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Target cell type-dependent differences in Ca^{2+} channel function underlie distinct release probabilities at hippocampal glutamatergic terminals

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33 **ABSTRACT**

34 Target cell type-dependent differences in presynaptic release probability (P_r) and short-term

35 plasticity are intriguing features of cortical microcircuits that increase the computational

36 power of neuronal networks. Here we tested the hypothesis that different voltage-gated Ca^{2+}

37 channel densities in presynaptic active zones (AZs) underlie different P_r. Two-photon Ca^{2+}

38 imaging, triple immunofluorescent labeling and three-dimensional electron microscopic (EM)

39 reconstruction of rat CA3 pyramidal cell axon terminals revealed approximately 1.7 – 1.9-

40 times higher Ca^{2+}inflow per AZ area in high P_r boutons synapsing onto parvalbumin positive

41 interneurons than in low P_r boutons synapsing onto mGluR1α positive interneurons. EM

42 replica immunogold labeling, however, demonstrated only 1.15-times larger Cav2.1 and

43 Cav2.2 subunit densities in high P_r AZs. Our results indicate target cell type-specific

44 modulation of voltage-gated Ca^{2+} channel function or different subunit composition as

45 possible mechanisms underlying the functional differences. In addition, high P_r synapses are

46 also characterized by a higher density of docked vesicles, suggesting that a concerted action of

47 these mechanisms underlies the functional differences.

48

49 **Significance Statement**

50 Target cell type-dependent variability in presynaptic properties is an intriguing feature of

51 cortical synapses. When a single cortical pyramidal cell establishes a synapse onto a

52 somatostatin-expressing interneuron (IN), the synapse releases glutamate with low

53 probability, whereas the next bouton of the same axon has high release probability when its

54 postsynaptic target is a parvalbumin-expressing IN. Here we used combined molecular,

55 imaging and anatomical approaches to investigate the mechanisms underlying these

56 differences. Our functional experiments implied a ~2-fold larger Ca^{2+} channel density in high

57 release probability boutons whereas freeze-fracture immunolocalization demonstrated only a

58 15% difference in Ca^{2+} channel subunit densities. Our results point toward a postsynaptic

59 target cell type-dependent regulation of Ca^{2+} channel function or different subunit

60 composition as the underlying mechanism.

61

62 **Introduction**

63 Target cell type-dependent differences in the probability of glutamate release (P_r) and the

64 consequent differences in short-term plasticity are well-established phenomena of cortical

65 microcircuits. Two decades ago, Thomson (1997) reported that neocortical pyramidal cell

66 (PC) to PC connections showed paired-pulse depression, whereas PC to certain interneuron

67 (IN) synapses displayed robust short-term facilitation. Later, a similar phenomenon was found

68 in the hippocampus (Ali et al., 1998; Ali and Thomson, 1998; Scanziani et al., 1998;

69 Losonczy et al., 2002), where combined functional and molecular neuroanatomical

70 approaches led to the identification of the postsynaptic IN types. Somatostatin and

71 metabotropic glutamate receptor 1α- (mGluR1α) expressing, oriens-lacunosum moleculare

72 (O-LM) and oriens-bistratified (O-Bi) INs of the CA1 area receive facilitating EPSCs with

73 low initial P_r, whereas synaptic inputs onto fast-spiking (FS) parvalbumin- (PV) expressing

74 INs (e.g. basket, axo-axonic, bistratified cells) display short-term depression and have high

75 initial P_r (Atwood and Karunanithi, 2002; Losonczy et al., 2002; Biro et al., 2005; Koester and

76 Johnston, 2005; Mercer et al., 2012). Simultaneous recordings between a presynaptic PC and

77 two distinct types of IN revealed that the axon of a single PC can transmit different aspects of

78 information coded in a complex spike train to distinct postsynaptic cell types (Markram et al.,

79 1998b, 1998a; Reyes et al., 1998; Pouille and Scanziani, 2004; Koester and Johnston, 2005).

80 It has also been demonstrated that distinct types of short-term plasticity enable neuronal

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81   networks to perform complex computations (Pouille and Scanziani, 2004), but almost nothing

82   is known about the mechanisms underlying these functional differences.

83   A candidate protein bestowing different P_r and short-term plasticity to axon terminals was

84   mGluR7, a metabotropic glutamate receptor that shows postsynaptic target cell type-

85   dependent differences in its presynaptic density (Shigemoto et al., 1996). However, a group

86   III mGluR-specific antagonist failed to abolish the differences in short-term plasticity of

87   synapses expressing or lacking mGluR7 (Losonczy et al., 2003). More recently, Sylwestrak

88   and Ghosh (2012) identified the extracellular leucine-rich repeat fibronectin containing

89   protein 1 (Elfn1) as a key molecule in bestowing short-term facilitation. This protein is

90   selectively expressed postsynaptically in O-LM cell somata and dendrites, and imposes

91   facilitating neurotransmitter release to the presynaptic axon terminals. Although, to date, there

92   is no data available regarding the mechanisms underlying the low initial P_r of these facilitating

93   synapses, Rozov et al. (2001) put forward an elegant hypothesis based on their experiments

94   involving fast and slow Ca^{2+} buffers. They postulated that the low initial P_r of facilitating

95   cortical PC synapses can be explained by a larger coupling distance between voltage-gated

96   Ca^{2+} channels and Ca^{2+} sensors on the docked vesicles compared to the high P_r PC synapses

97   on FS INs. Assuming similar Ca^{2+} sensors and docked vesicle distributions, this would

98   suggest a lower average Ca^{2+} channel density within the AZs of low P_r synapses.

99   Here we tested this hypothesis with two independent methods. 1) Using two-photon Ca^{2+}

100   imaging in hippocampal CA3 PC axon terminals, *post hoc* immunohistochemical

101   identification of their postsynaptic target cells, followed by three-dimensional (3D) EM

102   reconstructions of the imaged boutons we estimated the amount of Ca^{2+} entering the bouton

103   (peak concentration x the bouton volume) and divided it by the AZ area. We refer to this as

104   our 'functional Ca^{2+} channel density' estimate. 2) We also measured the densities and

105   distribution of Cav2.1 and Cav2.2 voltage-gated Ca^{2+} channel subunits in presynaptic AZs

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106   using SDS-digested freeze-fracture replica immunogold labeling (SDS-FRL) and we refer to it

107   as 'Ca^{2+} channel density'. Finally, we assessed the densities of docked vesicles in boutons

108   with different postsynaptic target cell types using EM tomography.

109

110   **Materials and Methods**

111   *Slice preparation and electrophysiological recordings*. Male Wistar rats (14 – 17 days old, n

112   = 97) were sacrificed by decapitation in accordance with the Hungarian Act of Animal Care

113   and Experimentation (1998, XXVIII, section 243/1998) and with the ethical guidelines of the

114   Institute of Experimental Medicine Protection of Research Subjects Committee. The animals

115   were housed in the vivarium of the Institute of Experimental Medicine in a normal 12 hour /

116   12 hour light / dark cycle. Young experimental animals were kept in a cage with their mother

117   and were used before weaning. Acute hippocampal slices were prepared as published

118   previously (Holderith et al., 2012). Briefly: after decapitation, the brain was quickly removed

119   and placed into an ice-cold cutting solution containing (in mM): sucrose, 205.2; KCl, 2.5;

120   NaHCO$_3$, 26; CaCl$_2$, 0.5; MgCl$_2$, 5; NaH$_2$PO$_4$, 1.25; glucose, 10; saturated with 95 % O$_2$ and

121   5 % CO$_2$. 300 μm thick horizontal slices were cut from the ventral part of the hippocampus

122   using a Leica Vibratome (Leica VT1200S; Leica Microsystems, Vienna, Austria), incubated

123   in an interface-type holding chamber in ACSF containing in mM: NaCl, 126; KCl, 2.5;

124   NaHCO$_3$, 26; CaCl$_2$, 2; MgCl$_2$, 2; NaH$_2$PO$_4$, 1.25; glucose, 10 saturated with 95% O$_2$ and 5%

125   CO$_2$ (pH = 7.2 – 7.4) at 36 °C, then kept at 22 – 24 °C. Recordings were carried out in the

126   same ACSF at 22 – 24 °C up to 6 hours after slicing.

127   Cells were visualized using a Femto2D microscope equipped with oblique illumination and a

128   water immersion lens (25X, NA = 1.05, Olympus, Japan or 25X, NA = 1.1, Nikon, Japan).

129   Whole-cell voltage- or current-clamp recordings were performed from CA3 PCs or INs

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130   located in the strata oriens and pyramidale using MultiClamp 700A and B amplifiers

131   (Molecular Devices). Recorded traces were filtered at 3 - 4 kHz and digitized on-line at 20

132   kHz. Patch pipettes (resistance 3 – 6 MΩ) were pulled from thick-walled borosilicate glass

133   capillaries with an inner filament. Intracellular solution contained in mM: K-gluconate, 110;

134   KCl, 5; creatine phosphate, 10; HEPES, 10; ATP, 2; GTP, 0.4; biocytin, 5 (pH = 7.3; 290 –

135   300 mOsm). For voltage-clamp recordings of evoked EPSCs in INs, the intracellular solution

136   contained picrotoxin (0.6 - 0.8 mM). For current-clamp recordings and Ca^{2+} imaging in PC

137   axons, 100 or 300 μM Fluo5F (Molecular Probes) and 20 μM Alexa594 (Molecular Probes)

138   was added to the intracellular solution.

139   The firing pattern of the INs was determined with a series of 500 ms long hyper- and

140   depolarizing current pulses with amplitudes of 125 - 500 pA. A cell was considered fast

141   spiking if the average firing frequency exceeded 70 Hz. For extracellular stimulation, a

142   unipolar stimulating electrode was placed in the stratum oriens at least 100 μm away from the

143   soma. Interneurons were held at -70 mV and 5 stimuli (0.2 – 0.3 ms duration, 20 - 200 μA) at

144   40 or 50 Hz with 30 second inter-stimulus interval (15 – 70 repetitions) were applied. Data

145   from 40 and 50 Hz stimulations were pooled (Fig. 1*I*). To be able to assess the Elfn1/2

146   expression in mGluR1α$^+$ cells with different short-term plasticity, cells with inputs exhibiting

147   short-term depression (~10% in the random mGluR1α$^+$ population) were selectively searched

148   for (Fig. 1*J*, *K*). Series resistance was monitored and was < 20 MΩ. Pyramidal cells were held

149   at -70 mV (with a maximum of −100 pA DC current) and single APs at 0.05 – 0.016 Hz were

150   evoked with 2 – 4 ms-long depolarizing current pulses (1 – 1.2 nA). Peak amplitude and full

151   width at half maximal amplitude of the APs were monitored and cells were rejected if any of

152   these parameters changed more than 10%.

153   *Two-photon [Ca^{2+}] imaging*. Experiments were performed with a Femto2D (Femtonics Ltd.,

154   Budapest, Hungary) laser scanning microscope equipped with a MaiTai femtosecond pulsing

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155 laser tuned to 810 nm (described in Holderith et al., 2012). Electrophysiological data and

156 image acquisition was controlled with a software written in MATLAB (MES, Femtonics Ltd.,

157 Budapest, Hungary). Cells were filled for 2 hours with a Ca^{2+}-insensive (20 μM Alexa594)

158 and a Ca^{2+}-sensitive fluorophore (100 or 300 μM Fluo5F). Boutons were selected at 150 – 300

159 µm distances from the soma on the second and third order collaterals of the main axon in the

160 stratum oriens at 35 – 80 µm slice depth. They were imaged in line scan mode (scan duration

161 500 or 1200 ms at 1 kHz, 1 - 3 per minute repetition, 2 - 3 scans averaged for each bouton)

162 with a laser intensity of 2 - 6 mW at the back aperture of the objective lens. Single AP-evoked

163 changes in fluorescence were quantified during the recording as $\Delta G/R(t) = (F_{green(t)} - F_{rest, green})$

164 $/ (F_{red} - I_{dark, red})$ where $F_{green(t)}$ represents the green fluorescence signal as a function of time,

165 $F_{rest,green}$ is the green fluorescence before stimulation, and $I_{dark,red}$ is the dark current in the red

166 channel. To normalize data across batches of dyes, G_{max}/R values were measured by imaging

167 a sealed (tip melted and closed by heating) pipette filled with intracellular solution containing

168 10 mM $CaCl_2$ for each cell at the same position where the boutons were imaged. $\Delta G/R$

169 measurements from boutons were divided by G_{max}/R, yielding the reported values of G/G_{max}.

