# SUBCELLULAR DISTRIBUTION OF VOLTAGE-GATED ION CHANNELS IN THE HIPPOCAMPUS

**PhD thesis outlines**

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# 1. Introduction

Our brain's ability to process, store and retrieve information about the continuously changing internal and external world, and to effectively perform widely different functions, is supported by a remarkable diversity of its building blocks (neurons) and an astonishingly complex dynamic connectivity (synaptic) between them. The activity of any individual neuron is shaped by excitatory and inhibitory inputs arriving from other cells. This picture is complicated by the fact that the impact of these inputs, the integration of the conveyed information and the transformation into output signals is governed by the orchestrated action of several types of voltage- and ligand-gated ion channels that are expressed in different patterns on the surface of the neuron.

Among these, voltage-gated $K^+$ channels ($K_v$) have received special attention due to their molecular and functional heterogeneity. Hippocampal pyramidal cells (PCs) express a wide variety of $K_v$ channel subunits, which might reside in distinct axo-somato-dendritic compartments. Electrophysiological experiments have identified the delayed rectifier $I_K$ current, mediated mainly by $K_v2.1$ channels, in the somato-dendritic region of CA1 PCs. These channels are believed to regulate excitability and $Ca^{2+}$ influx during periods of repetitive high-frequency firing, and might play a role in suppressing the pathological hyperexcitability of neurons. $K_v$ channels have also been found in axons, where they set the threshold and sculpt the shape of the action potentials in addition to regulating repetitive firing properties of PCs. Among these, the $K_v1.1$ channel is of particular significance, as dysfunctions or the absence of this channel have been associated with various types of neurological disorders including epilepsy and episodic ataxia. Nonetheless, despite extensive electrophysiological and anatomical investigations, the exact location and densities of these ion channels are still unknown.

Another fundamental issue regarding the subcellular distribution of ion channels is that excitatory and inhibitory synapses localized in the same subcellular compartment of a given neuron can display differences in the type, number, density and nanoscale distribution of their ion channels. Currently a lot of research is focused on presynaptic voltage-gated $Ca^{2+}$ channels ($Ca_v$), as differences in their distributions are assumed to underlie the heterogeneity in the temporal precision, efficacy and short-term plasticity of synaptic transmission. The opening of these channels mediates a local intracellular $Ca^{2+}$ influx. $Ca^{2+}$ then diffuses from the source ($Ca_v$ channels) to the vesicular sensor (synaptotagmins) and by activating it, triggers neurotransmitter release. As the $Ca^{2+}$ signal generated by a single open $Ca^{2+}$ channel declines steeply with distance, the spatial arrangement of $Ca_v$ channels and readily-releasable vesicles on the nanoscale is a crucial determinant of the release probability (*Pr*).

Distinct $Ca_v$ channel distribution was implicated as a mechanism underlying target cell type-dependent differences in the *Pr* of glutamate release and the consequent differences in short-term plasticity. Single PCs generate different responses in two distinct types of inhibitory interneurons (INs): somatostatin and $mGlu_{1a}$-expressing O-LM and O-Bi cells of the hippocampus and bitufted INs of the neocortex receive facilitating EPSCs with low initial *Pr*, whereas synaptic inputs onto fast-spiking parvalbumin (PV)-expressing INs display short-term depression and have high initial *Pr*. Although, to date, there are no data available regarding the mechanisms underlying the low initial *Pr* of these facilitating synapses, Rozov *et al.* (2001) put forward an elegant hypothesis based on their experiments involving fast and slow $Ca^{2+}$ buffers. They postulated that the low initial *Pr* of facilitating cortical PC synapses can be explained by a larger coupling distance between $Ca_v$ channels and $Ca^{2+}$ sensors on the readily-releasable vesicles

compared with the high *Pr* PC synapses on fast-spiking INs. Assuming similar $Ca^{2+}$ sensors and docked vesicle distributions, this would suggest a lower average $Ca_v$ channel density within the AZs of low *Pr* synapses. To confirm or reject this hypothesis high-resolution immunolocalization experiments will need to be carried out to compare the $Ca_v$ channel densities between high and low *Pr* boutons of the same axons.

Input cell type-dependent differences in the properties of synaptic release were also described, and similarly differences in $Ca_v$ channel distribution were suggested as underlying mechanism. For example, cholecystokinin (CCK) or PV-expressing basket cells terminals contacting hippocampal PCs differ substantially in their mechanisms of coupling of APs and exocytosis. While $PV^+$ output synapses release GABA in a tightly synchronized manner in response to presynaptic APs, $CCK^+$ INs generate a less timed, highly asynchronous input. From these cells GABA is released for up to several hundred milliseconds after high-frequency stimulation. Hefft and Jonas (2005) suggested that the asynchronous release at the $CCK^+$ IN output synapses could be a consequence of loose coupling between $Ca^{2+}$ source and sensor, that is to say a larger diffusional distance between $Ca_v$ channels and synaptotagmins that trigger GABA release. In contrast, tight coupling would promote synchronous release in $PV^+$ output synapses. Furthermore, they used blockers of specific types of $Ca^{2+}$ channels to show that $CCK^+$ INs use N-type $Ca^{2+}$ channels ($Ca_v2.2$) for transmitter release, whereas $PV^+$ INs rely on P/Q-type channels ($Ca_v2.1$). It was suggested that P/Q-type channels must be located in AZs, while N-type channels should be distributed throughout the presynaptic terminals to explain the differential coupling between $Ca_v$ channels and $Ca^{2+}$ sensors at the two types of synapses. To validate these assumptions direct anatomical proof will be needed.

