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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 δ-variant. 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 α₂δ₁-subunits at the cell surface. We found that α₂δ₁-subunits show higher surface mobility than α₁-subunits, and that they are only transiently confined together, suggesting a weak association between α₁- and α₂δ₁-subunits. Moreover, we observed that different α₁-subunits engage in different degrees of association with the α₂δ₁-subunit, revealing the tighter interaction of α₂δ₁ with CaV2.1 > CaV2.2 > CaV2.1 > CaV3.2. These data indicate a distinct regulation of the α₁/α₂δ₁ 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 α₂δ₁-subunits mainly depends on their expression density and confinement in the membrane. Based on the different motilities of particular α₁/α₂δ₁-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 a 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. 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 Ca²⁺-effectors for synaptic vesicle (SV) fusion 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. 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. 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 δ-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. Several distinct β- and δ-isofoms and splice variants are encoded in most vertebrate genomes, and changes in the combination of α₁, β- and δ-subunits within a particular VGCC have a substantial impact on kinetic properties and trafficking. For example, exchanging β-subunits altered the inactivation properties of presynaptic CaV2.1 and CaV2.2 and changed presynaptic transmitter release. While αδ-subunits can also alter the voltage-dependent channel inactivation, their major function is their ability to promote channel trafficking and to tune the number of synaptic VGCC. The latter property may account for pathological conditions characterized by an increase of surface expressed calcium channels. In addition, αδ-subunits may assume VGCC-independent functions during synaptogenesis by their interaction with extracellular proteins like thrombospondins. Genomic aberrations of α₂δ₁-subunits have been reported to cause epilepsy and intellectual disabilities as well as hyperinsulinism in humans caused by deletion of the CD36 gene. The extracellular von Willebrand A domain and cache domains of αδ-subunits have been implicated in the physical interaction with the first three segments of the α₁-subunit. Moreover, multiple glycosylation sites of the α₂δ appear to
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 argues 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

HEK293T 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, blasticidin resistance, GenBank No. AF055477), rat β3 (in pcDNA3.1, reovin resistance), and rat α2δ1 (in pcDNA3, hygromycin 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 F254, resulting in the duplication of C252—F254 after the epitope (HKACF254—HA-epitope—C252FFPS). Specifically, two α1B-PCR fragments were generated using the following pairs of primers: (1) ratN-NotI_fw: cta ggc ggc ctc ttt ggg ggc acr ratN-5P(HA) RV_rev; P-gtc ata tgg ata gaa gga gcc ctc atg gaa ttt g and (2) ratN-5P(HA)RF_fw: P-gtc cct gat tgc gtc ctc ccc aac aag aca gat ratN-syn_rev and ccc gta cgg cct cgg ctc tgt ctt cgc. With the first and second halves of the epitope-sequence included in the forward and reverse ratN-5P(HA) primers and NotI or BswI sites included in the outer primers, respectively, the two fragments were tail-to-head ligated and thereafter used to replace the respective NotI-BswI fragment in the original rat CaV2.2 construct.

Similarly, CaV2.2::GFP was based on rat sequence (CAC1B_RA) and generated by inserting eGFP 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 MQVFNIALDDGTSSINRHNNFRTFLQALMLFRS-ATGEAWHEIMLSCLGNRACDP-Gly-Thr-eGFP-Thr-Glu-Gly-Thr>HANASECGSFAYFY...N-terminal GFP-tagged CaV3.2 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. Non-specific 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, 50185-1ML-F) and anti-rabbit Abberior STAR 580 1:200 (Abberior; 2-0012-005-8) 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...
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 100×-STED objective (HCX PL APO 100×, 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 on 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 × 1024 pixel format with a pixel size of 25.2 nm (due to 6 × zoom). The scan speed was set to 700 Hz by using 48× 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 × 512 pixel image sections with the software OpenView (provided by Dr. N. Ziv, Tel Aviv, Israel). Here, fluorescent clusters positive for the GFP-tagged construct was described in a previous work.5 The tag was inserted on the same position as GFP in the N-terminus. Briefly, we used laser diodes to photoconvert 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 EM-CCD camera (Andor, EMCCD, IXon Ultra). The green fluorescence of mEOS2 (excitation 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 × 100 nm².