170 The effects of 50 μM $CdCl_2$ and 1 μM ω-CTX MVIIC (diluted in 1 mg/ml BSA; Tocris and

171 Alomone Labs) were tested by comparing the peak amplitudes of presynaptic $[Ca^{2+}]$ transients

172 in individual boutons averaged from 2-3 consecutive scans in control conditions and after 10

173 (for $CdCl_2$) or 30 min (for ω-CTX MVIIC) of wash-in of the drug.

174 *Tissue processing*. Following recordings, the slices were fixed in a solution containing 4 %

175 paraformaldehyde (PFA), 0.2% picric acid in 0.1 M phosphate buffer (PB; pH = 7.4) at 4 °C

176 for either 12 - 36 hours (PV or mGluR1α immunolabeling) or 1-3 hours (for Elfn1/2 labeling).

177 Slices containing filled PCs were incubated in 10 and 20% sucrose as cryoprotectant,

178 repeatedly freeze-thawed above liquid nitrogen, embedded in agarose (2%) and re-sectioned

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179   at 70 – 90 μm thickness. Slices containing filled INs were immunolabelled without re-

180   sectioning.

181   For quantification of colocalization of mGluR1α and Elfn1/2 six, and for mGluR1α and PV

182   three 14 – 17 days old male Wistar rats were deeply anaesthetized and were transcardially

183   perfused with ice cold fixative containing 2 or 4% PFA and 0.2% picric acid in 0.1 M PB for

184   25 minutes. The brains were then quickly removed from the skull and placed in 0.1 M PB. For

185   serial EM reconstructions and EM tomography the fixative contained 2% PFA and 1%

186   glutaraldehyde (GA) in 0.1 M sodium acetate buffer (pH = 6) for 2 minutes, followed by 45

187   minutes of perfusion with 2% PFA and 1% GA in 0.1 M borate buffer (pH = 9) in six 14 – 17

188   days old male Wistar rats. The brains were left in the skull overnight then removed and placed

189   in 0.1 M PB. 60 μm thick sections were cut from the ventral hippocampus.

190   *Light microscopy*. Sections / slices were washed in 0.1M PB and blocked in normal goat

191   serum (NGS, 10%) for 1 hour made up in Tris-buffered saline (TBS; pH 7.4), incubated in the

192   following primary antibodies: mouse anti-PV (1:1000, SWANT; RRID:AB_10000343);

193   rabbit anti-PV (1:1000 Synaptic Systems; RRID:AB_1210396), guinea pig anti-mGluR1α

194   (1:1000, Frontier Institute Co. Ltd; Mansouri et al., 2015; RRID:AB_2531897) or rabbit anti-

195   Elfn1/2 (1:500, Sigma; RRID:AB_1079280) diluted in TBS containing 2% NGS. After

196   several washes the following secondary antibodies were applied: Alexa488- or Cy5-

197   conjugated goat anti-mouse or goat anti-rabbit, and Cy3-conjugated donkey anti-guinea pig

198   IgGs. Biocytin was visualized with Alexa488- (Molecular Probes) or Cy5- (Jackson

199   Laboratories) conjugated streptavidin (1:500). Sections were mounted in Vectashield. Image

200   stacks were acquired with an Olympus FV1000 confocal microscope with 20x and 60x (oil

201   immersion) objectives. Contacts between PC boutons and IN dendrites were considered as

202   putative synapses if they had no apparent gap between them in the focal plane.

203   *Electron microscopy*. For standard transmission EM and EM tomography, biotinylated goat

204   anti-mouse, goat anti-rabbit and goat anti-guinea pig secondary antibodies were used (Vector

205   Laboratories, 1:50). Reactions and biocytin in the filled cells (after fluorescent examination)

206   were visualized using an avidin-biotin-horseradish peroxidase complex (Vector Laboratories)

207   and 3-3'- diaminobenzidine tetrahydrochloride (DAB) as chromogen. Sections were treated

208   with 1% OsO_4, stained in 1% uranyl acetate, dehydrated in a graded series of ethanol and

209   embedded in epoxy resin (Durcupan). Representative cells were reconstructed using the

210   Neurolucida system (Micro-BrightField Europe, Magdeburg, Germany) attached to a Zeiss

211   Axioscope2 microscope using 40x or 100x oil-immersion objectives. Imaged axon segments

212   were re-embedded and sectioned at 60 nm. Digital images from serial EM sections were taken

213   from the identified imaged boutons or randomly selected PV^+ or $mGluR1\alpha^+$ dendrites in the

214   stratum oriens at magnifications of 7500x or 10000x with a Jeol1011 EM. Boutons were 3D

215   reconstructed, and their volumes and the area of the AZ (equal to the corresponding PSD)

216   were measured using the Reconstruct software (http://synapses.clm.utexas.edu/). In the case

217   of multiple AZ boutons (12% in PV and 9% in mGluR1α targeting population), the total AZ

218   area was calculated by summing the individual AZ areas (Fig. 3*E*).

219   *EM tomography*. 200 nm thick sections were cut and proteinA-conjugated 10 nm gold

220   particles (Cytodiagnostic) were applied on both sides as fiducial markers. Immunolabelled

221   dendritic segments (n = 3 age-matched rats) were randomly selected in the stratum oriens.

222   Single-axis tilt series of perpendicularly oriented synapses were acquired using a Tecnai G2

223   Spirit BioTWIN transmission EM operating at 120 kV and equipped with an Eagle 4K HS

224   digital camera (Fei Europe Nanoport, Eindhoven, The Netherlands). Tilt series were recorded

225   between ± 65° (with 2° increments between ± 45° and with 1° increments between ±45-65°)

226   at 30 000x magnification using Fei Xplore3D. Tomographic volumes were reconstructed

227   using the IMOD package (Kremer et al., 1996; Imig et al., 2014), and exported without

228   binning as z-stacks for analysis (320 images per subvolume resulting an 0.6 nm virtual pixel

229   size in Z dimension). AZ area and vesicle distance from the presynaptic membrane were

230   measured with the Reconstruct software. A vesicle was considered to be docked if the outer

231   part of the lipid bilayer was in direct contact with the inner part of the AZ membrane bilayer.

232   A vesicle was considered to be pre-docked if the distance between these lipid bilayers did not

233   exceed 5 nm. The 'membrane-proximal' population was pooled from docked and pre-docked

234   populations.

235   *SDS-digested freeze-fracture replica-labeling (SDS-FRL).* Eight male Wistar rats (P15–17)

236   were deeply anaesthetized and transcardially perfused with a fixative containing 2% PFA and

237   0.2% picric acid in 0.1 M PB for 15 minutes. The brains were then quickly removed from the

238   skull and placed in 0.1 M PB. Horizontal sections of 80 μm thickness were cut from the

239   forebrain with a vibratome and were cryoprotected in 30% glycerol. Small blocks from the

240   ventral CA3 area were frozen with a high-pressure freezing machine (HPM100; Leica

241   Microsystems), fractured with a freeze-fracture machine (BAF060; Leica Microsystems), and

242   processed for SDS-FRL as described previously (Kerti et al., 2012). Tissue debris was

243   digested from the replicas in a TBS solution containing 2.5% SDS and 20% sucrose at 80°C

244   overnight. The replicas were then washed and blocked with 5% BSA in TBS for 1 hour

245   followed by an incubation in a solution of the following primary antibodies: rabbit anti-

246   Cav2.1 (1:500 or 1:600, Synaptic Systems, Cat No. 152 203; RRID:AB_2619841; this Ab

247   provides identical labeling to that of a guinea pig anti-Cav2.1, the specificity of which was

248   proven in Holderith et al., 2012) rabbit anti-Cav2.2 (1:400, Synaptic Systems; Cat No.: 152

249   303; RRID:AB_2619844; specificity of the reaction with this Ab is verified in Lenkey et al.,

250   2015), rabbit anti-Kv3.1b (1:200–1:250, Alomone; RRID:AB_2040166), guinea pig anti-

251   mGluR1α (1:100, Frontier Institute Co. Ltd; RRID:AB_2531897). Replicas then were washed

252   and incubated in a solution containing the following secondary antibodies: goat anti-rabbit

253   IgGs coupled to 5 or 10 nm gold particles (1:75 or 1:100; British Biocell) and goat anti-guinea

254   pig IgGs coupled to 15 nm gold particles (1:75 or 1:100; British Biocell). Replicas were

255   rinsed in TBS and distilled water before they were picked up on copper parallel bar grids and

256   examined with a Jeol1011 EM. All antibodies used in this study recognized intracellular

257   epitopes on their target proteins and consequently were visualized by gold particles on the P-

258   face. The nonspecific background labeling was measured on surrounding E-face structures.

259   To quantify the Cav2.1 and the Cav2.2 subunit densities in the AZs of axon terminals

260   targeting Kv3.1b$^+$ or mGluR1α$^+$ dendrites, all experiments were performed with the 'mirror

261   replica method' (Hagiwara et al., 2005). With this method, replicas are generated from both

262   matching sides of the fractured tissue surface, allowing the examination of the corresponding

263   E- and P-faces of exactly the same membranes. The AZs were delineated on the P-face based

264   on the underlying high density of intramembrane particles. Gold particles inside the synaptic

265   area and up to 30 nm away from its edge were counted. Axon terminals containing Cav

266   subunit labeling without an elevated density of intramembrane particles were discarded from

267   the analysis because this is a characteristic feature of inhibitory terminals (Lenkey et al.,

268   2015). All AZs, fractured partially or in their completeness, were quantified. When the

269   synaptic area was not flat, the replica was tilted. To eliminate reaction-to-reaction variability

270   in the Cav subunit labeling, synaptic, extrasynaptic bouton, and background Cav densities

271   were normalized to the mean of the Cav densities measured in the AZs targeting mGluR1α$^+$

272   profiles in each reaction.

273   *Analysis of the sub-AZ distribution of immunoreactive Cav2.1 and Cav2.2 subunits*. To

274   investigate whether the within-AZ distribution of gold particles labeling the Cav2.1 or Cav2.2

275   subunits is different from random distributions, we computed two measures with an in-house

276   developed software. First, we calculated the mean of the nearest neighbor distances of all gold

277   particles within an AZ ($\overline{\text{NND}}$) and that of random distributed gold particles within the same

278   AZ (same number of gold particles, 1000 repetitions). The $\overline{\text{NND}}$s were then statistically

279   compared with Wilcoxon signed-rank test. In our second approach, we computed a two-

280   dimensional spatial autocorrelation function (g(r)) for our experimental data and for their

281   random controls based on the methods of Veatch et al. (2012). The g(r) reports the probability

282   of finding a second gold particle at a given r distance away from a given gold (Veatch et al,

283   2012). For randomly distributed gold particles g(r) = 1, whereas spatial inhomogeneities result

284   in g(r) values > 1 at short distances. In our experiments, we computed the g(r) for $0 < r < 80$

285   nm, then their mean ($\overline{g(\text{r})}$) was calculated and compared to those obtained from random

286   distributions using the Wilcoxon signed-rank test.

287

288   *Quantification of mGluR1α and Elfn1/2, and mGluR1α and PV colocalization.* Colocalization

289   of immunolabelled somata and dendrites was assessed in image stacks acquired with an

290   Olympus FV1000 confocal microscope with 20x (somata) or 60x (dendrites) objectives in

291   randomly selected areas of the CA3 stratum oriens for Elfn1/2, and dorsal and ventral CA1

292   and CA3 areas for PV. Image stacks were acquired at 20X magnification and cells were

293   quantified in ROIs of 200x600x60 μm in the Fluoview software. Image stacks at 60X

294   magnification were imported into the Neurolucida software, and the total length of single and

295   double labeled dendrites were measured in ROIs of 70x70x36 μm.

296   *Data analysis and statistical tests.* Analysis of electrophysiological and two-photon imaging

297   data was performed using specialized Matlab-based software (MES, Femtonics Ltd. Budapest,

298   Hungary). Data were plotted using OriginPro. Normality of data was assessed using Shapiro-

299   Wilks test; statistical significance was assessed with *t*-test (independent two-sample with

300   unequal variances, Table 1) when the distribution was not statistically different from normal.

