaV}_{2.2}$ channel [Figs. 3(d) and 3(e)]. Since the occupation rate of the tagged subunits and the respective antibody is not determined and might differ between the used epitopes (GFP-tag and HA-tag), this experiment does not allow to quantify the absolute ratio between associated and dissociated subunits. However, the relative difference in the colocalization indicate a mismatch in the case of extracellular GFP-tag on the $\text{CaV}_{2.2}$ channel. Both dynamic and static imaging data indicate a fluctuation between associated and dissociated subunits. A potential mechanism to alter the kinetic properties of the surface expressed $\text{CaV}_{2.2}$ channel population could be the up- or downregulation of surface expressed $\alpha_2\delta_1$-subunits, as seen in the development of chronic pain by nerve injuries in the peripheral nervous system.44 However, an alteration in $\alpha_2\delta_1$-subunit expression is often accompanied by an overall change of the $\text{CaV}_{2.2}$ channel expression as well.

### 4.3 Surface Mobility of Tagged $\alpha_2\delta_1$ VGCC Subunits in Neurons

To mitigate the limitations of a heterologous expression system, in particular, (i) the weak resting membrane potential of HEK293 cells causing a majority of expressed VGCCs to be...
in an inactivated state, (ii) the different lipid composition of the HEK293 membrane with putative effects on diffusion, and (iii) the different glycosylation pattern and entirely different spectrum of potential extracellular binding partners for α2δ1, we aimed to perform a proof-of-principle experiment in neurons (Fig. 4). To examine whether CaV2.2 and α2δ1-subunits also differ in their mobility in neuronal membranes, we expressed CaV2.2::GFPintra and α2δ1-HA in cultured hippocampal neurons. As mentioned above, extracellular tagged CaV2.2 channels did not express reliably in the neuronal membrane and had slightly altered kinetic properties, which was not the case for intracellular tagged channels. The use of CaV2.2 has the advantage that overexpression leads to their accumulation in the presynaptic compartment, allowing us to probe whether α2δ1 show a tight association to pore-forming CaV2.2 subunits in a structurally defined membrane compartment. Intracellularly GFP-tagged CaV2.2 channels could be clearly detected in synapses [Fig. 4(a)]. We then used FRAP experiments to define the mobile fraction of CaV2.2::GFPintra, resulting in 25% fluorescence recovery within 6 min after photobleach [Figs. 4(a) and 4(b)]. In addition, we monitored the surface dynamics of QD-labeled α2δ1-HA with and without coexpression of CaV2.2::GFPintra. In neurons, the mobility of α2δ1-subunits in the axonal membrane was comparable to GPI::GFP [Fig. 4(d)], consistent with the HEK cell experiments [Fig. 2(f)]. In contrast to the heterologous expression system, the mobility of α2δ1-subunits on the axonal membrane was not influenced by coexpression of CaV2.2::GFPintra, even in the presynaptic membrane [Fig. 4(d); comparison to Figs. 2(e) and 2(f)]. More strikingly, however, the α2δ1-subunit dynamic is significantly different between the axonal and presynaptic membrane compartments [Fig. 4(d)], suggesting that in neurons, the localization of VGCCs affects the dynamics of subunits more than an association between subunits. In support, we observed that the majority of tagged molecules did not stabilize in the synapse but exchanged between the synaptic and extrasynaptic (axonal) membrane areas during the observation period [e.g., Fig. 4(c)]. As reported before, clustered CaV2.2 channels represent, to a large extent, presynaptic terminals. Thus, these data support the observation in HEK293 cells that VGCC subunits are not tightly associated with each other on the cellular membrane. The strong effect of the localization (synaptic versus axonal) on diffusion, in turn, indicates that additional parameters involved in neurons remain to be uncovered in the future.