### 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 Imaging 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 MetaMorph 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 Laboritories) 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 an inverted Zeiss microscope (AxioObserver) equipped with an EMCCD camera (Evolvë™ 512, Photometrics) using a 100× 1.4 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. [8].

### 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 (100×, 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 photobleach 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 EM-CCD camera (Andor, EMCCD, IXon Ultra). The green fluorescence of mEOS2 (excitation 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 × 100 nm².

### 2.9 Image Analysis

Localization and trajectory reconnection 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.

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 are shown either as median and an interquartil range, or as mean ± SEM. Significance 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 \(\alpha_2\delta\)-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 \(\alpha_2\delta\)-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 CaV1.2 and CaV2.2 channels without major impact on channel traffic and function. However, a similar approach was not successful for the CaV2.1 channel. A serious drawback as CaV2.1 is the predominant variant in most excitatory synapses. In this study, we, therefore, tested additional positions in the outer loops of CaV2.1 and CaV2.2 for the insertion of HA- and GFP-epitopes. The successful positions for both tags in the pore-forming subunit of CaV2.2 are indicated in Fig. 1(a).

Subunits carrying an epitope were expressed on thesurface 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 CaV2.2 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. For our investigation of the VGCC subunit interaction and surface dynamics, we, therefore, chose the heterologous cell expression system.

To characterize the extracellularly tagged CaV2.2 variants electrophysiologically, we performed whole-cell recordings of

![Fig. 1](https://www.spiedigitallibrary.org/journals/Neurophotonics Vol. 3(4) Dec 2016 - Oct-Dec 2016 • Vol. 3(4) Neurophotonics 041809-4)
transfected HEK293 cells, coexpressing subunits α\textsubscript{1B} with and without epitope tags, β\textsubscript{3}, and α\textsubscript{2δ1} [Figs. 1(c)–1(g)]. Representative traces [Fig. 1(c), 1/f-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 Ca\textsubscript{V}2.2 :: GFP, however, showed a >50% reduction in current density compared to Ca\textsubscript{V}2.2 or Ca\textsubscript{V}2.2 :: HA [Fig. 1(e)]. Interestingly, the Ca\textsubscript{V}2.2 :: GFP/β\textsubscript{3}/α\textsubscript{2δ1}-channel complex containing such a large epitope still showed similar current densities to WT Ca\textsubscript{V}2.2/β\textsubscript{3} VGCCs without coexpressed α\textsubscript{2δ1}-subunits.

We next probed activation and steady-state-inactivation properties of tagged Ca\textsubscript{V}2.2 and compared these parameters to barium currents recorded from a stable Ca\textsubscript{V}2.2 cell line [13] with no tags on α\textsubscript{1} - or α\textsubscript{2δ1}-subunits (Table 1). Since the association of α\textsubscript{2δ1} with α\textsubscript{1}-subunit not only promotes surface expression, but also influences the time course of voltage-dependent inactivation, we tested this parameter for all three channel constructs at different potentials. Similar to their current densities, the HA-tagged (Ca\textsubscript{V}2.2 :: HA) channel was not significantly different from the WT channel. The extracellular GFP-tagged Ca\textsubscript{V}2.2 (Ca\textsubscript{V}2.2 :: GFP\textsubscript{extra}) showed a slower inactivation, comparable to WT-Ca\textsubscript{V}2.2 channel expressed without α\textsubscript{2δ1} [Figs. 1(c) and 1(e)]. Coexpression of the α\textsubscript{1}-subunit with the β\textsubscript{3} alone led to similar current kinetics as seen with the Ca\textsubscript{V}2.2 :: GFP\textsubscript{extra} [Fig. 1(d)]. Thus, both current density and inactivation time course indicate that the large insert of an extracellular GFP limits the association of the α\textsubscript{2δ1}-subunits with the Ca\textsubscript{V}2.2 channel.