301   For non-normal distributions and when the sample size was small, either Mann Whitney U-

302   test (MW U-test; two unpaired groups, Fig. 2*J*, 3*D*, *E*, Fig. 4*O-Q*, Fig. 7*G*, *H*), or Wilcoxon

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303   signed-rank test (two paired groups, Fig. 5 *P*, *R*, 6 *K*, *L*), or Kruskal–Wallis test (multiple

304   unpaired groups) with MW U-test with Bonferroni adjustment (2*I* (3 groups), 5*O* (5 groups),

305   6*J* (5 groups)) were used. For the comparison of the short-term plasticity of evoked EPSCs,

306   two-way repeated measures ANOVA with Bonferroni *post hoc* test was used (Fig. 1). Power

307   analysis were performed and predicted a power of 0.8 for every dataset in Fig. 2 and 3 and

308   0.75 for data in Fig. 7. Data are presented as mean ± standard deviation (SD) for normally

309   distributed data or median and interquartile ranges (IQR) for non-normal distributions. Results

310   were considered significant when $p < 0.05$. In all figures, $*p < 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

311

312   **Results**

313   **Distinct short-term plasticity of EPSCs in different IN types of the hippocampal CA3**

314   **region**

315   We chose CA3 PC local axon collaterals as the subject of our study because they are

316   amenable to presynaptic $[Ca^{2+}]$ measurements (Holderith et al., 2012) and establish synaptic

317   contacts onto both FS, PV positive (PV^+) and mGluR1α positive ($mGluR1\alpha^+$) INs. First we

318   characterized the short-term plasticity of EPSCs recorded from PV^+ and $mGluR1\alpha^+$ INs in the

319   CA3 region of young Wistar rats. Whole-cell voltage-clamp recordings were performed from

320   the somata of GABAergic INs located in the stratum oriens with biocytin-containing

321   intracellular solutions and five EPSCs were evoked by extracellular stimulation of PC axons

322   (Fig. 1). The cells were characterized based on their firing patterns (Fig. 1*C*, *G*), *post hoc*

323   determined dendritic and axonal arbors (Fig. 1*A*, *E*) and their PV or mGluR1α

324   immunoreactivity (Fig. 1*B*, *F*). EPSCs evoked by a train of stimuli at 40 or 50 Hz in PV^+ INs

325   showed short-term depression ($EPSC^{5th} / EPSC^{1st}$: 0.48 ± 0.16, n = 10; Fig. 1*D*, *I*). In contrast,

326   five stimuli elicited facilitating EPSCs in $mGluR1\alpha^+$ INs ($EPSC^{5th} / EPSC^{1st} = 3.0 \pm 1.9$, n =

13

327   31; Fig. 1*H, I*). Although the mGluR1α^{+} cells showed marked heterogeneity based on their

328   dendritic and axonal arbors, firing patterns and short-term plasticity, their excitatory inputs

329   showed a different short-term plasticity pattern when compared to those recorded from PV[+]

330   cells (Fig. 1*I*). These results clearly demonstrate robust differences in the short-term plasticity

331   of evoked EPSCs in PV[+] and mGluR1α[+] INs in the CA3 area similar to those observed in the

332   CA1 area and the neocortex, offering the use of these molecules in this brain region as

333   markers to label the postsynaptic compartments of functionally distinct presynaptic axon

334   terminals. Because 75% of mGluR1α^{+} cells also contain low amounts of PV in the dorsal

335   hippocampal CA1 area (Ferraguti et al., 2004), we performed colocalization of these two

336   molecules in the dorsal and ventral CA1 and CA3 areas. Our results confirmed those of

337   Ferraguti et al. (2004) revealing 66 ± 4% and 65 ± 21% colocalization in the dorsal CA1 and

338   CA3 areas, respectively. However, we found that less than 4% of the mGluR1α^{+} cells are

339   immunopositive for PV in the ventral CA3 area. This low prevalence of colocalization was

340   observed in perfusion fixed brain sections (2.4 ± 1.7%, n = 3 rats) as well as in immersion

341   fixed hippocampal *in vitro* slices (3.3 ±3.5%, n = 4 rats).

342   As Elfn1 has been demonstrated to play a critical role in the short-term facilitation of EPSCs

343   in mGluR1α[+] O-LM cells of the CA1 region (Sylwestrak and Ghosh, 2012), we performed

344   double immunolocalization for mGluR1α and Elfn1/2 in the stratum oriens of the CA3 area of

345   perfusion fixed rats. We found that almost all (99 ± 4%) Elfn1/2 immunopositive (Elfn1/2[+])

346   dendrites also contained mGluR1α and that, conversely, 90 ± 5% of the mGluR1α[+] dendrites

347   were also Elfn1/2[+] (~6000 μm dendrite / animal, n = 3 animals). When we tested the short-

348   term plasticity of EPSCs in Elfn1/2 and mGluR1α double labelled INs, they showed a robust

349   short-term facilitation, the degree of which was significantly larger than that detected in

350   mGluR1α[+], but Elfn1/2 negative, cells (Fig. 1*J, K*). These results reveal a previously unseen

351   diversity among mGluR1α[+] INs and provide an explanation for the variance in the short-term

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352   plasticity of their inputs. Elfn1/2 seems to be an ideal molecular marker for postsynaptic INs

353   that receive strongly facilitating inputs, but its *post hoc* visualization requires very mild

354   fixation (see Methods) that is incompatible with *post hoc* recovery of axonal arbor and EM

355   analysis. However, because 90% of mGluR1α[+] IN dendrites are also Elfn1/2[+], we decided to

356   use mGluR1α as our molecular marker for facilitating synapses.

357   **Target cell type-dependent differences in Ca^{2+} inflow at the AZ**

358   First we opted for a combined functional and morphological approach to estimate the

359   'functional Ca^{2+} channel density' in the presynaptic AZs of CA3 PCs (Holderith et al., 2012).

360   This approach requires the measurements of [Ca^{2+}] in local axon terminals of PCs using two-

361   photon microscopy with an intracellularly applied Ca^{2+} sensitive dye (300 μM Fluo5F; Fig. 2).

362   Single action potential (AP)-evoked [Ca^{2+}] transients showed sizeable variability in their peak

363   amplitudes (coefficient of variation (CV) = 0.37, n= 692 boutons in 30 cells). To specifically

364   examine [Ca^{2+}] in axon terminals with identified postsynaptic target cell types, we fixed the

365   slices following the *in vitro* imaging experiments and then visualized the intracellular biocytin

366   and immunolabelled the tissue for mGluR1α and PV for confocal microscopy analysis (Fig.

367   2*D-G*). In most experiments we imaged 15-30 boutons, out of which few had apparently PV^{+}

368   or mGluR1α^{+} dendrites as postsynaptic targets. From the total of 692 imaged boutons, we

369   found 26 and 61 as potential presynaptic elements to PV^{+} and mGluR1α^{+} INs, respectively.

370   Our *post hoc* analysis revealed that the peak amplitude of the [Ca^{2+}] transient was 1.25-times

371   larger in PV^{+} dendrite-targeting boutons compared to their mGluR1α^{+} dendrite-targeting

372   counterparts (PV: median: 0.134 G/G$_{max}$, interquartile ranges (IQR): 0.105 – 0.168 G/G$_{max}$, n

373   = 26; mGluR1α: median: 0.107 G/G$_{max}$, IQR: 0.081 – 0.138 G/G$_{max}$, n = 61, unidentified

374   target: median: 0.114 G/G$_{max}$, IQR: 0.084 – 0.145 G/G$_{max}$, n = 605,; Fig. 2*I*; Table 1). To

375   minimize potential errors caused by slightly different dye concentrations in distinct PCs, we

376   calculated peak amplitude ratios with two other methods. First, we restricted our analyses to

15

377   cells that contained both PV$^+$ and mGluR1α$^+$ dendrite-targeting boutons and calculated

378   within-cell ratios, and found a similar 1.28-times higher value in boutons targeting PV$^+$

379   dendrites (n = 10 cells). Second, we normalized the peak amplitude of the [Ca^{2+}] transients in

380   each PV$^+$ and mGluR1α$^+$ dendrite-innervating bouton to the mean of peak amplitudes

381   recorded from all boutons of a given cell and again found a 1.21-times larger value in boutons

382   targeting PV$^+$ dendrites. In a separate set of experiments, we performed [Ca^{2+}] imaging with

383   100 μM Fluo5F and calculated the above mentioned peak amplitude ratios. The within-cell

384   ratio of peak [Ca^{2+}] in boutons innervating PV$^+$ or mGluR1α$^+$ dendrites was 1.32 (n = 11 cell),

385   whereas the [Ca^{2+}] transients in PV or mGluR1α-innervating boutons normalized to the mean

386   [Ca^{2+}] transients were 1.33 (n = 18) and 1.04 (n = 35), respectively, yielding a ratio of 1.27

387   (Table 1).

388   Because Sylwestrak and Ghosh (2012) reported the presence of kainate receptors in axon

389   terminals innervating mGluR1α$^+$ O-LM cells that could potentially contribute to the

390   presynaptic [Ca^{2+}], we examined the effect of a non-selective voltage-gated Ca^{2+} channel

391   blocker. We found that 50 μM Cd^{2+} caused a similar reduction in the peak [Ca^{2+}] in

392   mGluR1α$^+$ dendrite-innervating boutons (89 ± 10 % block n = 8) compared to that of

393   unidentified targets (87 ± 10% block, n = 36 in n = 4 cells), ruling out a significant cell type-

394   dependent differential contribution of kainate receptors to single AP-evoked presynaptic

395   [Ca^{2+}] transients. To assess the contribution of the N/P/Q-type Ca^{2+} channels to the [Ca^{2+}]

396   transients we applied ω-CTX MVIIC (a selective N- and P/Q-type Ca^{2+} channel blocker) in a

397   concentration (1 μM) that almost completely abolishes the evoked EPSCs in both PV$^+$ (96 ±

398   3% block, n = 4 cells) and mGluR1α$^+$ (93 ± 7% block, n = 6) INs in the str. oriens of the CA3

399   area. The toxin reduced the peak amplitude of the presynaptic [Ca^{2+}] transients by 45 ± 10%

400   (n = 12 in PV-innervating boutons) and 46 ± 18% (n = 19 mGluR1α-innervating boutons),

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401 demonstrating a similar contribution of these channels to the $[Ca^{2+}]$ transients in the two

402 bouton populations.

403 A larger $[Ca^{2+}]$ transient might be the consequence of lower Ca^{2+} buffering, a smaller bouton

404 volume, or a larger amount of Ca^{2+} entering the bouton. To assess potential differences in

405 Ca^{2+} buffering in these boutons, which were often only a few microns away from each other

406 (inter-bouton distance: 6.4 ± 3.5 μm) on the same axon branch, first we fitted the decay of the

407 grand total averaged $[Ca^{2+}]$ transients with single exponentials and found very similar time

408 constants (PV: 352 ms vs. mGluR1α: 413 ms). However, the high fluorescent dye

409 concentration (300 μM) used in these experiments might dominate the decay, masking

410 potential differences in the fixed buffer concentration. To circumvent this problem, we

411 recorded $[Ca^{2+}]$ transients with 100 μM Fluo5F and analyzed their decay times (Fig. 2*J*). The

412 $[Ca^{2+}]$ transients recorded with lower dye concentrations displayed a substantially faster decay

413 (300 μM: 463 ms vs. 100 μM: 210 ms, fitted to the first 260 ms of the averaged traces). When

414 the decay time constants of $[Ca^{2+}]$ transients were compared in boutons innervating PV$^+$ and

415 mGluR1α$^+$ dendrites, no significant difference was found (PV: median: 277 ms, IQR: 212 –

416 403 ms, n = 18; mGluR1α: median: 259 ms, IQR: 207 – 322 ms, n = 35; p = 0.61, MW U-test;

417 Fig. 2*J*), arguing against a robust difference in Ca^{2+} buffering. To test potential differences in

418 bouton volumes, we carried out 3D EM reconstructions of PV$^+$ or mGluR1α$^+$ dendritic

419 segments together with their presynaptic axon terminals in the stratum oriens of perfusion

420 fixed tissue (Fig. 3). Both PV$^+$ and mGluR1α$^+$ dendrites were densely innervated by axon

421 terminals, the majority of which formed asymmetrical (excitatory) synapses onto dendritic

422 shafts (Fig. 3*A*, *B*). These boutons mainly originate from CA3 PCs located either in the ipsi-

423 or contralateral hemisphere. Three-dimensional reconstructions (Fig. 3*C*) revealed no

424 significant difference in the volume of the boutons innervating these IN types (PV: median:

425 0.20 μm^3, IQR: 0.136 – 0.25 μm^3, n = 67; mGluR1α: median: 0.20 μm^3, IQR: 0.125 – 0.291

426 μm^3, n = 87; p = 0.65, MW U-test; Fig. 3*D*), demonstrating that distinct bouton volumes are

427 not the main cause of the observed differences in peak $[Ca^{2+}]$. Because previous studies from

428 our laboratory revealed that presynaptic Cav2.1 and Cav2.2 Ca^{2+} channel subunits are

429 confined to the AZ of hippocampal glutamatergic and GABAergic axon terminals (Holderith

430 et al., 2012; Lenkey et al., 2015), we measured the total AZ areas in the 3D reconstructed

431 boutons and found significantly smaller AZs in PV^+ dendrite-innervating boutons (PV:

432 median: 0.06 μm^2, IQR: 0.04 – 0.08 μm^2, n = 67; mGluR1α: median: 0.08 μm^2, IQR: 0.05 –

433 0.11 μm^2, n = 87; p = 0.0001, MW U-test; Fig. 3*E*). Finally, we calculated the total Ca^{2+} that

434 enters upon an AP by multiplying the peak $[Ca^{2+}]$ with the medians of the volume of

435 randomly selected and 3D reconstructed boutons. Assuming that this Ca^{2+} enters through Ca^{2+}

436 channels located in the AZs, we normalized this data to the median AZ area. This way we can

437 calculate the total amount of Ca^{2+} that enters the boutons through a unit AZ area (termed as

438 'functional Ca^{2+} channel density') in PV^+ and $mGluR1\alpha^+$ dendrite-targeting boutons and

439 found it to be 1.7-times larger in PV^+ dendrite-innervating boutons.

