

Supplemental Material

Nanoscale organization of Ca_v2.1 splice isoforms at presynaptic terminals: Implications for synaptic vesicle release and synaptic facilitation

Agnes Thalhammer^{1, 2,*}, Fanny Jaudon^{1, 3}, Jessica Muià¹, Gabriele Baj^{1, 2} and Lorenzo A. Cingolani^{1, 4,*}

¹ Department of Life Sciences, University of Trieste, 34127 Trieste, Italy

² Centro Interdipartimentale di Microscopia Avanzata (CIMA), University of Trieste, 34127 Trieste, Italy

³ IRCCS Ospedale Policlinico San Martino, 16132 Genoa, Italy

⁴ Center for Synaptic Neuroscience and Technology (NSYN), Fondazione Istituto Italiano di Tecnologia (IIT), 16132 Genoa, Italy

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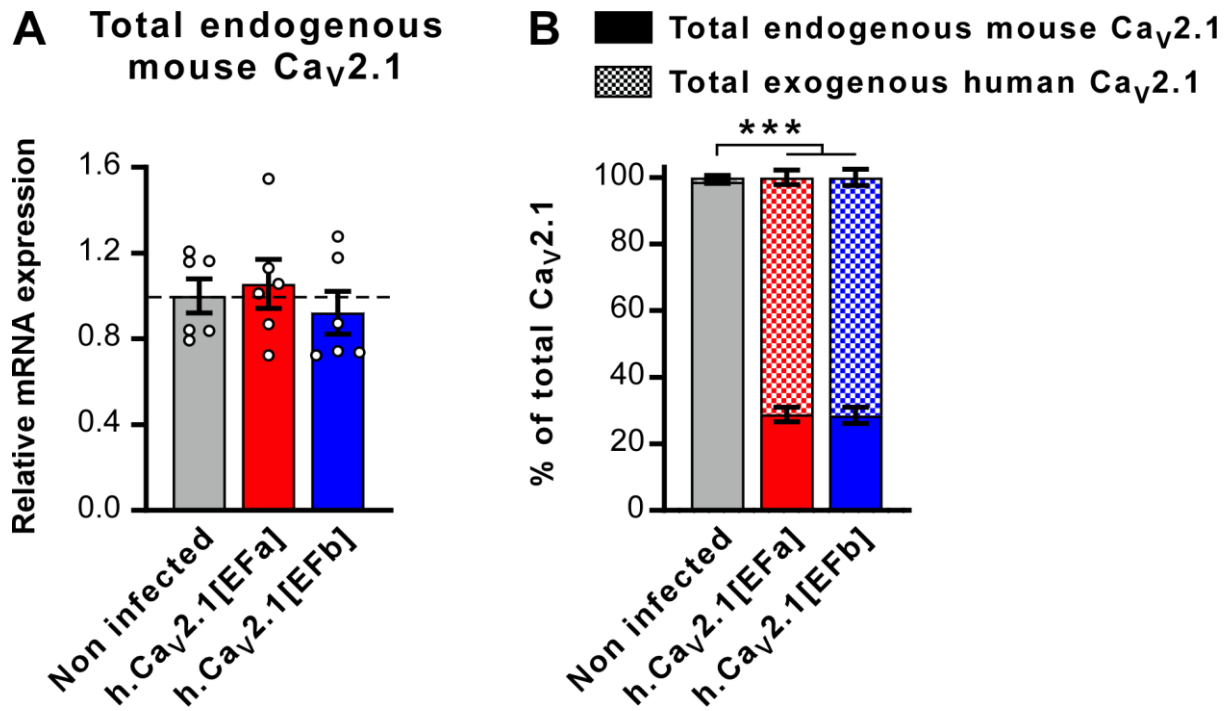


Figure S1. Levels of endogenous mouse $\text{Ca}_v2.1$ are not affected by exogenous expression of human $\text{Ca}_v2.1$. (A) RT-qPCR quantification of endogenous mouse $\text{Ca}_v2.1$ in 18 DIV primary cortical neurons expressing the indicated constructs ($p > 0.05$, one-way ANOVA; $n = 6$ from 2 independent cultures). (B) The relative abundance of endogenous mouse versus exogenous human $\text{Ca}_v2.1$ was determined by absolute quantification using calibration curves with known concentrations of recombinant plasmids ($***p < 0.001$, Chi-square test; $n = 6$ from 2 independent cultures). Total endogenous mouse $\text{Ca}_v2.1$ and total exogenous human $\text{Ca}_v2.1$ in panels A and B were detected with primers m. $\text{Ca}_v2.1$ _Fw/Rv (located in exons 2 and 3) and h. $\text{Ca}_v2.1$ _Fw/Rv (located in the N-terminal AU1 tag and exon 1), respectively (see Fig S3).