To further characterize the effect of the binding of Quantum dot (QD)-coupled antibodies on VGCC function, important for our study of surface dynamics (see below), we compared the kinetic properties of WT and tagged channels under addition of anti-HA and anti-GFP antibodies (Table 1). The extracellular HA- and GFP-tagged Ca\textsubscript{V}2.2 channels showed a shift of 5 mV to more positive potentials. The addition of tag specific antibodies also changed the steady-state inactivation of the tagged channel in comparison to nontagged channels (Table 1). The bias in the functional properties might have an impact on the proper function of Ca\textsubscript{V}2.2 channels in neuronal membrane compartments, specifically the synapse. Nevertheless, we assume that in HEK cells the majority of channels will be in steady-state inactivation. Thus, surface mobility of calcium channels in the membrane of HEK cells mainly represent an activation-independent feature of calcium channels due to the resting membrane potential of about −40 mV in HEK cells. In neurons, we employed an intracellular N-terminal tagged Ca\textsubscript{V}2.2 channel (see below) to avoid any interference with the kinetic properties of the expressed channel population.

### 4.2 Single-Particle Tracking of Calcium Channel Subunits

To evaluate whether the differences in Ca\textsuperscript{2+} currents and kinetics recorded from untagged and various tagged VGCCs reflect differences in the α\textsubscript{1}/α\textsubscript{2δ1} 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 Ca\textsubscript{V}2.2 pore-forming and α\textsubscript{2δ1}-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 Ca\textsubscript{V}2.2 channels and α\textsubscript{2δ1}-subunits expressed in different combinations show their distinct distributions in the membrane [Figs. 2(a), 2(b)]. We found that the surface expression of the extracellularly tagged Ca\textsubscript{V}2.2 :: GFP\textsubscript{extra} channels strictly depends on the coexpression of an α\textsubscript{2δ1}-subunit. Moreover, HA-tagged α\textsubscript{2δ1}-subunits showed a rather diffusive localization with only a small population of clustered molecules when expressed alone [Fig. 2(a)]. Revealing a diffusion coefficient almost identical to GPI-anchored GFP [Fig. 1(e)], items 1 and 2). These results provide support for the proposed GPI-anchorage of α\textsubscript{2δ1}-subunits [2]. In contrast, coexpression of α\textsubscript{2δ1} with either extra- or intracellularly GFP-tagged Ca\textsubscript{V}2.2 channels (including the β\textsubscript{3}-subunit as in all experiments) triggered the formation of surface clusters and partial confinement of α\textsubscript{2δ1}-subunits [Figs. 2(b)–2(d)]. While all Ca\textsubscript{V}2.2 variants were able to cluster α\textsubscript{2δ1}, the restrictive effect of Ca\textsubscript{V}2.2 on the mobility of α\textsubscript{2δ1}-subunit was stronger for the intracellularly tagged Ca\textsubscript{V}2.2 channels compared to the extracellular variants [Fig. 2(e), items 3 versus 4]. Moreover, the extracellular GFP-tagged Ca\textsubscript{V}2.2 channels themselves displayed significantly different surface mobility than intracellularly tagged Ca\textsubscript{V}2.2 channels [Figs. 2(c)–2(d)]. While all Ca\textsubscript{V}2.2 variants were able to cluster α\textsubscript{2δ1}, the restrictive effect of Ca\textsubscript{V}2.2 on the mobility of α\textsubscript{2δ1}-subunit was stronger for the intracellularly tagged Ca\textsubscript{V}2.2 channels compared to the extracellular variants [Fig. 2(e), items 3 versus 4]. Moreover, the extracellular GFP-tagged Ca\textsubscript{V}2.2 channels showed an isomorph specific effect on the diffusion properties of α\textsubscript{2δ1}-subunits with different pore-forming α\textsubscript{1}-subunits [Fig. 2(f)]. The low-voltage activated Ca\textsubscript{V}3.2 channel did not induce a reduction of α\textsubscript{2δ1}-subunit surface dynamics [Fig. 2(f), item 6], whereas the surface trafficking of Ca\textsubscript{V}3.2 channels is reported to be supported by the expression of α\textsubscript{2δ1}-subunits [2].