## 2. Objectives

The general aim of my work was to investigate the cell surface distribution of different voltage-gated $K^+$ and $Ca^{2+}$ channels and to reveal potential input- and target cell type-dependent differences in ion channel distributions that might underlie distinct functions. To address the aims of my dissertation, I adopted the highly-sensitive, high-resolution electron microscopic sodium dodecylsulphate-digested freeze-fracture replica labelling (SDS-FRL) method.

The specific aims in this study were:

(1) To determine the precise subcellular distribution pattern of two delayed-rectifier $K^+$ channel subunits ($K_v1.1$ and $K_v2.1$) in distinct axo-somato-dendritic compartments of CA1 PCs.

(2) To reveal target cell type-specific differences in the distribution and densities of voltage-gated $Ca^{2+}$ channels in CA3 PC axon terminals.

(3) To determine input cell type-dependent differences in the distribution of voltage-gated $Ca^{2+}$ channels in basket cell axon terminals of the hippocampal CA3 area.

**Contributions:**
The gold distribution analysis was performed with a software written by Miklós Szoboszlay.

## 3. Methods

### *3.1. Tissue preparation*

Wistar rats (adult: postnatal day (P) 30–66, n = 11 male; young: P15–17, n = 8 male), CCK-BAC/DsRedT3 (P19–P25, n = 5 male), $CB_1^{+/+}$ (P18, P26, n = 2 female) and $CB_1^{-/-}$ (P18, n = 2 female, kindly provided by Prof. Andreas Zimmer) mice were deeply anaesthetized then transcardially perfused. For light microscopic immunofluorescent reactions, animals were perfused with a fixative containing either 4% paraformaldehyde (PFA) and 15v/v% picric acid (PA) in 0.1 M phosphate buffer (PB) or with 2% PFA in 0.1 M Na acetate buffer for 15 minutes. Afterwards 70 μm thick coronal sections were cut from the forebrain with a vibratome. Some brain sections were treated with 0.2 mg/ml pepsin in 0.2 M HCl at 37°C for 18–20 minutes. For SDS-FRL, animals were perfused with a fixative containing 2% PFA and 15v/v% PA in 0.1 M PB for 15 minutes. Coronal or horizontal sections of 80 μm thickness were cut, then small tissue blocks from the dorsal CA1, dorsal and ventral CA3 were trimmed, and cryo-protected by overnight immersion in 30% glycerol. Replicas from $Ca_v2.2^{+/+}$ (P18, n = 1) and $Ca_v2.2^{-/-}$ mice (9 months old mouse, n = 1, provided by Prof. Yasuo Mori) were provided by Prof. Ryuichi Shigemoto.

### *3.2. Fluorescent immunohistochemistry*

Following several washes in 0.1 M PB and then in Tris-buffered saline (TBS), sections were blocked in 10% normal goat serum (NGS), followed by overnight incubation in primary antibodies diluted in TBS containing 2% NGS and 0.1% Triton X-100. After several washes in TBS, the sections were incubated in a mixture of secondary antibodies made up in TBS containing 2% NGS for 2 hours. Sections were washed in TBS, then in 0.1 M PB before mounting on slides in Vectashield. Images from the CA1 region were acquired using a confocal laser scanning microscope (FV1000; Olympus).

### *3.3. SDS-FRL*

Small blocks from the CA1 and CA3 areas were frozen in a high-pressure freezing machine (HPM100). The fractured tissue surfaces were coated with thin layers of carbon (2–5 nm), platinum (2 nm) and carbon (20 nm). Tissue debris was 'digested' from the replicas in a solution containing 2.5% SDS and 20% sucrose in TBS at 80°C overnight. Following several washes in TBS containing 0.05% bovine serum albumin (BSA), replicas were blocked in TBS containing 0.1–5% BSA for 1 hour, then incubated overnight at room temperature or for four days at 4˚C in the blocking solution containing the primary antibodies. Replicas were then incubated for 2 hours in TBS containing 5% BSA and gold coupled secondary antibodies. Specimens were analyzed with a transmission electron microscope (JEM-1011; JEOL Ltd). All antibodies recognized intracellular epitopes on their target proteins and consequently were visualized by gold particles on the protoplasmic face (P-face). Nonspecific background (BG) labeling was measured on the exoplasmic face (E-face) structures surrounding the measured P-faces.