### 4.4 Modeling of Subunit Association Within Different Membrane Compartments

To systematically investigate whether the association between VGCC subunits depends on the level of overall surface expression or their local densities, we developed a modeling approach based on our previous work. The model builds on...
Fig. 5 Modeling interaction time of α1- and α2δ1-subunits. (a) Part of a lattice grid used for the simulation with channel α1 units (red), α2δ units (blue), and interacting channels and α2δ units (red–blue units in the top left part). (b) Random interaction time distribution for \( t_{\text{interact}} \sim 1 \) s and \( t_{\text{interact}} \sim 10 \) s as indicated. (C1–4) Simulation of noninteracting channels and α2δ-subunits: Trajectories of channels (1) and α2δ-subunits (2), distribution of diffusion coefficient for channels (red) and α2δ units (blue) and averaged MSD of channels (red) and α2δ-subunits (blue) over simulation time of 3 s (4); (D1–4) Simulation with interacting channels and α2δ-subunits, interaction time corresponding to experimental observations, compare Fig. 3(b), black curve (display of data like in c); (E1–4) Simulation of strongly interacting channels and α2δ-subunits (display of data like in C); (F1–4) Simulation on a smaller lattice grid were channels remain confined (200 nm × 200 nm), whereas α2δ-subunits can enter and leave the area of channel confinement. The density of channels in the confinement is higher than in (C)–(E). The α2δ-subunits are inserted within the channel confinement in a 1:1 initial ratio, whereas outside the channel cluster α2δ-subunits are inserted in a similar density without channels. Association of the subunits is only possible in the confinement with box boundary conditions for the channels with interacting time between 0 and 10 s. (g) Number of pairs of interacting channel and α2δ-subunits over simulation time of 3 s for the 2 μm × 2 μm lattice grid like case in (d) (blue) and smaller 200 nm × 200 nm lattice grid like in case (f), with the interaction time between 0 and 10 s.
a two-dimensional regular lattice [gray, Fig. 5(a)], in which $\alpha_1$-[red, Fig. 5(a)] and $\alpha_2\delta_1$-[blue, Fig. 5(a)] subunits can occupy a number of positions with defined size of 10 nm × 10 nm. The subunits may move randomly in two dimensions [arrows, Fig. 5(a)], representing lateral diffusion within the cell membrane. The step time of the $\alpha_1$-subunit $\Delta_{\alpha_1C}$ and the step time of the $\alpha_2\delta_1$-subunits $\Delta_{\alpha_2\delta_1}$ correspond to the experimentally obtained diffusion coefficients (Fig. 2). Our model observes several limitations: (1) $\alpha_1$-subunits and $\alpha_2\delta_1$-subunits do not occupy a position twice; (2) $\alpha_1$ and $\alpha_2\delta_1$-subunits may occupy one lattice space together and then “interact” with each other [blue–red unit, Fig. 5(a)]; and (3) the system has open boundaries, thus $\alpha_1$ and $\alpha_2\delta_1$-subunits can move across the lattice border and re-enter at the other side. If $\alpha_1$ and $\alpha_2\delta_1$-subunits occupy the same position, they move together [green arrows, Fig. 5(a)] until a predefined interaction time $t_{\text{interact}}$ is reached and then they move again independently. We based our simulation in accordance with experimental observation data with 50 channels on a 2 $\mu$m × 2 $\mu$m system (corresponding to a 200 $\times$ 200 lattice grid) and added 50 units of a 0.001 $\mu$m [200 nm = 200 nm] step size between the channels and $\delta_1$-subunits. At the beginning of our simulation, the $\alpha_1$-subunits and $\alpha_2\delta_1$-subunits were also altered since the majority of the $\alpha_2\delta_1$-subunits can move freely without $\alpha_1$-subunit interactions. A small part of the $\alpha_2\delta_1$-subunits bond to channels partially in the central area [Fig. 5(f)]. This is also reflected in the small subpopulation of slower $\alpha_2\delta_1$-subunits in the distribution of the diffusion coefficient [Fig. 5(f)].

The simulation predicts that under all conditions tested there will be a population of channels in the synapse lacking an $\alpha_2\delta_1$-subunit. In order to illustrate the fraction of free channels, we counted the number of interacting pairs over time for two systems with the interaction time resulting in the diffusion properties as seen in our experiments ($t_{\text{interact}} = 0$ to 10 s). For the 2 $\mu$m × 2 $\mu$m system [Fig. 5(d)], the number of $\alpha_2\delta_1$-subunit-associated channels continuously decreased. For channels confined into the smaller 200 nm × 200 nm system [Fig. 5(f)] and with an excess of exchanging $\alpha_2\delta_1$-subunits, the population of $\alpha_2\delta_1$-subunit-associated channels reached a plateau after 1 to 2 s [Fig. 5(g)]. These results indicate that confinement of channels and the overlap population of $\alpha_2\delta_1$-subunits might be sufficient to regulate surface channel assembly, despite the low affinity of $\text{CaV}_2.2$ channel and $\alpha_2\delta_1$-subunits.

5 Discussion

Function and assembly of VGCCs have been studied intensely in heterologous expression systems and depend critically on the association of $\alpha_1$ pore-forming with auxiliary $\beta$- and $\alpha_2\delta$-subunits. While $\beta$-subunits are absolutely required for the trafficking of $\alpha_1$ pore-forming subunits to the plasma cell membrane, $\alpha_2\delta$-subunits are able to further enhance the forward trafficking to promote a stronger surface expression of the channel. In addition, isoforms of both $\beta$- and $\alpha_2\delta$-subunits have different impacts on the kinetic properties of VGCCs. The study of the role of auxiliary VGCC subunits has been confounded by the observation that in neurons, at least $\alpha_2\delta$-subunits have additional roles.