We next used the extracellular tagged Ca\textsubscript{V}2.2 channel to characterize the surface affinity between the two subunits. The labeling efficiency between extracellular HA-tagged and GFP-tagged Ca\textsubscript{V}2.2 channels was substantially different. First, only coexpression of Ca\textsubscript{V}2.2, β\textsubscript{3} - and α\textsubscript{2δ1}-subunits was sufficient to bring a population of extracellular GFP-tagged Ca\textsubscript{V}2.2 channels to the surface. Second, labeling efficiency was weaker for extracellular HA-tagged Ca\textsubscript{V}2.2 channels than GFP-tagged Ca\textsubscript{V}2.2 channels if all subunits are expressed. Third, the surface diffusion was significantly different between the two extracellular tagged Ca\textsubscript{V}2.2 channels [Fig. 2(b), items 5, 6]. Probing the surface dynamics of α\textsubscript{2δ1}-subunits coexpressed with the extracellular tagged Ca\textsubscript{V}2.2 channel is complementary to the dynamics of tagged Ca\textsubscript{V}2.2 channels. Expression of α\textsubscript{2δ1}-subunits with extracellular GFP-tagged Ca\textsubscript{V}2.2 channels slightly
Fig. 2. Surface dynamics of individual CaV2.2 channel subunits and αδβ-subunits expressed in different combinations. (a)–(d) Expressed combinations of CaV2.2 and αδβ-subunits in HEK cells and their localization density map as well as trajectory map are presented. The scale bar represents 600 nm. Arrows indicate clusters of the tracked subunit as indicated in the sketches on the left side. (e) Medians and interquartile range of diffusion coefficient of the mobile population of channel subunits in the cell membrane (D > 0.001 μm²/s) for proteins and combinations as indicated. Data are from 2 to 3 transfected HEK-cell cultures, the number of trajectories are αδβ:CaV2.2 : : GFPextra; 1951, αδβ : + CaV2.2 : : GFPextra; 1049, CaV2.2 : : HAextra; 279, CaV2.2 : : mEOS2intra; 8503 traj., significances were determined by a Kruskal–Wallis-test followed by a Dunn’s multiple comparison test: **, p < 0.005; ***. p < 0.0005. (f) Median and interquartile range of diffusion coefficient of the mobile population (D > 0.001 μm²/s) of αδβ-subunit expressed alone or in combination with different αδ-subunits as indicated. The number of trajectories are: GPI-GFP: 1172 traj., αδβ:1:8967 traj., +/β and CaV1.2: 4609 traj., +/β and CaV2.2:6661 traj., +/β and CaV2.1: 3558 traj., +/β, and CaV3.2:563 traj.; (g) The distribution histogram for the diffusion coefficients of subunit combinations as indicated reveal the differences between the different combinations. Note that only a very small fraction of channels and subunits is indeed immobile (D < 0.001 μm²/s).