### *3.4. Quantification of immunogold particles labeling the $K_v$1.1 and $K_v$2.1 subunits in the rat CA1 region*

Quantitative analysis of immunogold labeling for the $K_v$1.1 and $K_v$2.1 subunits was performed on CA1 PC somata, 11 different dendritic compartments, axon initial segments (AISs) and axon terminals in six CA1 sublayers (n = 3 rats for each subunit). In addition to stratum oriens (SO), stratum pyramidale (SP), stratum radiatum (SR), and stratum lacunosum-moleculare (SLM; above 360 μm), the SR was divided into proximal (0–120 μm), middle (120–240 μm) and distal SR (240–360 μm) parts based on the distance from SP. The main apical dendrites, oblique dendrites, spines and axon terminals were grouped according to this criterion. Oblique dendrites were identified

based on their small diameter and the presence of at least one emerging spine from the dendritic shaft. Structures were only considered to be spines if they emerged from a dendritic shaft. Axon terminals were identified either based on the presence of an active zone (AZ) facing a postsynaptic density (PSD) on the opposing E-face of a spine or dendrite, or the presence of gold particles labeling the SNAP-25. Images of AISs of CA1 PCs were taken in SP and SO. To quantify the $K_v2.1$ subunit in AISs (n = 3 rats), the $K_v1.1$ subunit was used as molecular marker.

### *3.5. Quantitative analysis of immunogold particles labeling the $Ca_v2.1$ and $Ca_v2.2$ subunits in rat CA3 PC axon terminals contacting $K_v3.1b$ or $mGlu_{1a}$ immunopositive cells*

To quantify the $Ca_v2.1$ and the $Ca_v2.2$ subunit densities in the AZs of axon terminals targeting $K_v3.1b^+$ or $mGlu_{1a}^+$ dendrites in the SO of the CA3 area, the 'mirror replica method' was used. One replica was immunolabeled for $K_v3.1b$ and $mGlu_{1a}$ to identify IN dendrites; its mirror surface was labeled for a $Ca_v$ channel subunit and all subcellular structures were identified in both replicas. The AZs were delineated on the P-face based on the underlying high density of intramembrane particles (IMPs). Axon terminals containing $Ca_v$ subunit labeling without an elevated density of IMPs were discarded because this is a characteristic feature of inhibitory terminals. To eliminate reaction-to-reaction variability in the $Ca_v$ subunit labeling, synaptic, extrasynaptic bouton, and background $Ca_v$ densities were normalized to the mean of the $Ca_v$ densities measured in the AZs targeting $mGlu_{1a}^+$ profiles in each reaction.

### *3.6. Quantification of $CB_1$, Rim1/2, $Ca_v2.1$ and $Ca_v2.2$ subunits in axon terminals targeting the somatic region of PCs in the distal CA3 area of the rat and mouse hippocampus*

To quantify the $CB_1$, Rim1/2, $Ca_v2.1$ and $Ca_v2.2$ subunit densities on axon terminals, electron micrographs of PC somatic

E-face membranes with attached P-face axon terminal fragments were taken from SP of the distal CA3 area of a rat and two mice. I have restricted the analysis to those profiles that had an area > 0.01 and < 0.21 $\mu m^2$, corresponding to the range of AZ sizes obtained from 3D electron microscopic reconstructions performed by N. Holderith. The gold particle densities were then calculated in these P-face membranes without assuming that the entire membrane is an AZ. I provided indirect evidence for the potential enrichment of $Ca_V2.1$ and $Ca_V2.2$ in AZs in the following way. Using Rim1/2 labeling, I demonstrated that 31.7% and 23.6% of the P-face membrane fragments fractured to large E-face somatic membranes contain AZs in rats and mice, respectively. In $Ca_V2.1$ and $Ca_V2.2$ double-labeling experiments, this proportion was similar (43% and 19.8%).

### *3.7. Analysis of immunogold particle distribution patterns*

To investigate whether the distribution of a given protein within a certain subcellular compartment is compatible with a random process or not I used a software developed by Miklós Szoboszlay. First, I calculated the mean of the nearest neighbor distances ($\overline{NND}$) of all gold particles within the area in question and that of randomly distributed gold particles (same number of gold particles placed in the same area, 200 or 1000 repetitions). The $\overline{NND}$s were then compared statistically using the Wilcoxon signed-rank test. In our second approach, I computed a 2D spatial autocorrelation function ($g(r)$) for my experimental data and for their corresponding random controls based on Veatch *et al.* (2012). The $g(r)$ reports the probability of finding a second gold particle at a given distance $r$ away from a given gold particle. For randomly distributed gold particles $g(r) = 1$, whereas spatial inhomogeneities result in $g(r)$ values > 1 at short distances. I computed the $g(r)$ for $0 < r < 80$ nm and then their mean ($\overline{g(r)}$) was calculated and compared with those obtained from random distributions using the Wilcoxon signed-rank test.