440 To provide a more direct measure of the 'functional Ca^{2+} channel density' in these AZs, we

441 randomly selected a subset of our *in vitro* two-photon imaged boutons with LM

442 immunofluorescent identification of their postsynaptic target cells (n = 12 PV^+ and n = 18

443 $mGluR1\alpha^+$-targeting boutons from 9 PCs) and performed serial section EM of the imaged

444 boutons (Fig. 4*A-M*). This method provides a more accurate 'functional Ca^{2+} channel density'

445 estimate, but it has the disadvantage of being so labor intensive that a smaller data set was

446 obtained. The peak amplitude of the $[Ca^{2+}]$ transient in EM-verified PV^+ dendrite-targeting

447 boutons was 1.32-times larger compared to that recorded in $mGluR1\alpha^+$ dendrite-targeting

448 boutons (PV: 0.18 ± 0.06 G/G_{max}, median: 0.17 G/G_{max}, n = 10; mGluR1α: 0.13 ± 0.03

449 G/G_{max}, median: 0.13 G/G_{max}, n = 13, p = 0.02, MW U-test). We could faithfully measure the

450 AZ area in 7 and 10 boutons targeting PV^+ and $mGluR1\alpha^+$ dendrites, respectively. In this

18

451 subset, the peak $[Ca^{2+}]$ in PV^+ dendrite-targeting boutons was 1.34-times larger compared to

452 that in mGluR1α$^+$ dendrite-targeting ones (PV: 0.17 ± 0.05 G/G$_{max}$, median: 0.17 G/G$_{max}$, n =

453 7; mGluR1α: 0.13 ± 0.02 G/G$_{max}$, median: 0.13 G/G$_{max}$, n = 10, p = 0.045, MW U-test) similar

454 to the difference observed for the population data (c.f. Fig. 2*I*, 4*N-O*). EM analysis of these

455 boutons revealed that they established synapses onto dendritic shafts that received many

456 convergent asymmetrical synapses (implying that they were IN dendrites). None of these

457 boutons had any ultrastructural sign of photodamage; they had only a single AZ and appeared

458 qualitatively similar to those reconstructed from perfusion-fixed tissue. When the AZ area to

459 bouton volume ratio was calculated for this subset of boutons (PV: 0.32 ± 0.08 1/μm, median:

460 0.31 1/μm, n = 7; mGluR1α: 0.47 ± 0.13 1/μm, median: 0.50 1/μm, n = 10; p = 0.04, MW U-

461 test, Fig. 4*P*), a similar significant difference was observed compared to the larger population

462 obtained from the perfusion-fixed tissue with better ultrastructural preservation (PV: $0.32 \pm$

463 0.13 1/μm, median: 0.29 1/μm, n = 59; mGluR1α: 0.42 ± 0.18 1/μm, median: 0.40 1/μm, n =

464 79; p < 0.001, MW U-test, single AZ boutons only). Finally, we calculated the total amount of

465 Ca^{2+} per AZ area for these imaged and EM analyzed boutons and obtained a 1.9-times larger

466 value for the PV^+ dendrite-innervating axon terminals (PV: 0.54 ± 0.12, median: 0.56, n = 7;

467 mGluR1α: 0.28 ± 0.06, median: 0.27, n = 10; p = 0.002, MW U-test; Fig. 4*Q*). These two

468 combined functional–structural approaches indicated 1.7- and 1.9-times differences in the

469 'functional Ca^{2+} channel density' in the AZs of these two bouton populations. Finally, our EM

470 analysis revealed a similar accuracy in predicting synaptic contacts with confocal microscopy

471 between biocytin filled axon terminals and PV^+ (10 out of 12 boutons) or mGluR1α$^+$ (13 out

472 of 18 boutons) dendrites.

473 **Cav2.1 and Cav2.2 subunit densities in presynaptic AZs as determined with replica**

474 **immunolabeling**

475   In the next set of experiments, we performed SDS-FRL to obtain an independent measure of

476   presynaptic Ca^{2+} channel densities. In order to quantitatively compare the immunogold

477   labeling for Ca^{2+} channel subunits in presynaptic AZs that synapse onto distinct IN types, the

478   postsynaptic targets of the axon terminals need to be identified. This requires the use of the so

479   called face-matched mirror replica technique (Hagiwara et al., 2005) and the molecular

480   identification of the target IN types, because the type of IN from small fractured membrane

481   segments cannot be determined based on morphological features. Following rapid freezing

482   under high pressure, we randomly fractured tissue sections from the CA3 area and created

483   carbon-platinum-carbon replicas of both fractured surfaces (Figs. 5, 6). One replica was

484   immunoreacted to identify IN dendrites and its mirror surface was labeled for a Ca^{2+} channel

485   subunit, and all subcellular structures (dendrites, AZs) were identified in both replicas.

486   Metabotropic GluR1α is a transmembrane protein that is expressed in the somato-dendritic

487   plasma membrane of hippocampal INs and specific antibodies are available that can be used

488   for replica labeling (Mansouri et al., 2015). PV is a cytoplasmic protein that cannot be

489   detected with SDS-FRL, therefore we identified somato-dendritic regions of PV[+] INs based

490   on the presence of immunogold labeling for the Kv3.1b voltage-gated K^+ channel subunit (Du

491   et al., 1996). Both mGluR1α and Kv3.1b antibodies recognize intracellular epitopes,

492   therefore, label the protoplasmic-face (P-face) of dendritic plasma membranes (Figs. 5, 6).

493   Many membrane segments are attached to these IN dendrites that represent the extracellular-

494   face (E-face) of presynaptic axon terminals. As our antibodies against Ca^{2+} channel subunits

495   recognize intracellular epitopes (label on the P-face), they cannot be used to localize these

496   channels in these attached axonal E-face membranes. The P-faces of these dendrite-attached

497   presynaptic membranes are present in the 'mirror replicas' (Figs. 5, 6). Because the release of

498   glutamate from these axon terminals are mainly mediated by P/Q- and N-type Ca^{2+} channels

499   (see above), we localized both Cav2.1 and Cav2.2 subunits. The dendrite-attached P-face

500 membrane segments were often labelled for the Cav2.1 subunit and the gold particles were

501 concentrated over areas that had an elevated density of intra-membrane particles,

502 corresponding to the AZs (Fig. 5*A*-*N*). The normalized density of gold particles within the

503 AZs was significantly larger than that found in the surrounding E-face plasma membranes

504 (defined as background), while the Cav2.1 subunit density in extrasynaptic bouton membranes

505 was similar to that of the background (Fig. 5*O*). We performed these experiments in five

506 animals and analyzed a total of 112 and 172 AZs attached to Kv3.1b[+] and mGluR1α[+]

507 dendrites, respectively, in the stratum oriens of the CA3 area. Quantitative comparison of the

508 two AZ populations revealed an overall 1.15-times higher density in Kv3.1b[+] dendrite-

509 targeting AZs (Fig. 5*O*; for non-normalized gold densities see Table 1)

510 Next, we conducted the same investigation for the other major Ca^{2+} channel subunit (Cav2.2;

511 Fig. 6). Presynaptic P-face plasma membranes attached to Kv3.1b[+] (n = 52) or mGluR1α[+] (n =

512 114) dendrites were also heavily labeled for the Cav2.2 subunit (Fig. 6*A*-*I*). Gold particles

513 were confined to the AZs, where their densities were significantly higher than the background

514 (Fig. 6*J*). Cav2.2 subunit densities also showed a 1.20-fold higher values in Kv3.1b[+]

515 compared to mGluR1α[+] dendrite-targeting AZs, but the difference did not reach significance

516 (Fig. 6*J*; for non-normalized gold densities see Table 1). Finally, we investigated whether the

517 sub-AZ distribution of the gold particles labeling the Cav subunits is compatible with a

518 random process. To test this, we computed two measures (nearest neighbor distance (NND),

519 and a two-dimensional spatial autocorrelation function (ACF), see Methods) and compared

520 the real anti-Cav2.1 immunogold distribution data in 43 Kv3.1b[+] and 72 mGluR1α[+] dendrite-

521 targeting AZs (fractured in their completeness) to those of random gold distributions. Both

522 NND and ACF analysis revealed that the actual data was significantly different from random

523 distributions (Fig. 5*P*, *R*). A similar result was obtained for the Cav2.2 subunit in 21 (Kv3.1b)

524 and 40 (mGluR1α) AZs (Fig. 6*K*, *L*). The significantly lower mean NNDs in both Kv3.1b[+]

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525   and mGluR1α[+] dendrite-innervating AZs demonstrate spatial inhomogeneities of gold

526   particles.

527   **Difference in docked vesicle density in PV[+] and mGluR1α[+] dendrite-innervating boutons**

528   Not only the Ca^{2+} channel density influences the distance between the presynaptic Ca^{2+}

529   channels and the Ca^{2+} sensors on the synaptic vesicles, but the density of vesicles docked at

530   the AZ is also a key parameter. The best currently known method to analyze the distribution

531   of synaptic vesicles around the AZ at high resolution is EM tomography (Siksou et al., 2007;

532   Imig et al., 2014). We therefore carried out EM tomography on 200 nm thick resin embedded,

533   perfusion fixed tissue immunoreacted for PV or mGluR1α (Fig. 7) and estimated the densities

534   of vesicles associated with these two AZ populations. Examining the tomographic sub-

535   volumes at a resolution of 0.6 nm allowed the selection of the Z directional planes where each

536   vesicle is closest to the presynaptic AZ membrane, from which the measurements were

537   performed. We defined a vesicle as docked if it directly contacted the AZ membrane (Fig. 7*B*,

538   *E*) or as pre-docked if the vesicle to AZ membrane distance was < 5 nm (Fig. 7*C*, *F*).

539   Quantitative comparisons revealed a 1.5- and 1.4-times higher density of docked (Fig. 7*G*)

540   and membrane-proximal (docked and pre-docked vesicles pooled together; Fig. 7*H*, Table 1.)

541   vesicles in AZs contacting PV[+] INs, compared to mGluR1α[+] INs, respectively.

542

543   **Discussion**

544   In the present manuscript, we performed two series of experiments to estimate the densities of

545   Ca^{2+} channels in presynaptic AZs of CA3 PCs contacting two distinct types of IN. First, we

546   carried out a combined functional and morphological investigation to estimate what we call

547   'functional Ca^{2+} channel density' and found an almost 2-fold higher density in the AZs

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548 innervating PV$^+$ compared to mGluR1α$^+$ INs. This is the consequence of a larger presynaptic

549 [Ca^{2+}] transient, a smaller AZ area and a similar volume of boutons innervating PV$^+$ INs. We

550 also determined the densities of immunogold particles labeling the Cav2.1 and Cav2.2 Ca^{2+}

551 channel subunits using SDS-FRL and found that PV$^+$ dendrite-innervating terminals exhibited

552 only a 1.15-times higher Ca^{2+} channel subunit density. Finally, a 1.5-times higher density of

553 docked vesicles in PV$^+$ dendrite-innervating AZs was revealed by EM tomography.