reduced the mobility of αδβ-subunits. Whereas in combination with the intracellular GFP-tagged CaV2.2 channel, the reduction of αδβ-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 αδβ-subunits. Second, the association of the αδβ-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 αδβ, we expressed CaV2.2 : : HAextra channels together with an FLAG-tagged αδβ-subunit. The diffusion properties of the latter were similar to the HA-tagged αδβ-subunit in combination with the intracellularly tagged subunit 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 αδβ. Thus, the size of the extracellular label has a significant impact on the association of CaV2.2 channels and αδβ-subunits to each other. In addition, stPalm experiments with N-terminal mEOS2 tagged CaV2.2 channels support the diffusion dynamics recorded by the use of anti-HA-QD-labeled CaV2.2 : : HAextra channels [Fig. 2(e), item 7]. These experiments exclude the possibility that antibody precoated QDs might have local cross-linking activity, which could influence surface dynamics of calcium channel subunits. The clustered distribution and slower diffusion of CaV2.2 channels indicate a stronger confinement of the pore-forming subunit than the extracellular associated lipid anchored αδβ-subunits.
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 CaV2.2 channels [Fig. 3(b)]. Plotting the MSD versus time interval [Fig. 3(c)] confirmed the idea of general stronger confinement of CaV2.2 channels versus \( \alpha_2 \delta_1 \)-subunits. Fitting the MSD of transient confined periods of diffusion [Fig. 3(d)], 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 CaV2.2 channel and extracellular tagged CaV2.2 channels [Fig. 3(e)]. 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 CaV2.2 channel and \( \alpha_2 \delta_1 \)-subunits, dependent on the position of the GFP-tag in the CaV2.2 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 CaV2.2 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 CaV2.2 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. However, an alteration in \( \alpha_2 \delta_1 \)-subunit expression is often accompanied by an overall change of the CaV2.2 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. [3]). 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. Ha]. 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. Ha and Hb]. 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. Hc] and consistent with the HEK cell experiments [Fig. Hf]. 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. Hg] comparison to Figs. He and Hf. More strikingly, however, the α2δ1-subunit dynamic is significantly different between the axonal and presynaptic membrane compartments [Fig. Hh]. 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 $\alpha_1$- and $\alpha_2\delta_1$-subunits. (a) Part of a lattice grid used for the simulation with channel $\alpha_1$ units (red), $\alpha_2\delta$ units (blue), and interacting channels and $\alpha_2\delta$ units (red–blue units in the top left part). (b) Random interaction time distribution for $t_{\text{interact}} \sim 1 \text{ s}$ and $t_{\text{interact}} \sim 10 \text{ s}$ as indicated. (C$_1$–C$_4$) Simulation of noninteracting channels and $\alpha_2\delta$-subunits: Trajectories of channels (1) and $\alpha_2\delta$-units (2), distribution of diffusion coefficient for channels (red) and $\alpha_2\delta$ units (blue) and averaged MSD of channels (red) and $\alpha_2\delta$ units (blue) over simulation time of 3s (4); (D$_1$–D$_4$) Simulation with interacting channels and $\alpha_2\delta$-subunits, interaction time corresponding to experimental observations, compare Fig. 3(b), black curve (display of data like in C); (E$_1$–E$_4$) Simulation of strongly interacting channels and $\alpha_2\delta$-subunits (display of data like in C); (F$_1$–F$_4$) Simulation on a smaller lattice grid were channels remain confined (200 nm $\times$ 200 nm), whereas $\alpha_2\delta$-subunits can enter and leave the area of channel confinement. The density of channels in the confinement is higher than in (C)–(E). The $\alpha_2\delta$-subunits are inserted within the channel confinement in a 1 : 1 initial ratio, whereas outside the channel cluster $\alpha_2\delta$-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 $\alpha_2\delta$-subunits over simulation time of 3 s for the 2 $\mu$m $\times$ 2 $\mu$m lattice grid like case in (d) (blue) and smaller 200 nm $\times$ 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 $\times$ 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 $\Delta t_{\alpha_1}$ and the step time of the $\alpha_2\delta_1$-subunits $\Delta t_{\alpha_2\delta_1}$ correspond to the experimentally obtained diffusion coefficients (Fig. 5). 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 nm $\times$ 2 nm system (corresponding to a 200 $\times$ 200 lattice grid) and added 50 units of a 1:1 ratio of $\alpha_1$-subunits-$\alpha_2\delta_1$-subunits, initially as interacting partners on the same squares. We set the interaction time $t_{\text{interact}}$ between a $\alpha_1$ and $\alpha_2\delta_1$ pairs as an equal-distributed number with a defined mean value [Fig. 5(b) shows $t_{\text{interact}}$ distributions for mean values of $t_{\text{interact}}$ $\sim$ 10 s and $t_{\text{interact}}$ $\sim$ 1 s]. Thus, the variable parameter in our Monte Carlo simulation is the interaction time of channels and $\alpha_2\delta_1$-subunits.