## 4. Results

### *4.1. Subcellular distribution of the $K_v$1.1 subunit in the hippocampal CA1 area*

First, I investigated the distribution of the $K_v$1.1 subunit in the hippocampal CA1 area of adult rats using light microscopic immunofluorescent localizations. The identical labeling pattern obtained with two antibodies labeling different, non-overlapping parts of the $K_v$1.1 protein strongly suggests the specificity of the immunolabeling. At low magnifications, an intense punctate neuropil labeling was seen in the SO and SR in agreement with published data, corresponding to either presynaptic terminals or dendritic spines. At higher magnifications, AISs and the juxta-paranodal region of myelinated axons were also observed. Double-labeling experiments with known AIS markers such as Ankyrin-G and pan-$Na_v$ verified that the intensely labeled processes were indeed AISs. In order to unequivocally identify the origin of the punctate neuropil labeling of the SO and SR, and to assess the densities of the $K_v$1.1 subunit in 18 axo-somato-dendritic compartments of CA1 PCs, I turned to the SDS-FRL method.

Electron microscopic analysis of the replicas revealed elongated structures in the SP and SO strongly immunolabeled for the $K_v$1.1 subunit. These structures were then molecularly identified as AISs by the high density of pan-Neurofascin labeling. In AISs, gold particles consistently avoided the PSD of axo-axonic GABAergic synapses identified as dense IMP clusters. In the alveus, strongly $K_v$1.1 subunit immunoreactive profiles were found surrounded by cross-fractured myelin sheets. These structures are likely to correspond to the juxta-paranodal region of myelinated axons.

Next, I assessed the origin of the neuropil labeling of the SO and SR. Small P-face membrane profiles containing an AZ and facing a PSD on the opposing spine or dendritic shaft membrane were consistently labeled. Double-labeling

experiments for the $K_v1.1$ subunit and SNAP-25, a member of the SNARE protein complex restricted to axon terminals, confirmed that these profiles were axon terminals.

After this qualitative assessment of the reactions, I calculated the densities of the $K_v1.1$ subunit in 18 distinct compartments by counting gold particles on P-face membranes and divided these numbers by the total measured membrane areas (Table 1).

**Table 1. Densities of gold particles labeling $K_v1.1$ and $K_v2.1$ subunits in distinct subcellular compartments of CA1 PCs.** Density values are provided in gold/$\mu m^2$ mean ± SD (between animals). In parentheses, the number denotes the number of counted gold particles. Bold indicates density values that are significantly above BG. # indicates that the AIS densities were measured in separate, double-labeling experiments in which the AISs were molecularly identified with $K_v1.1$. In this reaction the BG labeling was 0.6 ± 0.1 gold/$\mu m^2$.

| Quantified subunit | $K_v1.1$ | $K_v2.1$ |
|---|---|---|
| SO bouton | **4.0** ± 1.2 (195) | 1.1 ± 0.5 (47) |
| AIS | **26.2** ± 4.9 (2758) | **11.5** ± 1.8 (900) # |
| SP soma | 0.7 ± 0.2 (704) | **10.3** ± 1.1 (10501) |
| SR proximal apical dendrite | 0.7 ± 0.2 (98) | **9.4** ± 0.5 (3868) |
| SR middle apical dendrite | 0.5 ± 0.2 (80) | 1.6 ± 0.1 (377) |
| SR distal apical dendrite | 0.6 ± 0.4 (107) | 1.1 ± 0.1 (415) |
| SR proximal oblique dendrite | 0.5 ± 0.3 (30) | 1.4 ± 0.2 (126) |
| SR middle oblique dendrite | 0.9 ± 0.1 (50) | 1.0 ± 0.1 (95) |
| SR distal oblique dendrite | 0.8 ± 0.4 (39) | 1.2 ± 0.1 (97) |
| SR proximal spine | 0.5 ± 0.6 (2) | 1.2 ± 1.0 (10) |
| SR middle spine | 0.8 ± 0.7 (4) | 0.5 ± 0.9 (2) |
| SR distal spine | 0.2 ± 0.3 (2) | 1.1 ± 0.2 (8) |
| SR proximal bouton | **4.0** ± 0.5 (128) | 1.4 ± 0.4 (42) |
| SR middle bouton | **3.6** ± 0.7 (158) | 1.3 ± 0.2 (45) |
| SR distal bouton | 3.2 ± 0.6 (137) | 1.6 ± 0.6 (42) |
| SLM tuft dendrite | 1.1 ± 0.6 (74) | 1.4 ± 0.2 (221) |
| SLM tuft spine | 0.7 ± 0.7 (10) | 1.3 ± 0.2 (22) |
| SLM bouton | 3.2 ± 0.6 (104) | 1.1 ± 0.2 (42) |
| BG | 0.3 ± 0.1 (97) | 0.7 ± 0.1 (333) |