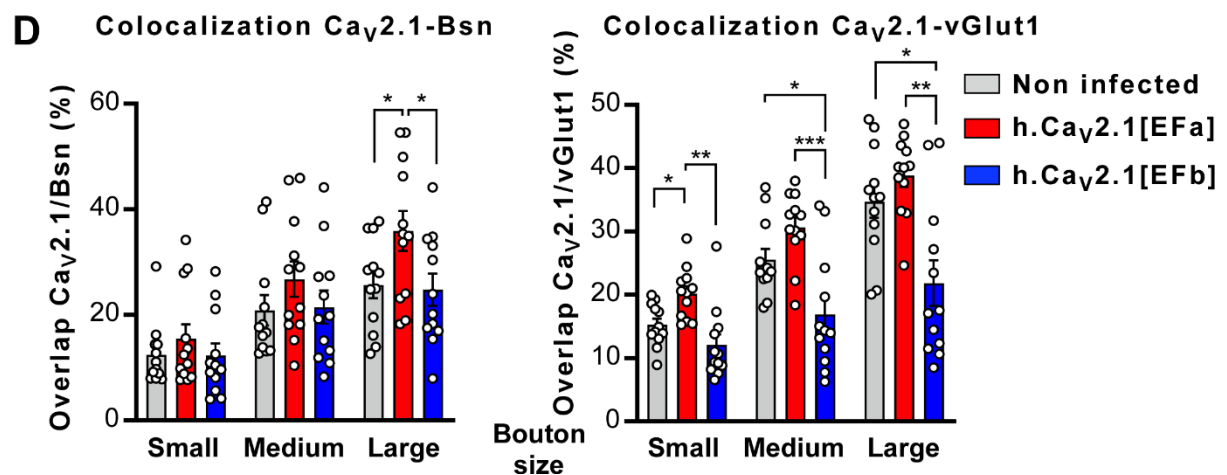
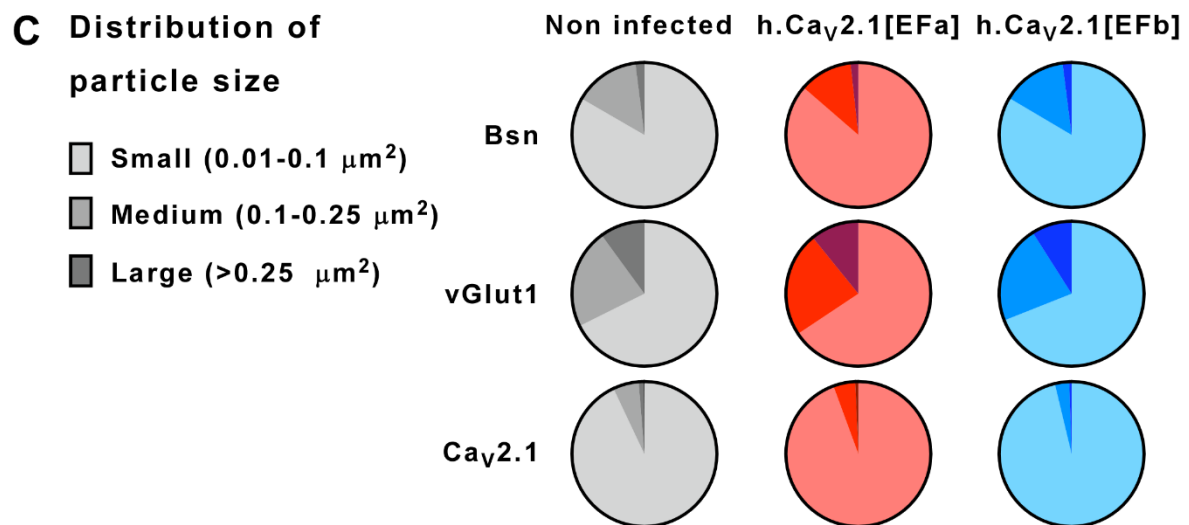
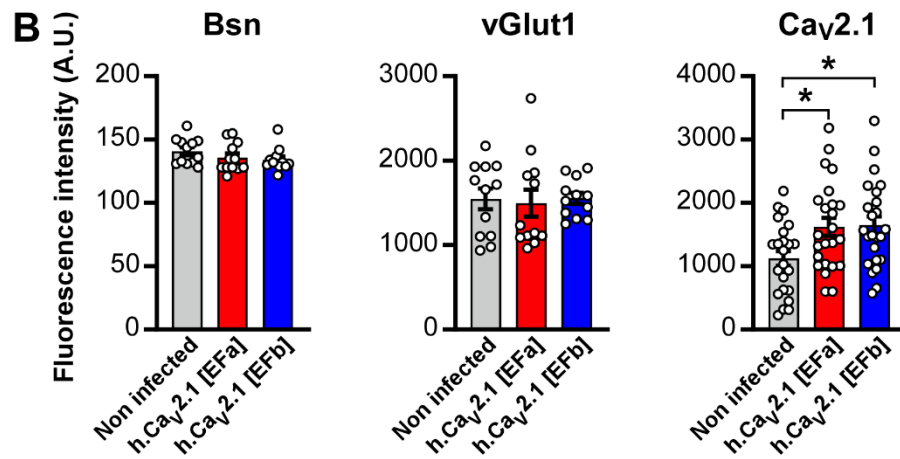
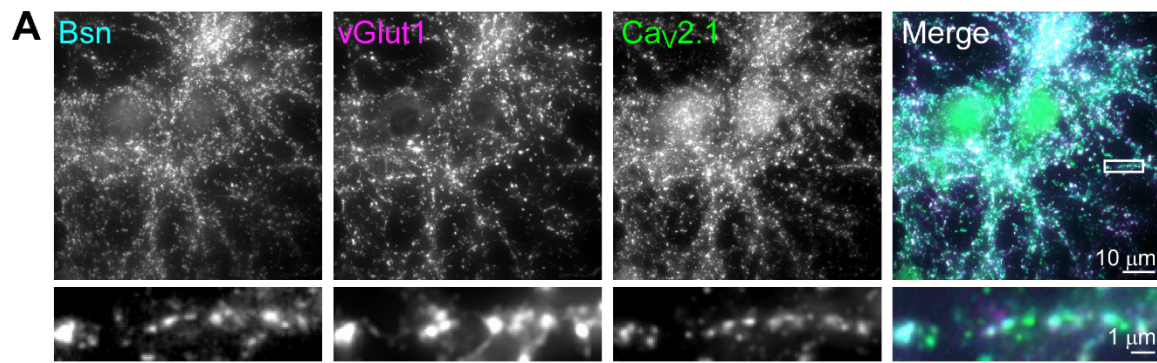
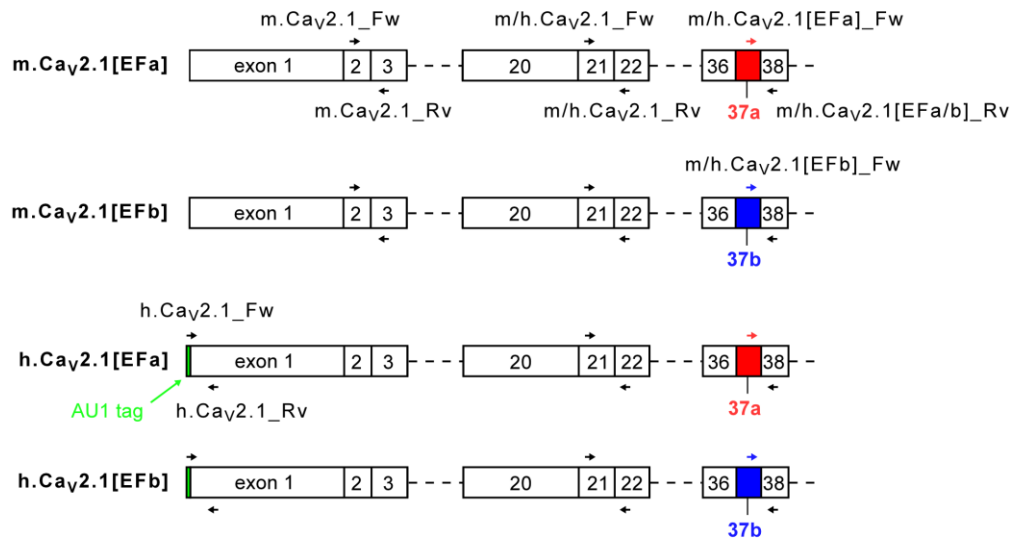