The first simulation shows the results for noninteracting $\alpha_1$ and $\alpha_2\delta_1$-subunits, i.e., a system with an interaction time $t_{\text{interact}}$ $=$ 0 s [Fig. 5(c)]. The display represents 10 trajectories of channels [Fig. 5(d)] and 10 $\alpha_2\delta_1$-subunits [Fig. 5(e)]. The trajectory selection is done to enhance the visibility of particular channel trajectories but is random. The color code change is from orange (starting position) to red (final position) for channels, and from yellow (starting position) to blue (final position) for $\alpha_2\delta_1$-subunits. The Monte Carlo simulations were repeated 20 times, a sufficient number to obtain a robust distribution of diffusion coefficients. As shown in Fig. 5[c]-diffusion coefficients and MSD curves are very different from channels and $\alpha_2\delta_1$-subunits and comparable to the mobility properties if both proteins are expressed separately.

We next assumed an interaction time between 0 and 10 s for channels and $\alpha_2\delta_1$-subunits, resulting in a simulation of diffusion coefficients and MSD for $\alpha_2\delta_1$-subunit and channels that overlap and are close to the experimental situation [Fig. 5(e)]. [see Fig. 5(b) blue line and Fig. 5(b) blue curve]. Interestingly, increasing the range of the interaction time 0 to 20 s led to an almost identical mobility of channels and $\alpha_2\delta_1$-subunits [Fig. 5(f)]. Thus, we kept the value of $t_{\text{interact}}$ between 0 and 10 s to simulate our experimental conditions. Short interaction times between 0 and 1 s between $\alpha_2\delta_1$-subunits and channels lead to a diffusion coefficient distribution which was broader. The empirically found time window for subunit interactions (0 to 10 s) should help to simulate the distribution and interaction times in the presynaptic membrane.