The gold particle density values were not significantly higher than BG in somata, apical dendrites, tuft dendrites in the SLM, oblique dendrites and dendritic spines ($P < 0.001$ One-way analysis of variance ANOVA (OWA), $P = 0.999$ Dunnett's *post hoc* test; n = 3 rats). In contrast, gold particle densities on axon terminals were significantly above BG ($P < 0.001$ OWA, $P < 0.05$ Dunnett's *post hoc* test; n = 3 rats) in SO, proximal and

middle SR. In distal SR and SLM gold particle densities on axon terminals were very similar, but the difference from BG did not reach significance ($P < 0.001$ OWA, $P = 0.07$ Dunnett's *post hoc* test; n = 3 rats). These densities on axon terminals were seven- to eightfold lower (ratios calculated after BG subtraction; $P < 0.001$ OWA, $P < 0.001$ Dunnett's *post hoc* test; n = 3 rats) than that found in AISs.

### *4.2. Subcellular distribution of the $K_v$2.1 subunit in the hippocampal CA1 area*

For immunofluorescent localization of the $K_v$2.1 subunit in the CA1 area of adult rat, two antibodies recognizing non-overlapping epitopes of the protein were used to ensure that the immunosignal was the consequence of a specific antigen–antibody interaction. At low magnification, strong immunolabeling of the SP and proximal part of the SR was observed, in line with the results of previous work. High-magnification images revealed plasma membrane-like labeling of somata, proximal apical and basal dendrites. Double-labeling experiments for $K_v$2.1 and $Na_v$1.6 revealed a clustered $K_v$2.1 labeling of AISs, confirming the results of Sarmiere *et al.* (2008). Interestingly, high-magnification single confocal images indicated that these $K_v2.1^+$ clusters did not overlap with the $Na_v$1.6 containing parts of AISs.

Using SDS-FRL, I detected strong immunogold labeling for the $K_v$2.1 subunit on PC somata and proximal dendrites, which were significantly higher than BG (Table 1; $P < 0.001$ OWA, $P < 0.001$ Dunnett's *post hoc* test; n = 3 rats). The labeling consisted of scattered and clustered gold particles in the plasma membrane. Both $\overline{\mathrm{NND}}$ and $\overline{g(r)}$ measurements indicated that the distribution of $K_v$2.1 subunit on CA1 PC soma is significantly different from random (Wilcoxon-signed rank test $P < 0.001$, n = 21 somata from 1 rat).

I also observed that some of the $K_v2.1^+$ gold clusters were located over loose patches of IMPs, which might be GABAergic perisomatic synapses. To molecularly identify GABAergic synapses, I performed double-labeling experiments for $K_v2.1$ and neuroligin-2 (NL-2), a specific marker of inhibitory synapses. These reactions revealed that the $K_v2.1$ subunit was absent from NL-2-containing areas, but occasionally the $K_v2.1^+$ and NL-$2^+$ clusters were close to each other. The non-uniform distribution of gold particles labeling for the $K_v2.1$ subunit was also characteristic for AISs (molecularly identified with either $Na_v1.6$ or $K_v1.1$), but $K_v2.1$ clusters never overlapped with presumed axo-axonic synapses and also showed a segregation from the $Na_v1.6$ and $K_v1.1$ labeling. AISs contained, on average, $11.5 \pm 1.8$ gold/$\mu m^2$, which did not differ significantly from the gold particle content of somata ($P = 0.97$, unpaired Student's t-test), but was significantly above the BG ($P < 0.01$ OWA, $P < 0.01$ Dunnett's *post hoc* test; n = 3 rats).

The densities of the $K_v2.1$ subunit in apical dendrites in the middle and distal SR, SLM tuft dendrites, oblique dendrites, dendritic spines, and axon terminals were not significantly different from the nonspecific BG labeling ($P < 0.001$ OWA, $P > 0.26$ Dunnett's *post hoc* test; n = 3 rats).

### *4.3. Target cell type-dependent localization of voltage-gated $Ca^{2+}$ channels in CA3 PC axon terminals*

In the next set of experiments, I tested the hypothesis that different $Ca_v$ channel densities in presynaptic AZs underlie different $P_r$ values. I chose CA3 PC local axon terminals in the SO of young rats as the subject of my study. These axons establish synapses onto fast-spiking PV-containing INs, which show high initial $P_r$ and short-term depression, whereas next bouton of the same axon has low initial $P_r$ and displays short-term facilitation when its postsynaptic target is $mGlu_{1a}$ and somatostatin expressing IN. The functional characterization of