Figure S2. Further analysis of fluorescence intensity and colocalization for Cav2.1, Bsn and vGlut1. (A) Wide-field fluorescence images of the same primary cortical neurons shown in Fig 2A before SIM reconstruction. **(B)** Quantification of image fluorescence intensity for Bassoon (Bsn), vGlut1 and Cav2.1 for images as in (A). Fluorescence intensity of Cav2.1 increases to the same extent upon expression of either Cav2.1[EFa] or Cav2.1[EFb] (* $p < 0.05$, one-way ANOVA followed by Tukey's post hoc test; $n = 12, 12$ and 24 fields of view for Bsn, vGlut1 and Cav2.1, respectively). **(C)** Proportions of small ($0.01 - 0.1 \mu\text{m}^2$), medium ($0.1 - 0.25 \mu\text{m}^2$) and large ($> 0.25 \mu\text{m}^2$) Bsn, vGlut1 and Cav2.1 puncta for the three indicated experimental conditions. The relative proportions of puncta size are not affected by exogenous expression of either Cav2.1[EFa] or Cav2.1[EFb] ($p > 0.05$, Chi-square test; $n = 12, 12$ and 24 fields of view for Bsn, vGlut1 and Cav2.1, respectively). **(D)** Left, analysis of colocalization between Cav2.1 and Bsn as for Fig 2D, E but done separately for boutons of different size, as indicated. Right, analysis of colocalization between Cav2.1 and vGlut1 as for Fig 2F, G but done separately for boutons of different size, as indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, 2-way ANOVA followed by Tukey's post hoc test; $n = 12$ fields of views per condition).