554

Target cell type-dependent differential modulation of Ca^{2+} channel function

556 Our Ca^{2+} imaging experiments are consistent with the results of Koester and Johnston (2005)

557 who have performed simultaneous paired whole-cell recordings and imaged [Ca^{2+}] transients

558 in presynaptic boutons mediating either facilitating or depressing EPSPs in the neocortex.

559 They also observed a larger presynaptic [Ca^{2+}] transient in depressing PC to multipolar cell

560 synapses compared to those mediating facilitating EPSPs in bitufted cells. However, the

561 difference in the recorded cortical boutons was more robust than that found in our present

562 study in CA3 PCs. Koester and Johnston (2005) did not provide ultrastructural information

563 regarding the bouton volume or AZ size, so the basis for the difference in [Ca^{2+}] between the

564 depressing and facilitating synapses could not be determined. Our data thus extend the current

565 knowledge by demonstrating that the larger [Ca^{2+}] in high P$_r$, FS PV$^+$ IN-innervating boutons

566 is not the consequence of a smaller bouton volume, but is caused by a larger amount of Ca^{2+}

567 entering the bouton upon an AP. By determining the size of the AZs (where Ca^{2+} channels are

568 concentrated) we can predict the [Ca^{2+}] in the vicinity of the release sites. Assuming similar

569 Ca^{2+} channel properties in different P$_r$ boutons, our data predict a 1.7 – 1.9-times higher

570 density of Ca^{2+} channels in high P$_r$ AZs. However, quantitative evaluation of several hundreds

571 of AZs with SDS-FRL for both Cav2.1 (P/Q) and Cav2.2 (N-type) Ca^{2+} channel subunits in

23

572 AZs that remained physically attached to Kv3.1b$^+$ (PV$^+$) or mGluR1α$^+$ somato-dendritic

573 membranes revealed only a ~15% higher Cav subunit density in the Kv3.1b$^+$ dendrite-

574 innervating AZs.

575 A potential explanation for the discrepancy between our 'functional channel density' and

576 SDS-FRL Cav subunit density estimates is a preferential enrichment of Cav2.3 (R-type)/Cav1

577 (L-type)/Cav3 (T-type) subunits in PV$^+$ dendrite-innervating boutons (Parajuli et al., 2012;

578 Carbone et al., 2014). However, our experiments with 1 μM ω-CTX MVIIC (a concentration

579 that almost fully block the evoked EPSCs in both IN types) revealed an almost identical block

580 of [Ca^{2+}] transients in boutons targeting these distinct IN types, arguing against differential

581 contribution of R-, T-, and L-type Ca^{2+} channels to the [Ca^{2+}] transients. Another possible

582 explanation for this discrepancy is a differential fixed Ca^{2+} buffer concentration in these two

583 bouton populations. However, the similar decay of the [Ca^{2+}] transients (recorded with either

584 300 or 100 μM Fluo5F) in these bouton populations argues against this possibility. We

585 suggest that differential target cell type-dependent regulation of Ca^{2+} channel function is the

586 most likely mechanism underlying the differences.

587 There are a number of ways to regulate Ca^{2+} channel function. Association with different β

588 subunits promotes different voltage-dependent activation and inactivation (reviewed by

589 Buraei and Yang, 2010). Interactions with SNARE proteins such as syntaxin and SNAP25 at

590 the so called 'synprint' motif reduce the channel open probability, whereas additional co-

591 expression of synaptotagmin reverses this effect (Zhong et al., 1999). This suggests a

592 regulatory switch by which presynaptic Ca^{2+} channels bound to Ca^{2+} sensors are functionally

593 enabled, whereas Ca^{2+} channels decoupled from Ca^{2+} sensors are disabled (Eggermann et al.,

594 2012). The AZ protein Munc13, which is involved in vesicle priming processes, has also been

595 found to alter Ca^{2+} inflow by modulating the kinetic properties of Ca^{2+} channels without

24

596 changing their density (Calloway et al., 2015). Probably the most widely studied modulation

597 of Ca^{2+} channel function is its regulation by presynaptic G-protein-coupled receptors (e.g.

598 mGluRs, A1 adenosine-, α2 noradrenergic, $GABA_B$ or endocannabinoid receptors; Bean,

599 1989; Dittman and Regehr, 1996; Takahashi et al., 1996; Leao and Von Gersdorff, 2002;

600 Brown et al., 2004; Szabo et al., 2014; Kupferschmidt and Lovinger, 2015). P/Q- and N-type

601 Ca^{2+} channel function is reduced via direct binding of G-protein β/γ-subunits to Ca^{2+} channel

602 β subunits. In a recent study, Anderson et al. (2015) demonstrated that presynaptic β

603 neurexins can transsynaptically reduce tonic endocannabinoid production and increase the P_r

604 of CA1 PC axons by alleviating presynaptic $[Ca^{2+}]$ from CB1-mediated inhibition. Another

605 way of modulating Ca^{2+} channel function is phosphorylation: CDK5 (kinase) / calcineurin

606 (phosphatase) equilibrium has been shown to set the phosphorylation state of the α1 subunit of

607 N-type Ca^{2+} channels, which influences the voltage dependence of the open probability of the

608 channel (Su et al., 2012; Kim and Ryan, 2013). Whatever the mechanisms are, they must be

609 able to modulate the function of presynaptic Ca^{2+} channels in a postsynaptic target cell type-

610 dependent manner.

611 The amount of Ca^{2+} entering through presynaptic voltage-gated Ca^{2+} channels is very

612 sensitive to the shape/waveform of the AP (Geiger and Jonas, 2000), and therefore a

613 postsynaptic target cell type-dependent difference in the AP waveform could also explain our

614 results. It remains to be seen whether the AP waveform in boutons (Rowan et al., 2014) that

615 are segregated by only a few microns along the same axon could be sufficiently different to

616 account for the ~30% difference in the $[Ca^{2+}]$ transient observed in our experiments.

617 The larger amount of Ca^{2+} together with a higher docked vesicle density in high P_r boutons,

618 indicates a higher $[Ca^{2+}]$ at the Ca^{2+} sensors. Rozov et al. (2001) tested the transmission

619 between cortical PCs and two distinct IN types (multipolar and bitufted) with fast and slow

620 Ca^{2+} buffers. The more robust effect of EGTA (slow buffer) on neurotransmitter release from

25

621 PC to bitufted compared to multipolar cells predicted a larger physical distance between the

622 Ca^{2+} channels and Ca^{2+} sensors (larger coupling distance) in the low P_r synapse. Our

623 'functional Ca^{2+} channel density' estimate is consistent with this prediction and supports the

624 hypothesis that the mechanisms underlying the low initial P_r and the subsequent short-term

625 facilitation is a large Ca^{2+} channel to Ca^{2+} sensor distance (Neher, 1998; Atwood and

626 Karunanithi, 2002; Eggermann et al., 2012).

627 Another level of complexity might arise from the potential target cell type-dependent

628 differences in the sub-AZ distribution of Ca^{2+} channels (Holderith et al., 2012; Nakamura et

629 al., 2015). Our NND and ACF analysis revealed that Cav2.1 and Cav2.2 subunits show

630 within-AZ distributions that are significantly different from random distributions. Note,

631 however, that the fact that the distribution of gold particles in both AZ populations differ from

632 random does not mean that the sub-AZ distribution of Cav channels is identical in both AZ

633 populations.

634

635 **Target cell type-dependent molecular differences in presynaptic axon terminals**

636 So far, the only known protein with a dramatic difference in its density between low- and

637 high-P_r synapses of a single PC axon is mGluR7 (Shigemoto et al., 1996), making it an ideal

638 candidate through which a low initial P_r and a consequent short-term facilitation could be

639 achieved. The pharmacological blockade of group III mGluRs (including mGluR7) increases

640 the amplitude of evoked EPSCs, but does not change the facilitating phenotype of EPSCs

641 recorded from CA1 mGluR1α^+ INs (Losonczy et al., 2003), suggesting a tonic, mGluR-

642 mediated reduction of transmitter release from these axon terminals. Similarly, knocking

643 down Elfn1 from somatostatin-positive INs also led to an increase in the amplitude of the 1st

644 EPSC of a train and a reduction in the degree of short-term facilitation, consistent with the

26

645   results of pharmacological block of mGluR7. Indeed, it has recently been shown that the

646   postsynaptically located Elfn1 has a key role in the selective recruitment of mGluR7 to the

647   presynaptic AZs of PC axons that contact somatostatin/mGluR1α^+ INs (Tomioka et al., 2014).

648   However, the short-term plasticity of the mGluR1α^+ dendrite-targeting boutons in mGluR7

649   antagonist or following Elfn1 knock-down is still facilitating, very different from that

650   observed in PV$^+$ IN-targeting boutons, suggesting that other mechanisms must be involved.

651   These might include the regulation of Ca^{2+} channel function mentioned above or the selective

652   presence of molecules that might impose facilitation on synapses (e.g. NMDA receptors,

653   Buchanan et al., 2012; kainate receptors, Sylwestrak and Ghosh, 2012; synaptotagmin-7,

654   Jackman et al., 2016). Additional factors could contribute to differences in initial P_r by

655   changing the sensitivity of the release machinery to $[Ca^{2+}]$. Proteins like Rab3A-D and

656   Munc13-3 increase (Schluter et al., 2004; Schluter et al., 2006; Ishiyama et al., 2014) whereas

657   others such as mover decrease (Korber et al., 2015) P_r without affecting the readily releasable

658   pool. Unc13 isoforms have been implicated in the preferential targeting of vesicles to docking

659   sites that are formed at varying distances from the Ca^{2+} channels (Bohme et al., 2016). Any of

660   these mechanisms may also contribute to the differences in initial P_r in addition to the above

661   described differences in $[Ca^{2+}]$.

662   **References**

663   Ali AB, Thomson AM (1998) Facilitating pyramid to horizontal oriens-alveus interneurone

664   inputs: dual intracellular recordings in slices of rat hippocampus. J Physiol (London)

665   507:185-199.

666   Ali AB, Deuchars J, Pawelzik H, Thomson AM (1998) CA1 pyramidal to basket and

667   bistratified cell EPSPs: dual intracellular recordings in rat hippocampal slices. J

668   Physiol (London) 507:201-217.

669   Anderson GR, Aoto J, Tabuchi K, Foldy C, Covy J, Yee AX, Wu D, Lee SJ, Chen L,

670   Malenka RC, Sudhof TC (2015) beta-Neurexins Control Neural Circuits by

671   Regulating Synaptic Endocannabinoid Signaling. Cell 162:593-606.

672   Atwood HL, Karunanithi S (2002) Diversification of synaptic strength: presynaptic elements.

673   Nature Rev Neurosci 3:497-516.

674   Bean BP (1989) Neurotransmitter inhibition of neuronal calcium currents by changes in

675   channel voltage dependence. Nature 340:153-156.

676   Biro AA, Holderith NB, Nusser Z (2005) Quantal size is independent of the release

677   probability at hippocampal excitatory synapses. J Neurosci 25:223-232.

678   Bohme MA, Beis C, Reddy-Alla S, Reynolds E, Mampell MM, Grasskamp AT, Lutzkendorf

679   J, Bergeron DD, Driller JH, Babikir H, Gottfert F, Robinson IM, O'Kane CJ, Hell SW,

680   Wahl MC, Stelzl U, Loll B, Walter AM, Sigrist SJ (2016) Active zone scaffolds

681   differentially accumulate Unc13 isoforms to tune Ca^{2+} channel-vesicle coupling. Nat

682   Neurosci 19:1311-1320.

683   Brown SP, Safo PK, Regehr WG (2004) Endocannabinoids inhibit transmission at granule

684   cell to Purkinje cell synapses by modulating three types of presynaptic calcium

685   channels. J Neurosci 24:5623-5631.

686   Buchanan KA, Blackman AV, Moreau AW, Elgar D, Costa RP, Lalanne T, Tudor Jones AA,

687   Oyrer J, Sjostrom PJ (2012) Target-specific expression of presynaptic NMDA

688   receptors in neocortical microcircuits. Neuron 75:451-466.

689   Buraei Z, Yang J (2010) The β subunit of voltage-gated Ca^{2+} channels. Physiological Reviews

690   90:1461-1506.

691   Calloway N, Gouzer G, Xue M, Ryan TA (2015) The active-zone protein Munc13 controls

692   the use-dependence of presynaptic voltage-gated calcium channels. eLife 4.