Finally, we studied the situation of calcium channels in the presynaptic membrane where their density is presumably much higher. We simulated a confined presynaptic membrane (200 nm $\times$ 200 nm) within the area used before (2 nm $\times$ 2 nm) and concentrated all 50 channels into the confinement [Fig. 5(h)]. We assumed that channels are confined in this small area, but allowed $\alpha_2\delta_1$-subunits to move independently in and out of the confined area. The start point for the simulation was that both channels and $\alpha_2\delta_1$-subunits are placed in the confined area. Due to the liberty of $\alpha_2\delta_1$-subunits, no $\alpha_2\delta_1$-subunit remains bound to a channel, which is a situation similar to 0 s interaction time (data not shown). We balanced this effect by increasing the number of $\alpha_2\delta_1$-subunits in the periphery of the channel cluster to equalize the density outside the confinement of the channels [Fig. 5(i)]. Now, the mobility of channels was slightly reduced within the confinement [Fig. 5(j)] compared to the larger 2 $\mu m \times 2 \mu m$ system [Figs. 5(k)]. The diffusion properties of $\alpha_2\delta_1$-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 bound to channels remain partially in the central area [Fig. 5(l)]. This is also reflected in the small subpopulation of slower $\alpha_2\delta_1$-subunits in the distribution of the diffusion coefficient [Fig. 5(m)]. 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 $\mu m \times 2 \mu m$ system [Fig. 5(d)], the number of $\alpha_2\delta_1$-subunit-associated channels continuously decreased. For channels confined into the smaller 200 $\mu m \times 200 \mu m$ system [Fig. 5(a)], 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 overpopulation of $\alpha_2\delta_1$-subunits might be sufficient to regulate surface channel assembly, despite the low affinity of CaV2.2 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 $\alpha_1$-subunit formation with auxiliary $\beta$- and $\alpha_2\delta$-subunits. While $\beta$-subunits are absolutely required for the trafficking of $\alpha_1$-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 effects on the kinetic properties of VGCCs. The study of the role of auxiliary VGCC subunits has been confronted 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 $\alpha_2\delta$-subunits during the development of chronic pain and synaptogenesis where the CaV2.2 channels are particularly the dominant calcium channel in immature hippocampal glutamatergic synapses. Probing the dynamics of the two subunits showed that CaV2.2 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 CaV2.2 channels and $\alpha_2\delta_1$-subunits revealed the existence of three populations: free CaV2.2 channels, free $\alpha_2\delta_1$-subunits, and associated CaV2.2 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. 5(c) and 5(d)] confirmed a substantial difference in
the confinement of CaV2.2 channels and αδ₁-subunits [Figs. 3(b)
and 3(c), 4(a)–4(d), 5(a)–5(d)]. This observation was further sub-
stantiated by determining the colocalization of calcium channel
subunits in the plane of the cell membrane using STED micros-
copy [Figs. 4(b) and 5(c)]. The physiological consequences are
proposed by the altered voltage-dependent inactivation of
evoked barium currents from tagged channels where the interac-
tion between CaV2.2 channels and αδ₁-subunits is altered
CaV2.2 : ∶ GFPextra, Fig. 4(d)]. Whether the dynamic subunit
associations will be a mechanism that influences channel activity
in their natural environment needs to be further explored. Interest-
ingly, the affinity of α₁-subunits and αδ₁-subunits seems to be
different between different calcium channels as observed in the
altered diffusion coefficient of αδ₁-subunits when expressed with
CaV1.2, CaV2.2, CaV2.1, or CaV3.2 channels [Fig. 2(b)]. The
strongest association seems to exist between CaV1.2 and
αδ₁-subunits, whereas CaV3.2 channels do not seem to interact
with αδ₁-subunits at the cell surface. With respect to the physio-
logical channel function and subunit association, αδ₁-subunits are
identified to tune the voltage-dependent opening of CaV1.2 channels.
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 αδ₁-subunit is between the extracellu-
lar loops of the transmembrane repeats I−III and the von
Willebrand A domain and cache1 domain of the αδ₁-subunit. Using voltage clamp fluorometry, it has been shown that the channel
αδ₁-subunit interaction with the voltage sensor domains
within the transmembrane repeats I−III is relevant for the activation of the channel. In light of these recent structure-function data, we
cannot fully exclude that the tagging of the αδ₁-subunit on the N-
terminus will influence the association of the subunits. However,
the functional tests with and without HA-tagged αδ₂-subunits (Fig. 1)
speak against a major impact of the chosen tag position. Whether
the proposed dynamic association between αδ₁-subunits and
CaV2.2 channels will be of functional relevance in neurons has
to be tested. In specific compartments, the synapse channel den-
sities can be tenfold higher as in the axon controlled by many
molecular interactions. Our first data in neurons suggest that
αδ₂-subunits are more confined in synapses, which is rather
due to other interactions than a specific affinity to CaV2.2 channels
(Fig. 3). The interaction with extracellular matrix protein might
be effective to confine αδ₁-subunits in the synapse. Our
simulations suggest that such local molecular crowding has an
impact on the association of channel subunits. The simulation fur-
ther indicated that the copy numbers for channels and αδ₂-sub-
units should be very different to have a certain population of
αδ₁-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. Whether other molecules, e.g., synaptic adhesion pro-
teins such as neurexins, participate in the confinement of
channels or αδ₁-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 αδ-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 inter-
actions are in the range of milliseconds to seconds and could
represent a potential variable tuning voltage-dependent cal-
cium signaling.

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