A**B**

Target	Fw primer name	Forward primer sequence (5' → 3')	Rv primer name	Reverse primer sequence (5' → 3')
mouse Ca _v 2.1	m.Ca _v 2.1_Fw	CCTGATGATGACAAGACACC	m.Ca _v 2.1_Rv	TTCCAGCCTCAAAACAGAAG
Ca _v 2.1 (mouse + human)	m/h.Ca _v 2.1_Fw	AACAAAAACGCCAACCCAGA	m/h.Ca _v 2.1_Rv	AAGTAGCGCAGGTTCCAGGAT
human Ca _v 2.1	h.Ca _v 2.1_Fw	CCTACAGATACATCGGAGGAGC	h.Ca _v 2.1_Rv	GGGGATGGGGTTGTAGAGTG
Ca _v 2.1[EFa] (mouse + human)	m/h.Ca _v 2.1[EFa]_Fw	GTCCTCATAGGGTTGCTTGC	m/h.Ca _v 2.1[EFa/b]_Rv	GGCAGGTCCATCCGCAG
Ca _v 2.1[EFb] (mouse + human)	m/h.Ca _v 2.1[EFb]_Fw	CCTGGGTCTGGGAAGAAGT	m/h.Ca _v 2.1[EFa/b]_Rv	GGCAGGTCCATCCGCAG
β-Actin	β-Actin_Fw	TTGCTGACAGGATGCAGAAG	β-Actin_Rv	AGTCCGCCTAGAAGCACTTG
GAPDH	GAPDH_Fw	TGTGTCCGTCGTGGATCTGA	GAPDH_Rv	CCTGCTTCACCACCTTCTTGA

Figure S3. RT-qPCR primers. (A) Primer location. (B) Primer sequences.

Supplemental Methods

Lentiviral vector construction

The pLL-Syn-h.AU1-Cav2.1[EFa] and pLL-Syn-h.AU1-Cav2.1[EFb] lentiviral constructs were made by inserting h.Cav2.1[EFa/b] (Thalhammer et al., 2017) into the pLL-Syn vector (Jaudon et al., 2022) using the NheI and XbaI restriction sites.

Primary cortical cultures

Cortical neuronal cultures were prepared from P0 C57BL/6J pups as previously described (Jaudon et al., 2022; Thalhammer et al., 2017). Cortices were dissected in ice-cold HBSS, digested with papain (30 U; cat. no. 3126, Worthington) for 40 min at 37°C, washed, and triturated in attachment medium (BME medium supplemented with 10% FBS, 3 mg/mL glucose, 1 mM sodium pyruvate, and 10 mM HEPES-NaOH [pH 7.40]) with a flame-polished glass Pasteur pipette. For RT-qPCR and Western blot experiments, cells were seeded at a concentration of 750,000/well onto 6-well plates coated with 2.5 mg/mL poly-D-lysine (PDL; P7405, Sigma) and 1 mg/mL laminin (L2020, Sigma); for superresolution microscopy experiments, cells were seeded at 75,000/well onto 1.2 cm diameter glass coverslips coated with PDL/laminin as above. After 4 hrs, the attachment medium was replaced with maintenance medium (neurobasal medium supplemented with 2.6% B27, 6 mg/mL glucose, 2 mM GlutaMax, 90 U/mL penicillin, and 0.09 mg/mL streptomycin). To prevent glia overgrowth, cytosine β -D-arabinofuranoside (AraC; 0.5 mM) was added at 5 DIV.