693   Carbone E, Calorio C, Vandael DH (2014) T-type channel-mediated neurotransmitter release.

694   Pflugers Arch 466:677-687.

695   Dittman JS, Regehr WG (1996) Contributions of calcium-dependent and calcium-independent

696   mechanisms to presynaptic inhibition at a cerebellar synapse. J Neurosci 16:1623-

697   1633.

698   Du J, Zhang L, Weiser M, Rudy B, McBain CJ (1996) Developmental expression and

699   functional characterization of the potassium-channel subunit Kv3.1b in parvalbumin-

700   containing interneurons in the rat hippocampus. J Neurosci 16:506-518.

701   Eggermann E, Bucurenciu I, Goswami SP, Jonas P (2012) Nanodomain coupling between

702   Ca(2+) channels and sensors of exocytosis at fast mammalian synapses. Nature Rev

703   Neurosci 13:7-21.

704   Ferraguti F, Cobden P, Pollard M, Cope D, Shigemoto R, Watanabe M, Somogyi P (2004)

705   Immunolocalization of metabotropic glutamate receptor 1α (mGluR1α) in distinct

706   classes of interneuron in the CA1 region of the rat hippocampus. Hippocampus

707   14:193-215.

708   Geiger JR, Jonas P (2000) Dynamic control of presynaptic Ca^{2+} inflow by fast-inactivating K^+

709   channels in hippocampal mossy fiber boutons. Neuron 28:927-939.

710   Hagiwara A, Fukazawa Y, Deguchi-Tawarada M, Ohtsuka T, Shigemoto R (2005)

711   Differential distribution of release-related proteins in the hippocampal CA3 area as

712   revealed by freeze-fracture replica labeling. J Comp Neurol 489:195-216.

713   Holderith N, Lorincz A, Katona G, Rozsa B, Kulik A, Watanabe M, Nusser Z (2012) Release

714   probability of hippocampal glutamatergic terminals scales with the size of the active

715   zone. Nature Neurosci 15:988-997.

716   Imig C, Min SW, Krinner S, Arancillo M, Rosenmund C, Sudhof TC, Rhee J, Brose N,

717   Cooper BH (2014) The morphological and molecular nature of synaptic vesicle

718   priming at presynaptic active zones. Neuron 84:416-431.

719   Ishiyama S, Schmidt H, Cooper BH, Brose N, Eilers J (2014) Munc13-3 superprimes synaptic

720   vesicles at granule cell-to-basket cell synapses in the mouse cerebellum. J Neurosci

721   34:14687-14696.

722   Jackman SL, Turecek J, Belinsky JE, Regehr WG (2016) The calcium sensor synaptotagmin 7

723   is required for synaptic facilitation. Nature 529:88-91.

724   Kerti K, Lorincz A, Nusser Z (2012) Unique somato-dendritic distribution pattern of Kv4.2

725   channels on hippocampal CA1 pyramidal cells. Eur J Neurosci 35:66-75.

726   Kim SH, Ryan TA (2013) Balance of calcineurin Aalpha and CDK5 activities sets release

727   probability at nerve terminals. J Neurosci 33:8937-8950.

728 Koester HJ, Johnston D (2005) Target cell-dependent normalization of transmitter release at
729   neocortical synapses. Science 308:863-866.

730 Korber C, Horstmann H, Venkataramani V, Herrmannsdorfer F, Kremer T, Kaiser M,
731   Schwenger DB, Ahmed S, Dean C, Dresbach T, Kuner T (2015) Modulation of
732   Presynaptic Release Probability by the Vertebrate-Specific Protein Mover. Neuron
733   87:521-533.

734 Kremer JR, Mastronarde DN, McIntosh JR (1996) Computer visualization of three-
735   dimensional image data using IMOD. J Struct Biol 116:71-76.

736 Kupferschmidt DA, Lovinger DM (2015) Inhibition of presynaptic calcium transients in
737   cortical inputs to the dorsolateral striatum by metabotropic $GABA_B$ and mGlu2/3
738   receptors. J Physiol (London) 593:2295-2310.

739 Leao RM, Von Gersdorff H (2002) Noradrenaline increases high-frequency firing at the calyx
740   of Held synapse during development by inhibiting glutamate release. J Neurophysiol
741   87:2297-2306.

742 Lenkey N, Kirizs T, Holderith N, Mate Z, Szabo G, Vizi ES, Hajos N, Nusser Z (2015) Tonic
743   endocannabinoid-mediated modulation of GABA release is independent of the CB1
744   content of axon terminals. Nat Comm 6:6557.

745 Losonczy A, Somogyi P, Nusser Z (2003) Reduction of excitatory postsynaptic responses by
746   persistently active metabotropic glutamate receptors in the hippocampus. J
747   Neurophysiol 89:1910-1919.

748 Losonczy A, Zhang L, Shigemoto R, Somogyi P, Nusser Z (2002) Cell type dependence and
749   variability in the short-term plasticity of EPSCs in identified mouse hippocampal
750   interneurones. J Physiol (London) 542:193-210.

751 Mansouri M, Kasugai Y, Fukazawa Y, Bertaso F, Raynaud F, Perroy J, Fagni L, Kaufmann
752   WA, Watanabe M, Shigemoto R, Ferraguti F (2015) Distinct subsynaptic localization
753   of type 1 metabotropic glutamate receptors at glutamatergic and GABAergic synapses
754   in the rodent cerebellar cortex. Eur J Neurosci 41:157-167.

755 Markram H, Wang Y, Tsodyks M (1998a) Differential signaling via the same axon of
756   neocortical pyramidal neurons. Proc Natl Acad Sci USA 95:5323-5328.

757 Markram H, Gupta A, Uziel A, Wang Y, Tsodyks M (1998b) Information processing with
758   frequency-dependent synaptic connections. Neurobiol Learn Mem 70:101-112.

759 Mercer A, Eastlake K, Trigg HL, Thomson AM (2012) Local circuitry involving
760   parvalbumin-positive basket cells in the CA2 region of the hippocampus.
761   Hippocampus 22:43-56.

762   Nakamura Y, Harada H, Kamasawa N, Matsui K, Rothman JS, Shigemoto R, Silver RA,

763   DiGregorio DA, Takahashi T (2015) Nanoscale distribution of presynaptic Ca^{2+}

764   channels and its impact on vesicular release during development. Neuron 85:145-158.

765   Neher E (1998) Vesicle pools and Ca^{2+} microdomains: new tools for understanding their roles

766   in neurotransmitter release. Neuron 20:389-399.

767   Parajuli LK, Nakajima C, Kulik A, Matsui K, Schneider T, Shigemoto R, Fukazawa Y (2012)

768   Quantitative regional and ultrastructural localization of the Cav2.3 subunit of R-type

769   calcium channel in mouse brain. J Neurosci 32:13555-13567.

770   Pouille F, Scanziani M (2004) Routing of spike series by dynamic circuits in the

771   hippocampus. Nature 429:717-723.

772   Reyes A, Lujan R, Rozov A, Burnashev N, Somogyi P, Sakmann B (1998) Target-cell-

773   specific facilitation and depression in neocortical circuits. Nature Neurosci 1:279-285.

774   Rowan MJ, Tranquil E, Christie JM (2014) Distinct Kv channel subtypes contribute to

775   differences in spike signaling properties in the axon initial segment and presynaptic

776   boutons of cerebellar interneurons. J Neurosci 34:6611-6623.

777   Rozov A, Burnashev N, Sakmann B, Neher E (2001) Transmitter release modulation by

778   intracellular Ca2+ buffers in facilitating and depressing nerve terminals of pyramidal

779   cells in layer 2/3 of the rat neocortex indicates a target cell-specific difference in

780   presynaptic calcium dynamics. J Physiol (London) 531:807-826.

781   Scanziani M, Gahwiler BH, Charpak S (1998) Target cell-specific modulation of transmitter

782   release at terminals from a single axon. Proc Natl Acad Sci USA 95:12004-12009.

783   Schluter OM, Basu J, Sudhof TC, Rosenmund C (2006) Rab3 superprimes synaptic vesicles

784   for release: implications for short-term synaptic plasticity. J Neurosci 26:1239-1246.

785   Schluter OM, Schmitz F, Jahn R, Rosenmund C, Sudhof TC (2004) A complete genetic

786   analysis of neuronal Rab3 function. J Neurosci 24:6629-6637.

787   Shigemoto R, Kulik A, Roberts JD, Ohishi H, Nusser Z, Kaneko T, Somogyi P (1996) Target-

788   cell-specific concentration of a metabotropic glutamate receptor in the presynaptic

789   active zone. Nature 381:523-525.

790   Siksou L, Rostaing P, Lechaire JP, Boudier T, Ohtsuka T, Fejtova A, Kao HT, Greengard P,

791   Gundelfinger ED, Triller A, Marty S (2007) Three-dimensional architecture of

792   presynaptic terminal cytomatrix. J Neurosci 27:6868-6877.

793   Su SC, Seo J, Pan JQ, Samuels BA, Rudenko A, Ericsson M, Neve RL, Yue DT, Tsai LH

794   (2012) Regulation of N-type voltage-gated calcium channels and presynaptic function

795   by cyclin-dependent kinase 5. Neuron 75:675-687.

31

796 Sylwestrak EL, Ghosh A (2012) Elfn1 regulates target-specific release probability at CA1-
797   interneuron synapses. Science 338:536-540.
798 Szabo GG, Lenkey N, Holderith N, Andrasi T, Nusser Z, Hajos N (2014) Presynaptic calcium
799   channel inhibition underlies CB1 cannabinoid receptor-mediated suppression of
800   GABA release. J Neurosci 34:7958-7963.
801 Takahashi T, Forsythe ID, Tsujimoto T, Barnes-Davies M, Onodera K (1996) Presynaptic
802   calcium current modulation by a metabotropic glutamate receptor. Science 274:594-
803   597.
804 Thomson AM (1997) Activity-dependent properties of synaptic transmission at two classes of
805   connections made by rat neocortical pyramidal axons in vitro. J Physiol (London)
806   502:131-147.
807 Tomioka NH, Yasuda H, Miyamoto H, Hatayama M, Morimura N, Matsumoto Y, Suzuki T,
808   Odagawa M, Odaka YS, Iwayama Y, Won Um J, Ko J, Inoue Y, Kaneko S, Hirose S,
809   Yamada K, Yoshikawa T, Yamakawa K, Aruga J (2014) Elfn1 recruits presynaptic
810   mGluR7 in trans and its loss results in seizures. Nat Comm 5:4501.
811 Veatch SL, Machta BB, Shelby SA, Chiang EN, Holowka DA, Baird BA (2012) Correlation
812   functions quantify super-resolution images and estimate apparent clustering due to
813   over-counting. PLoS One 7:e31457.
814 Zhong H, Yokoyama CT, Scheuer T, Catterall WA (1999) Reciprocal regulation of P/Q-type
815   Ca^{2+} channels by SNAP-25, syntaxin and synaptotagmin. Nature Neurosci 2:939-941.
816

817 **Table1:** Properties of $[Ca^{2+}]$ transients, bouton volumes, AZ areas and Cav
818 immunoreactivities in PV^+ and $mGluR1\alpha^+$ dendrite-innervating axon terminals.