these synapses was performed by T. Éltes and N. Holderith in our laboratory. I performed SDS-FRL to quantitatively compare the immunogold labeling for $Ca_v$ subunits in presynaptic AZs that synapse onto distinct IN types. To achieve this, the postsynaptic targets of the axon terminals need to be identified. This requires the use of face-matched 'mirror replica' technique and the molecular identification of the target IN types because the type of IN from small fractured membrane segments cannot be determined based on morphological features. One replica was immunoreacted to identify IN dendrites; its mirror surface was labeled for a $Ca_v$ subunit and all subcellular structures were identified in both replicas. The $mGlu_{1a}$ receptor is a transmembrane protein that is expressed in the somato-dendritic plasma membrane of hippocampal INs and specific antibodies are available that can be used for replica labeling. PV is a cytoplasmic protein that cannot be detected with SDS-FRL, so I identified somato-dendritic regions of fast-spiking $PV^+$ INs based on the presence of immunogold labeling for the $K_v3.1b$ voltage-gated $K^+$ channel subunit. Many membrane segments are attached to these IN dendrites that represent the E-face of presynaptic axon terminals. Because my antibodies against $Ca_v$ subunits recognize intracellular epitopes, they cannot be used to localize these channels in these attached axonal E-face membranes. The P-faces of these dendrite-attached presynaptic membranes are present in the mirror replicas. Because the release of glutamate from these axon terminals are mainly mediated by $Ca_v2.1$ and $Ca_v2.2$ subunits, I localized both subunits. The rabbit anti-$Ca_v2.1$ antibody used in the experiments provides identical labeling to that observed with another $Ca_v$ antibody raised in guinea pig, which specificity was verified in tissue obtained from $Ca_v2.1^{-/-}$ mice. The specificity of the $Ca_v2.2$ immunolabeling was validated on hippocampal tissue of $Ca_v2.2^{+/+}$ and $Ca_v2.2^{-/-}$ mice. The labeling pattern in $Ca_v2.2^{+/+}$ mice tissue was similar to that observed in rats.

In rats, the dendrite-attached P-face membrane segments were often labeled for the $Ca_v2.1$ subunit and the gold particles were concentrated over areas that had an elevated density of IMPs, corresponding to the AZs. The normalized density of gold particles within the AZs was significantly larger than the BG ($P < 0.0001$ Kruskal–Wallis (KW) test, $P < 0.0001$ *post hoc* Mann-Whitney *U* (MW) tests with Bonferroni correction), whereas the $Ca_v2.1$ subunit density in extrasynaptic bouton membranes was similar to that of the BG ($P < 0.0001$ KW test, $P > 0.05$ *post hoc* MW tests with Bonferroni correction). I performed these experiments in five animals and analyzed a total of 112 and 172 AZs attached to $K_v3.1b^+$ and $mGlu_{1a}{}^+$ dendrites, respectively, in the SO of the CA3 area. Quantitative comparison of the two AZ populations revealed an overall 1.15 times higher density in $K_v3.1b^+$ dendrite-targeting AZs ($P < 0.0001$ KW test, $P < 0.001$ *post hoc* MW tests with Bonferroni correction; for non-normalized gold densities see Table ). Presynaptic P-face plasma membranes attached to $K_v3.1b^+$ (n = 52) or $mGlu_{1a}{}^+$ (n = 114) dendrites were also heavily labeled for the $Ca_v2.2$ subunit. Gold particles were confined to the AZs, where their densities were significantly higher than the BG ($P < 0.0001$ KW test, $P < 0.0001$ *post hoc* MW tests with Bonferroni correction). $Ca_v2.2$ subunit densities also showed a 1.20-fold higher value in $K_v3.1b^+$ compared with $mGlu_{1a}{}^+$ dendrite-targeting AZs, but the difference did not reach significance ($P < 0.0001$ KW test, $P > 0.02$ *post hoc* MW tests with Bonferroni correction; for non-normalized gold densities, see Table ).

Finally, I investigated whether the sub-AZ distribution of the gold particles labeling the $Ca_v$ subunits is compatible with a random process. To test this, I computed the $\overline{\mathrm{NND}}$ and the $\overline{g(r)}$ then compared the real anti-$Ca_v2.1$ immunogold distribution data in 43 $K_v3.1b^+$ and 72 $mGlu_{1a}{}^+$ dendrite-targeting AZs (fractured in their completeness) with their corresponding random distributions (same number of gold particle placed

randomly in the same area, 1000 repetitions). Both measures revealed that the actual data were significantly different from random distributions ($P < 0.0001$ Wilcoxon signed-rank test). A similar result was obtained for the $Ca_V2.2$ subunit in 21 ($K_V3.1b$) and 40 ($mGlu_{1a}$) AZs ($P < 0.0001$ Wilcoxon signed-rank test). The significantly lower mean $\overline{NND}$s and the $\overline{g(r)} > 1$ in both $K_V3.1b^+$ and $mGlu_{1a}{}^+$ dendrite-innervating AZs demonstrate spatial inhomogeneities of gold particles within these AZs.