Most of the experiments in our study focused on the surface interaction of VGCC with $\alpha_2\delta_1$-subunits. Their interaction is particularly important for the activation of VGCC by calcium in immature hippocampal glutamatergic synapses. Probing the dynamics of the two subunits showed that $\text{CaV}_2.2$ channels and $\alpha_2\delta_1$-subunits are not permanently associated but rather transiently associate in both HEK293 cells and neurons. Using extracellular and intracellular tagged $\text{CaV}_2.2$ channels and $\alpha_2\delta_1$-subunits revealed the existence of three populations: free $\text{CaV}_2.2$ channels, free $\alpha_2\delta_1$-subunits, and associated $\text{CaV}_2.2$ channels with $\alpha_2\delta_1$-subunits. If there will indeed be a 1:1:1 stoichiometry between the channel subunits, the weak affinity (dwell time $t_{\text{interact}}$) might be compensated by different expression levels and confinement of subunits in the membrane, particular in synapses. Comparing the dynamics of both subunits [Figs. 2(e) and 2(g), 4(a)–4(d), 5(a)–5(d)] confirmed a substantial difference in
the confinement of CaV2.2 channels and α2δ1-subunits [Figs. 3(b) and 3(c), 4(a)–4(d), 5(a)–5(d)]. This observation was further substantiated by determining the colocalization of calcium channel subunits in the plane of the cell membrane using STED microscopy [Figs. 3(d) and 3(e)]. The physiological consequences are proposed by the altered voltage-dependent inactivation of evoked barium currents from tagged channels where the interaction between CaV2.2 channels and α2δ1-subunits is altered [CaV2.2::GFPextra, Fig. 1(d)]. Whether the dynamic subunit associations will be a mechanism that influences channel activity in their natural environment needs to be further explored. Interestingly, the affinity of α2-subunits and α2δ1-subunits seems to be different between different calcium channels as observed in the altered diffusion coefficient of α2δ1-subunits when expressed with CaV1.2, CaV2.2, CaV2.1, or CaV3.2 channels [Fig. 2(f)]. The strongest association seems to exist between CaV1.2 and α2δ1-subunits, whereas CaV3.2 channels do not seem to interact with α2δ1-subunits at the cell surface. With respect to the physiological function and subunit association, α2δ1-subunits are identified to tune the voltage-dependent opening of CaV1.2 channels.20,47,50 Recently, the first crystal structure of the CaV1.1 channel complex has been described by the use of cryo-electron microscopy. Here, the authors demonstrate that the interface of binding between CaV1.1 and α2δ1-subunit is between the extracellular loops of the transmembrane repeats I–III and the von Willebrand A domain and cache1 domain of the α2δ1-subunit.18

Using voltage clamp fluorometry, it has been shown that the channel α2δ1-subunit interaction with the voltage sensor domains within the transmembrane repeats I–III is relevant for the activation of the channel.20 In light of these recent structure-function data, we cannot fully exclude that the tagging of the α2δ1-subunit on the N-terminus will influence the association of the subunits. However, the functional tests with and without HA-tagged α2δ1-subunits (Fig. 1) speak against a major impact of the chosen tag position. Whether the proposed dynamic association between α2δ1-subunits and CaV2.2 channels will be of functional relevance in neurons has to be tested. In specific compartments, the synapse channel densities can be tenfold higher5,51,52 as in the axon controlled by many molecular interactions.53 Our first data in neurons suggest that α2δ1-subunits are more confined in synapses, which is rather due to other interactions than a specific affinity to CaV2.2 channels (Fig. 4). The interaction with extracellular matrix proteins16,54 might be effective to confine α2δ1-subunits in the synapse. Our simulations suggest that such local molecular crowding has an impact on the association of channel subunits. The simulation further indicated that the copy numbers for channels and α2δ1-subunits should be very different to have a certain population of α2δ1-subunits bound to the channels. The confinement and increased density of CaV2.2 channels themselves can be assigned to many intracellular interaction partners, particularly in the synapse.55–58 Whether other molecules, e.g., synaptic adhesion proteins such as neurexins,59 participate in the confinement of channels or α2δ1-subunits remains to be tested.

Thus, the described differences in surface dynamics of channel subunits give potential new insights into the function of calcium channels. The labile interaction between channel and α2δ-subunits suggests a local and fast mechanism to alter the signaling capacity of calcium channels based on their subunit composition. The timescale for such altered interactions are in the range of milliseconds to seconds and could represent a potential variable tuning voltage-dependent calcium signaling.

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Voigt et al.: Dynamic association of calcium channel subunits at the cellular membrane

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