	PV+ dendrite-targeting boutons					mGluR1α+ dendrite-targeting boutons						
	Mean	SD	Median	n	# animal	Mean	SD	Median	n	# animal	Test	p
Peak amplitude of $[Ca^{2+}]$ transient (300 µM Fluo5F) (G/G$_{max}$)	0.14	0.05	0.13	26*	15	0.11	0.04	0.11	61*	23	MW U-test	0.012
Normalized peak amplitude of $[Ca^{2+}]$ transient (300 µM Fluo5F)[1]	1.20	0.41	1.12	26*	15	1.00	0.28	0.98	61*	23	t-test	0.024
Peak amplitude of $[Ca^{2+}]$ transient (100 µM Fluo5F) (G/G$_{max}$)	0.21	0.10	0.22	14*	9	0.17	0.11	0.14	21*	13	MW U-test	0.100
Normalized peak amplitude of $[Ca^{2+}]$ transient (100 uM Fluo5F)[1]	1.33	0.51	1.36	18*	12	1.04	0.25	1.03	35*	18	t-test	0.037
Bouton volume in perfused tissue (µm^3)	0.20	0.10	0.20	67*	3	0.25	0.18	0.20	87*	3	MW U-test	0.650
Bouton volume *in vitro* slices (µm^3)	0.31	0.12	0.26	7	5	0.20	0.12	0.18	10	4		
Total AZ area in perfused tissue (µm^2)	0.06	0.03	0.06	67*	3	0.09	0.05	0.08	87*	3	MW U-test	0.000
AZ area *in vitro* slices (µm^2)	0.10	0.04	0.09	7	5	0.09	0.03	0.09	10	4		
Cav2.1 subunit density in AZs (gold/µm^2)[2]	373	47	370	112	5	321	46	325	172	5		
Cav2.2 subunit density in AZs (gold/µm^2)[2]	151	30	139	52	4	130	39	130	114	4		
Cav2.1 subunit density in extrasynaptic membranes (gold/µm^2)[2]	2.14	2.46	2.21	93	5	2.88	2.64	2.23	174	5		
Cav2.2 subunit density in extrasynaptic membranes (gold/µm^2)[2]	2.69	3.15	2.35	48	4	1.02	1.26	0.75	113	4		
Background Cav2.1 subunit density (gold/µm^2)[2]	2.27	1.93	2.94	132	5	2.27	1.93	2.94	132	5		
Background Cav2.2 subunit density (gold/µm^2)[2]	0.66	0.33	0.62	104	4	0.66	0.33	0.62	104	4		
Cav2.1 NND distance (nm)	24.7	4.0	23.3	43	5	24.3	4.6	23.1	72	5		
Cav2.2 NND distance (nm)	32.1	7.2	30.3	21	4	32.0	8.3	28.7	40	4		
Docked vesicle density (vesicle/µm^2)	33.1	21.1	35.5	47*	3	21.9	17.5	22.2	49*	3	MW U-test	0.010
Membrane proximal vesicle density (vesicle/µm^2)	64.9	42.0	59.1	47*	3	47.3	26.6	49.9	49*	3	MW U-test	0.037

819
820
821 * indicates the 'n' used for the statistical comparisons. [1]: $[Ca^{2+}]$ transients were
822 normalized to the mean of all measured $[Ca^{2+}]$ transients of the given cell. [2]: Values
823 calculated from the medians of 5 (Cav2.1) and 4 (Cav2.2) rats.

824 **Figure Legends**

825 **Figure 1.** Short-term plasticity of CA3 pyramidal cell synapses contacting PV^+ or $mGluR1\alpha^+$

826 interneurons. ***A***, Neurolucida reconstruction of an *in vitro* recorded basket cell in stratum

827 pyramidale (str. pyr.) of the CA3 region of the hippocampus (soma and dendrites orange, axon

828 black). ***B***, Confocal image of the biocytin filled interneuron (IN, left) showing

829 immunoreactivity for PV (right). Arrows indicate PV immunoreactivity of the biocytin filled

830 boutons. ***C***, Membrane potential responses upon de- and hyperpolarizing current injections.

831 The depolarizing suprathreshold response shows fast spiking (FS) characteristics. ***D***,

832 Excitatory postsynaptic currents (EPSC, average of 20 traces) evoked by extracellular

833 stimulation in the stratum oriens (str. ori.) display short-term depression. ***E***, Neurolucida

834 reconstruction of an *in vitro* recorded and biocytin filled oriens-bistratified IN (soma and

835 dendrites blue, axon black) in the str. ori. ***F***, The biocytin filled cell (left) is intensely labeled

836 for mGluR1α (right). ***G***, Membrane potential responses to hyper- and depolarizing current

837 pulses. Firing pattern shows moderate spike frequency adaptation and amplitude

838 accommodation. Note the prominent sag and the slow membrane time constant in response to

839 the hyperpolarizing current step. ***H***, Extracellular stimulation-evoked EPSCs display short-

840 term facilitation (average of 20 traces). ***I***, The short-term plasticity of evoked EPSCs onto PV

841 and mGluR1α positive INs differs significantly ($p < 0.01$ for cell type, $p = 0.2$ for stimulus

842 number and $p = 0.01$ for cell type and stimulus number interaction, two-way repeated

843 measures ANOVA, Bonferroni *post hoc* test). (**J**) A biocytin filled IN is intensely labeled for

844 mGluR1α and Elfn1/2. ***K***, Peak amplitudes of evoked EPSCs onto mGluR1α and Elfn1/2

845 double positive INs are significantly larger ($p < 0.05$ for cell type, $p = 0.31$ for stimulus

846 number and $p = 0.17$ for cell type and stimulus number interaction, two-way repeated

847 measures ANOVA, Bonferroni *post hoc* test) than those recorded from mGluR1α positive, but

848   Elfn1/2 negative cells. str. luc., stratum lucidum; str. rad., stratum radiatum; str. l-m, stratum

849   lacunosum-moleculare. Data are presented as mean ± SD.

850

851   **Figure 2.** Larger [Ca^{2+}] transients in PV^+ than in $mGluR1\alpha^+$ dendrite-contacting boutons. ***A***,

852   Two-photon (2P) image stack of a CA3 PC basal dendritic tree and axonal arbor filled with 20

853   μM Alexa Fluor 594 (Alexa594, red), 300 μM Fluo5F and biocytin. Boxed area is shown at

854   higher magnification in panel ***C***. Cartoon at the upper left corner illustrates the position of the

855   cell in the CA3 area. ***B***, Neurolucida reconstruction of the cell shown in panel ***A***. Boxed areas

856   correspond to panels ***A*** and ***C***. ***C-D***, High magnification two-photon (***C***) and confocal (***D***)

857   images of the scanned axon collaterals following fixation and visualization of the biocytin.

858   Numbers indicate the boutons that have been line scanned. Inset in ***C*** shows bouton #1 at a

859   higher magnification. The line indicates the position of the line scan. ***E-G***, Some of the

860   imaged boutons are in contact with PV (***E-F***, boutons #6 and #12) or mGluR1α (***G***, boutons

861   #15 and #16) immunolabeled dendrites (biocytin: white, PV: orange, mGluR1α: cyan). ***H***,

862   Single action potential-evoked [Ca^{2+}] transients (n = 16 transients from 16 boutons, each trace

863   is the average of 2 scans) recorded in the axon terminals shown in panel ***C***. Orange and cyan

864   traces are transients from PV+ and $mGluR1\alpha^+$ dendrite-contacting boutons, respectively. Inset

865   shows the same traces after Gauss filtering on an extended time scale. ***I***, The peak [Ca^{2+}]

866   transient amplitudes are significantly larger (Kruskal-Wallis test: p = 0.01, MW U *post hoc*

867   test, p = 0.01 between unidentified and PV and p = 0.01 between PV and mGluR1α) in PV^+

868   (orange) than in $mGluR1\alpha^+$ dendrite-contacting (cyan) or all other boutons (grey, n = 605

869   boutons from 30 cells). Black symbols correspond to individual data points obtained from the

870   cell shown in panels ***A***-***G***. ***J***, Averaged [Ca^{2+}] transients, recorded with 100 μM Fluo5F, in

871   PV^+ (orange) or $mGluR1\alpha^+$ (cyan) dendrite-targeting boutons. Mono-exponential fits show

872   similar decay kinetics. Inset shows the individual decay time constant values in 18 PV^+ and 35

35

873   mGluR1α^{+} dendrite-innervating boutons. The two populations are not significantly different

874   (p = 0.61, MW U-test). Circles indicate individual boutons, the boxes represent interquartile

875   ranges with the horizontal bars showing the medians.

876

877   **Figure 3**. Three dimensional reconstructions of PC axon terminals targeting PV^{+} or

878   mGluR1α^{+} dendrites. *A, B*, Electron micrograph of a PV^{+} (orange, *A*) and an mGluR1α^{+}

879   (cyan, *B*) dendrite (labeled with pre-embedding peroxidase reaction, dark precipitate)

880   receiving several excitatory inputs from presynaptic boutons (b). Arrowheads demarcate the

881   postsynaptic densities. *C*, Representative dendritic segments are reconstructed in 3D. Boutons

882   are white and postsynaptic dendrites are orange (PV^{+}) or cyan (mGluR1α^{+}). *D, E*, Bouton

883   volume is not (p = 0.65 MW U-test, *D*), but the total active zone area is significantly (p =

884   0.0001 MW U-test, *E*) different between PV^{+} and mGluR1α^{+} dendrite-targeting terminals.

885   Circles indicate individual boutons, the boxes represent interquartile ranges with the

886   horizontal bars showing the medians.

887

888   **Fig. 4**. Combined functional and morphological analyses of individual boutons show

889   differences in the amount of Ca^{2+} in PC axon terminals innervating PV^{+} or mGluR1α^{+} INs. *A*,

890   Neurolucida reconstruction of a CA3 PC filled with 20 µM Alexa 594, 300 µM Fluo5F and

891   biocytin. *B*, Low magnification 2P microscopic image stack of the basal dendritic tree and the

892   axonal arbor of the cell. *C*, Low magnification confocal image stack of the same cell. Boxed

893   areas on panels *A-C* indicate the axon collaterals where 21 boutons were imaged. *D-I*, High

894   magnification views of the two imaged axon collaterals (2P images: *D* and *G*, corresponding

895   confocal images: *E* and *H*, corresponding transmission LM images: *F* and *I*). Inset in panel *E*

896   shows the same axonal arbor at a lower magnification. Arrows indicate those boutons that

897   contacted either mGluR1α (#1-2 and #4) or PV (#3) immunopositive dendrites. *J*,

36

898   Immunofluorescent labeling for mGluR1α (cyan), PV (orange) and biocytin (white) of the

899   imaged area. Arrows point to boutons that form putative synapses on the labeled dendrites. *K*,

900   Correlated electron micrographs of the boutons shown in panel *J*. Arrowheads demarcate the

901   edges of the morphologically identified postsynaptic densities. *L*, Three-dimensional

902   reconstructions of the boutons shown in panels *J* and *K*. Light blue indicates the active zone,

903   dark grey deciphers mitochondria. *M*, Individual $[Ca^{2+}]$ transients (average of 2 scans)

904   recorded in the same boutons. *N, O*, Calcium transients in boutons with EM reconstructed

905   AZs (*N*: individual transients: semitransparent, mean: bold; PV: orange, n = 7, mGluR1α:

906   cyan, n = 10). The peak amplitude of the $[Ca^{2+}]$ transients is 1.3 times larger in PV (orange),

907   compared to mGluR1α (cyan) targeting boutons (p = 0.045 MW U-test, black circles

908   correspond to data points obtained from the cell shown in panels *A-M*). Grey symbols indicate

909   average $[Ca^{2+}]$ transients of the two bouton populations that were verified with confocal

910   microscopy only (values are from **Figure 2*I***). *P*, The active zone area normalized to the

911   bouton volume was significantly (p = 0.04 MW U-test) larger in mGluR1α^{+} (n = 10) than in

912   PV^{+} (n = 7) dendrite-targeting boutons similar to the population averages (grey symbols, p <

913   0.0001 MW U-test). *Q*, Calculated total Ca^{2+} per AZ area is twice as large in PV^{+} (n = 7) as in

914   mGluR1α^{+} (n = 10) dendrite-targeting boutons (p = 0.002, MW U-test). Data are presented as

915   mean ± SD.