Lentivirus production and infection

Lentiviruses were produced at the Viral Core Facility of the Charité - Universitätsmedizin Berlin (<https://vcf.charite.de>). Titers were 2.96×10^9 for Cav2.1[EFa] and 2.2×10^9 for Cav2.1[EFb]. Neuronal cultures were infected at 7 DIV with the lowest infectious dose capable of transducing >95% of neurons (dilution range: 1:300 to 1:700) and used for experiments after > 10 days.

RNA extraction and RT-qPCR

Total RNA was extracted with TRIzol™ Reagent (Cat. No. 15596026, Thermo Fisher Scientific) at 18 DIV, as previously described (Jaudon et al., 2022; Thalhammer et al., 2018). cDNA was prepared by reverse transcription of 1 μ g of RNA using the QuantiTect Reverse Transcription Kit (Cat. No. 205311, Qiagen). RT-qPCR was performed in triplicate with 10 ng of template cDNA using iQTM SYBR® Green Supermix (Cat. No. 1708886, Biorad) on a CFX96 Real-Time PCR Detection System (Biorad) with the following universal conditions: 5 min at 95°C, 45 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 45 s. The relative quantification of gene expression was determined using the $\Delta\Delta$ Ct method. Data were

normalized to glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and β -actin (Actb) by the multiple internal control gene method with GeNorm algorithm (Vandesompele et al., 2002). Absolute quantification was performed using the standard curve method. Calibration curves were constructed using five 10-fold serial dilutions of recombinant plasmids harboring target sequence. Sequences and location of all the primers used are listed in Figure S3.

Western blotting

Membrane protein-enriched fractions were prepared from cortical neurons at 18 DIV. Briefly, cells were washed once in ice-cold PBS and scraped in 100 μ l buffer A (25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM KCl, 2.5 mM EDTA) supplemented with protease and phosphatase inhibitors (complete EDTA-free protease inhibitors [cat. no. 1187358001, Roche]; serine/threonine and tyrosine phosphatase inhibitors [cat. nos. P0044 and P5726, Sigma]). After removal of the cell debris at 1,000 g, 4°C, for 10 min, the supernatant was centrifuged at 15,000 g, 4°C, for 15 min. The resulting pellet was dissolved in 100 μ l RIPA buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and centrifuged at 15,000 g, 4°C, for 15 min. The resulting supernatant was used for Western blot analysis. Protein concentration was quantified with the BCA Protein Assay kit (cat. no. 23227, Thermo Fisher Scientific). Proteins were separated by SDS-PAGE using 7.5% acrylamide gels and transferred on polyvinylidene fluoride (PVDF) membranes. After incubation with primary rabbit anti-Cav2.1 (1:500; cat. no. 152203, Synaptic Systems) or rabbit anti- β -tubulin III (1:1,000; cat. no. T2200, Sigma) antibodies, membranes were incubated with secondary HRP-conjugated goat anti-rabbit antibody (1: 5,000; cat. no. 31460, Thermo Fisher Scientific) and immunocomplexes were detected with the chemiluminescent substrate (cat. no. RPN2106, ECL Prime Western Blotting System, GE Healthcare). Chemiluminescent signals were acquired using a ChemiDoc imaging system (Biorad).

Immunostaining, SIM microscopy and Image analysis

Cultures were fixed at 20 DIV with Glyoxal (Richter et al., 2018) and blocked at RT in blocking buffer (4% Goat serum, 0.1% BSA in PBS) for 1 hr before o/n incubation with antibodies against Cav2.1 (rabbit; 1:1000; Synaptic Systems, #152203), vGlut1 (guinea pig; 1:1000; Synaptic Systems; #135304), Bassoon (mouse; 1:250; Synaptic Systems; #141011) and/or Munc13-1 (guinea pig; 1:500; Synaptic Systems; #126115). Coverslips were washed in blocking buffer (8 x 5 min), incubated for 1 hr at RT with goat secondary antibodies (1:800; ThermoFisher; Alexa 405 anti-mouse, #A31553; Alexa 488 anti-rabbit, #A11034; Alexa 647 anti-guinea pig, #A21450), washed in PBS and mounted using Prolong Gold (ThermoFisher).

Images were acquired on a Zeiss Elyra 7 structured illumination microscope (SIM) using a 63x objective with NA 1.46; 0.210 μ m step size in the Z direction and 13 phases for SIM acquisition, then reconstructed using the SIM default parameters in Zen Black 3.0. Using FIJI (<https://imagej.net/software/fiji/>), size and colocalization of puncta were automatically determined after maximum projection of the Z-stack and image thresholding with the Otsu and watershed algorithms.

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