**Table 2. Properties of $Ca_V$ immunoreactivities in $K_V3.1b^+$ and $mGlu_{1a}{}^+$ dendrite-innervating axon terminals.** Density values are calculated from the medians of 5 ($Ca_V2.1$) and 4 ($Ca_V2.2$) rats. For the NND calculations AZs fractured in their completeness were subselected from the same rats.

| | $K_V3.1b^+$ dendrite-targeting boutons | | | | $mGlu_{1a}{}^+$ dendrite-targeting boutons | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Median | n | Mean | SD | Median | n |
| $Ca_V2.1$ subunit density in AZs (gold/$\mu m^2$) | 373 | 47 | 370 | 112 | 321 | 46 | 325 | 172 |
| $Ca_V2.2$ subunit density in AZs (gold/$\mu m^2$) | 151 | 30 | 139 | 52 | 130 | 39 | 130 | 114 |
| $Ca_V2.1$ subunit density in extrasynaptic membranes (gold/$\mu m^2$) | 2.14 | 2.46 | 2.21 | 93 | 2.88 | 2.64 | 2.23 | 174 |
| $Ca_V2.2$ subunit density in extrasynaptic membranes (gold/$\mu m^2$) | 2.69 | 3.15 | 2.35 | 48 | 1.02 | 1.26 | 0.75 | 113 |
| BG $Ca_V2.1$ subunit density (gold/$\mu m^2$) | 2.27 | 1.93 | 2.94 | 132 | 2.27 | 1.93 | 2.94 | 132 |
| BG $Ca_V2.2$ subunit density (gold/$\mu m^2$) | 0.66 | 0.33 | 0.62 | 104 | 0.66 | 0.33 | 0.62 | 104 |
| $Ca_V2.1$ NND distance (nm) | 24.7 | 4.0 | 23.3 | 43 | 24.3 | 4.6 | 23.1 | 72 |
| $Ca_V2.2$ NND distance (nm) | 32.1 | 7.2 | 30.3 | 21 | 32.0 | 8.3 | 28.7 | 40 |

### *4.4. Input cell type-dependent localization of voltage-gated $Ca^{2+}$ channels in basket cell axon terminals of the hippocampal CA3 area*

Finally, I examined the distribution of $Ca_V$ channels in IN axon terminals targeting the soma of CA3 PCs. The somatic region of PCs is contacted almost exclusively by basket cells

expressing either PV or CCK/$CB_1$. Previous experiments employing subtype-specific $Ca_v$ channel blockers indicated that GABA release from CCK/$CB_1^+$ neurons is almost exclusively mediated by N-type channels, whereas $PV^+$ INs rely on P/Q-type channels. However, direct experimental data is not available concerning the localization of these subunits on these terminals. Therefore, I carried out high-resolution SDS-FRL of the $Ca_v2.1$ and $Ca_v2.2$ subunits in axon terminals targeting the soma of CA3 PCs in adult rats. As the antibodies against the $Ca_v$ subunits recognize intracellular domains of the target molecules, I focused on P-face segments of axon terminals attached to E-face somatic membranes. To differentiate between the two populations of axon terminals contacting the somatic region of PCs, I identified the axon terminals as CCK-containing boutons if they were immunolabeled for $CB_1$, and $PV^+$ if they were $CB_1$ immunonegative. To prove this assumption, first I preformed double-labeling experiments for $CB_1$ and VGAT, to show that the P-face membrane segments attached to E-face somatic membranes are GABAergic, while a subset of them is $CB_1$ immunoreactive. The specificity of the $CB_1$ immunosignal was validated in replicas from $CB_1^{+/+}$ and $CB_1^{-/-}$ mice.

When I performed colocalization of $CB_1$ and $Ca_v2.2$, I found that many $CB_1$ immunopositive terminals attached to E-face somatic membranes contained the $Ca_v2.2$ subunit, but the $Ca_v2.2^+$ profiles were almost always $CB_1^+$ (94%). Double-labeling experiments for the $CB_1$ and $Ca_v2.1$ subunit revealed that the $CB_1$ immunopositive boutons almost never contained the $Ca_v2.1$ subunit (95% were $Ca_v2.1$ immunonegative). Finally, double immunolabeling for the $Ca_v2.1$ and $Ca_v2.2$ subunits further confirmed the segregation of these $Ca_v$ subunits in axon terminal membrane segments fractured onto PC somata: 89% of the fractured membranes had either $Ca_v2.1$ or $Ca_v2.2$ labeling. Taken together, these experiments provide morphological evidence for the exclusive role of these $Ca_v$ subunits in $PV^+$ and

$CCK^+$ basket cell axon terminals. It is important to note that the segregation of $Ca_v$ channels could also be observed in mice (92% of the fractured membranes had either $Ca_v2.1$ or $Ca_v2.2$ labeling), where the specificity of the $Ca_v$ antibodies was tested.

The gold particles labeling the $Ca_v2.2$ and $Ca_v2.1$ subunits were restricted to sub-areas of the axon terminal segments, which might correspond to AZs. In contrast to excitatory AZs, where these $Ca_v$ subunits were enriched in AZs identified by the high density of IMPs, in soma-targeting GABAergic axon terminals this morphological feature was absent. This observation entails that AZs on these boutons can only be identified by using molecular markers for AZ proteins. Therefore, I performed labeling of Rim1/2, a protein restricted to excitatory and inhibitory AZs. My results show that 32% of P-face membranes fractured to large E-face somatic membranes contained AZs (n = 1 rat; this ratio was 24% in mice, data pooled from n =2 mice); in all cases without underlying high density of IMPs. Furthermore, the Rim1/2 immunolabeled AZs showed great variability in size, shape and number, similarly to the $Ca_v2.2$-enriched areas. This finding is consistent with the shapes, sizes and number of AZs obtained from 3D electron microscopic reconstructions of $CCK^+$ perisomatic boutons performed by N. Holderith in our laboratory. In $Ca_v2.1$ and $Ca_v2.2$ double-labeling experiments, the proportion of labeled profiles was similar to the Rim1/2 experiments (43% in rats and 20% mice), further supporting the notion that $Ca_v$ subunits are localized within AZs on these boutons.

Finally, the double-labeling experiments for $CB_1$ and the presynaptic AZ marker Rim1/2 also revealed an extensive colocalization of the two proteins in rats and mice, providing evidence for the presence of $CB_1$ in presynaptic AZs. My immunoreactions also revealed heterogeneity in the $CB_1$ content of AZs; some $Rim1/2^+$ AZs were strongly labeled, whereas others were apparently immunonegative.

## 5. Conclusions

My results from the first two parts of the dissertation reveal that the two studied delayed-rectifier $K^+$ channel subunits take up different subcellular locations in CA1 PCs, resulting in unique, subunit-specific labeling patterns. The $K_v1.1$ subunit is present in AISs and axon terminals of CA1 PCs, with an eightfold lower density in boutons. In contrast, the $K_v2.1$ subunit is detected at similar densities in AISs, somata and proximal dendrites, but not elsewhere. This subunit has a non-uniform plasma membrane distribution; Kv2.1 clusters are frequently adjacent to, but never overlap with, GABAergic synapses. Within the AIS the $K_v1.1$/$Na_v1.6$ and $K_v2.1$ subunits are segregated into distinct subdomains, all separate from GABAergic synapses, demonstrating that the surface of the AIS is molecularly more complex than previously anticipated. These results suggest that $K^+$ channels modulate neuronal excitability in a compartment-specific manner.

In the third part of the study, I found that both $Ca_v2.1$ (P/Q-type) and $Ca_v2.2$ (N-type) $Ca^{2+}$ channel subunits are present in functionally distinct CA3 PC axon terminals targeting the $K_v3.1b^+$ and $mGlu_{1a}{}^+$ dendrites. In both populations, the studied $Ca_v$ subunits are restricted to putative AZs, and their within-AZ distribution is significantly different from random distributions. Furthermore, high *Pr* $K_v3.1b^+$ dendrite-innervating AZs contain 15% higher $Ca_v$ subunit density than low *Pr* $mGlu_{1a}{}^+$ dendrite-contacting AZs. This target-dependent difference in $Ca_v$ channel density is much smaller than the almost twofold difference implied by functional experiments of T. Éltes and N. Holderith. My results suggest a target cell type-dependent regulation of $Ca^{2+}$ channel function or distinct subunit composition as the mechanism underlying the functional differences.

Finally, I provide morphological evidence for the segregation of distinct $Ca_v$ channel subunits in CCK/$CB_1{}^+$ and

$PV^+$ basket cell boutons contacting the somatic region of CA3 PCs. The $Ca_v2.2$ subunit is restricted to $CCK/CB_1^+$ axon terminals, while the $Ca_v2.1$ subunit is almost exclusively expressed by $PV^+$ boutons. In addition, $Ca_v$ channels are enriched over subareas of axon terminals, which also contain the Rim1/2 indicating their localization within AZs. As both $Ca_v$ subunits are restricted to putative AZs, the well-known differences in synaptic transmission between these boutons are likely to be the consequence of slight, but crucial within-AZs differences in the arrangement of synaptic vesicles and $Ca^{2+}$ channels. Moreover, AZs of $CCK/CB_1^+$ axon terminals contain the $CB_1$ in various quantities, which I propose to be the basis of the heterogeneity in endocannabinoid-mediated modulation of GABA release.

These findings reveal previously unseen cell-surface distribution patterns of two delayed-rectifier $K^+$ channel subunits, as well as input- and target cell type-dependent differences in $Ca_v$ channel distribution in axon terminals. These data furthers our understanding of ion channel localization and functional consequences of distinct distribution patterns.

## 6. Bibliography of the candidate's publications


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3. Éltes T[1], **Kirizs T**[1], Nusser Z, Holderith N. (2017) Target cell type-dependent differences in Ca(2+) channel function underlie distinct release probabilities at hippocampal glutamatergic terminals. J Neurosci, 37: 1910–1924.


[1] equal contribution