916

917   **Figure 5.** The density of Cav2.1 subunits is 1.15-times larger in the AZs of Kv3.1b^{+}

918   compared to mGluR1α^{+} dendrite-targeting boutons. *A*, *B,* Corresponding EM protoplasmic-

919   (PF) and exoplasmic-face (EF) images of a Kv3.1b^{+} (expressed in PV^{+} cells) cell receiving

920   several excitatory inputs (orange on panel *B*). *C*, *D,* Higher magnification images of the boxed

921   areas in *A* and *B* show a perisomatic EF membrane with attached PF fragments of axon

922   terminals (b$_{PF}$) containing AZs (orange in *D*). Active zones are indicated by the loose cluster
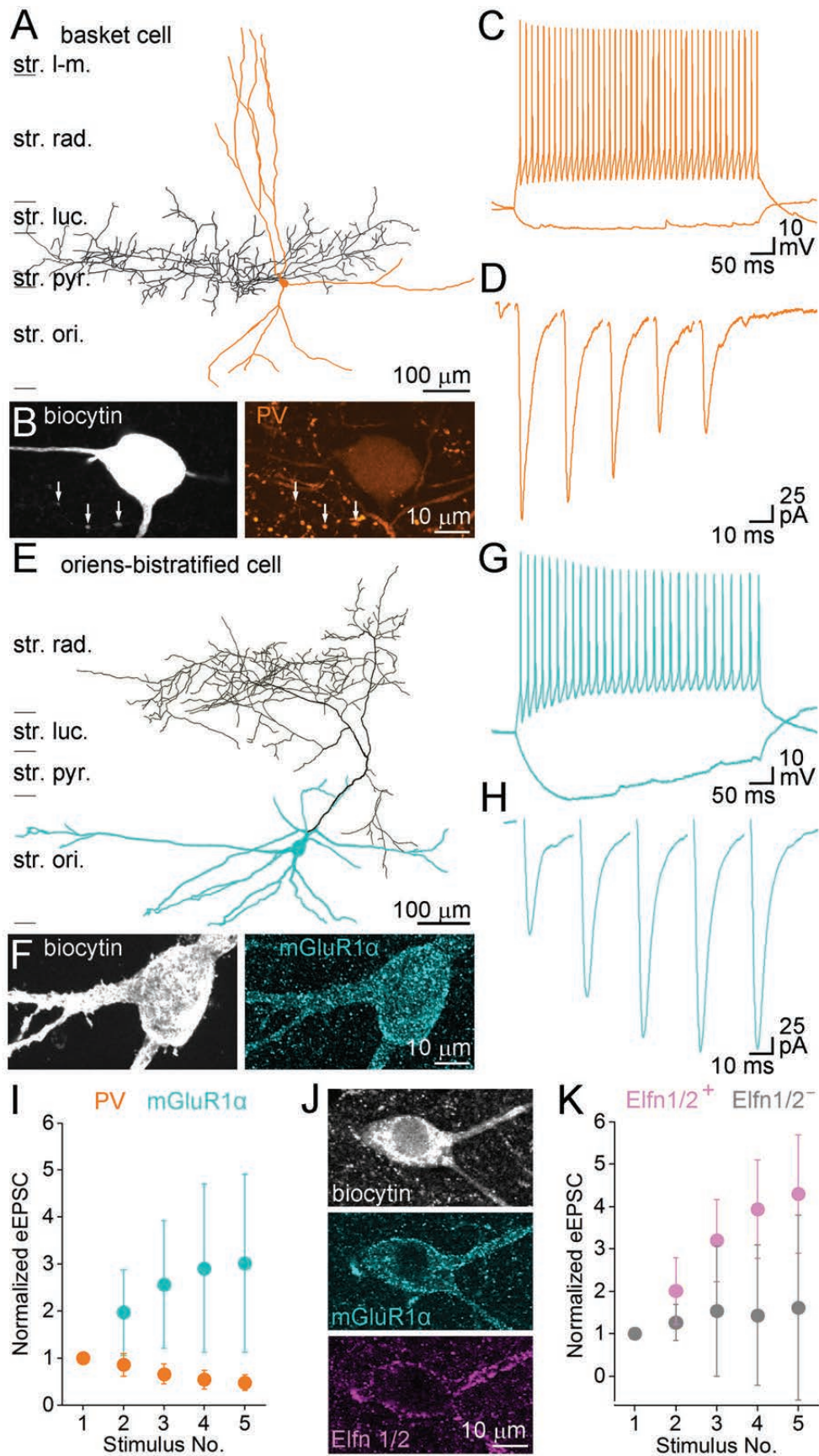
923   of intramembrane particles. *E*, Higher magnification view of the boxed areas in *D*. *F-H*,

924   Another example of an intensely Cav2.1 labelled AZ attached to a Kv3.1b$^+$ dendrite. *I*, *J*, PF

925   and EF images of an mGluR1α immunoreactive dendrite contacted by axon terminals with

926   AZs (cyan in *J*). *K*, A higher magnification image of the boxed area in *J*. *L-N*, Another

927   mGluR1α$^+$ dendrite-attached AZ with intense Cav2.1 labeling. *O*, Normalized densities of

928   gold particles labeling the Cav2.1 subunit within presynaptic AZ (syn.), extrasynaptic bouton

929   membranes (extrasyn.) and surrounding EF membranes (background: bg., grey) obtained from

930   5 rats. *Post hoc* MW U-tests with Bonferroni correction after Kruskal-Wallis test (p <

931   0.0001) demonstrated a significant difference between the synaptic and background labeling

932   (p < 0.0001) and between the synaptic compartments of Kv3.1b$^+$ and mGluR1α$^+$ dendrite

933   targeting boutons (p < 0.001). Circles indicate individual measurements of AZs, the boxes

934   represent interquartile ranges with the horizontal bars showing the medians. *P*, The nearest

935   neighbor distance (NND) was calculated for each gold particle within an AZ and the mean

936   value ($\overline{NND}$) is plotted against the $\overline{NND}$ of randomly placed gold particles (repeated 1000

937   times) for each AZ contacting Kv3.1b$^+$ (n = 43) or mGluR1α$^+$ (n = 72) dendrites. Wilcoxon

938   signed-rank test revealed significant difference (p < 0.0001) between the data and the random
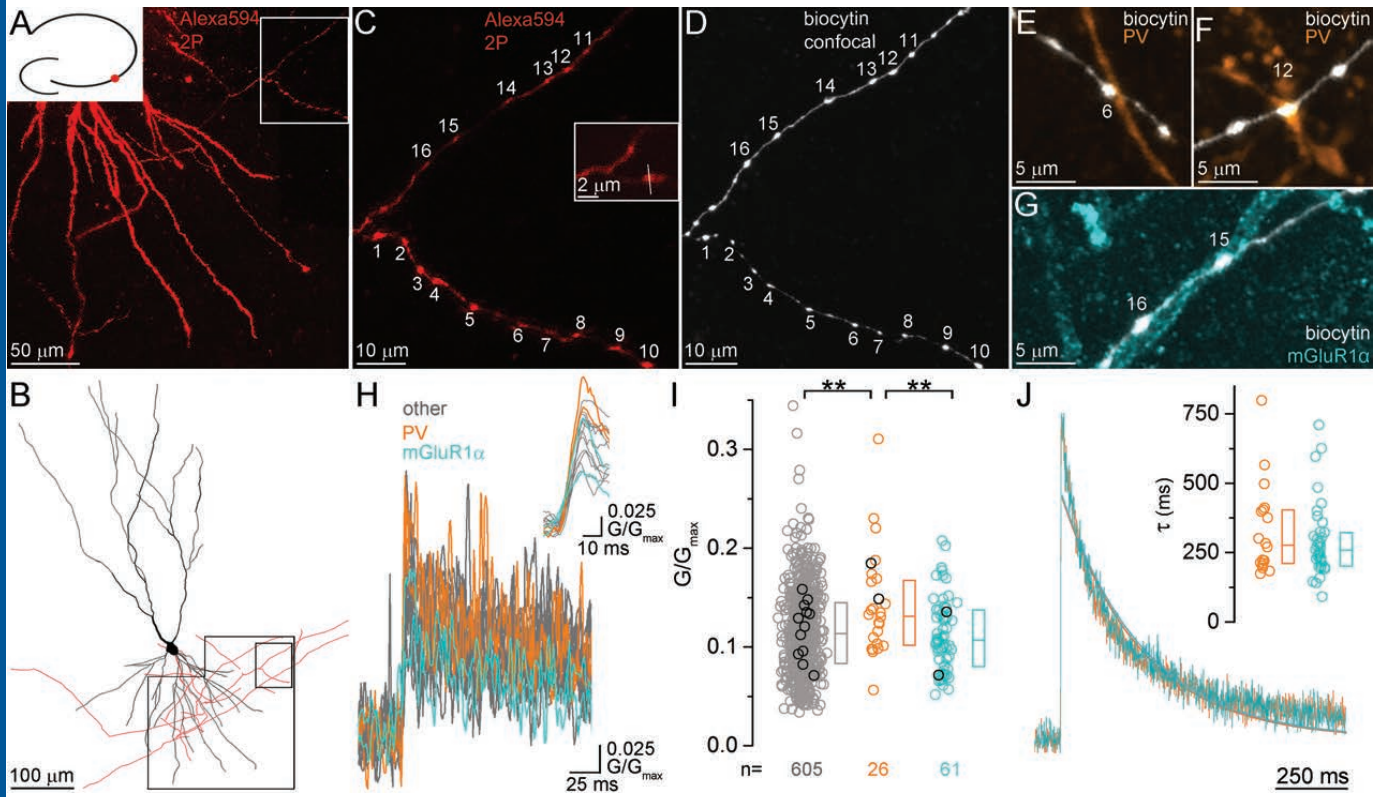
939   distributions for both AZ populations. *R*, Comparison of the mean two dimensional spatial

940   autocorrelation functions ($\overline{g(r)}$) measured in individual Kv3.1b$^+$ (n = 43) or mGluR1α$^+$ (n =

941   72) dendrite-targeting AZs to their simulated random controls (repeated 1000 times).

942   Wilcoxon signed-rank test revealed significant differences (p <0.0001) between the data and

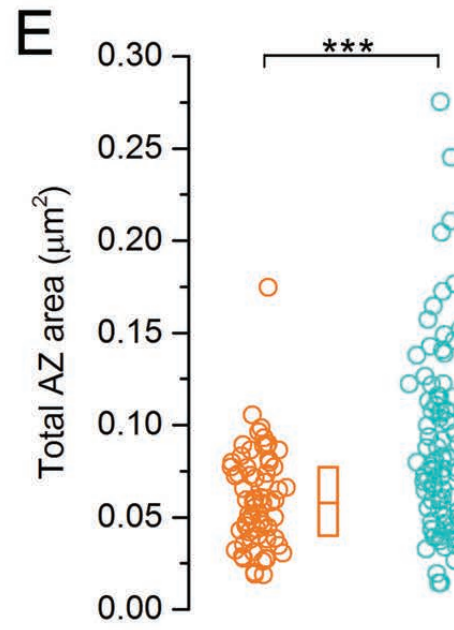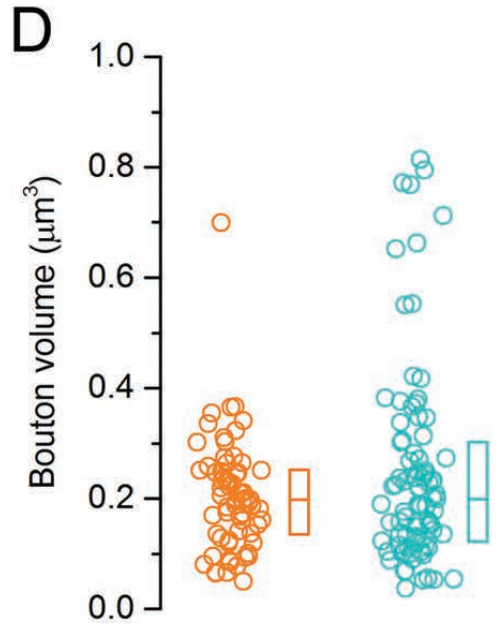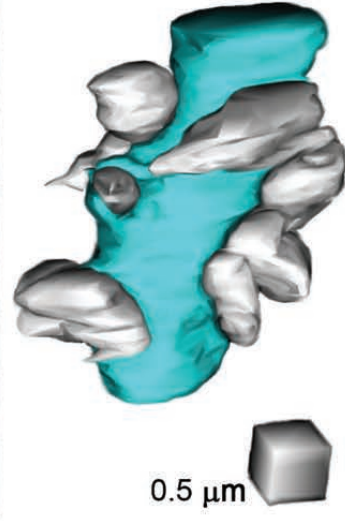943   the random distributions for both AZ populations.
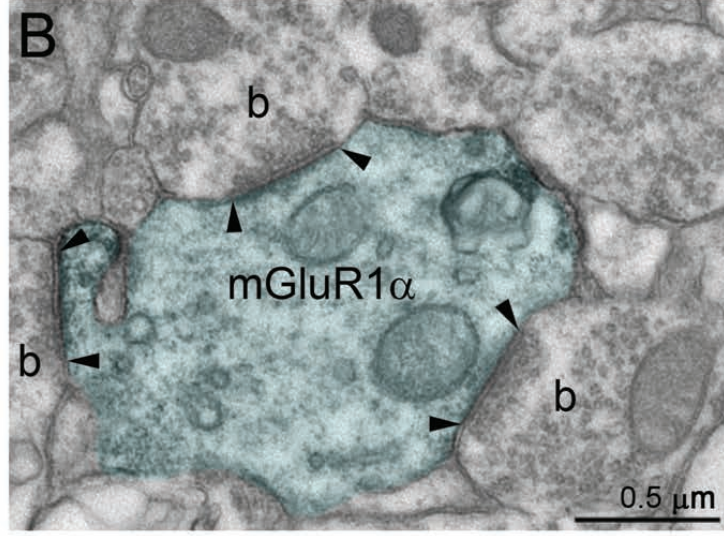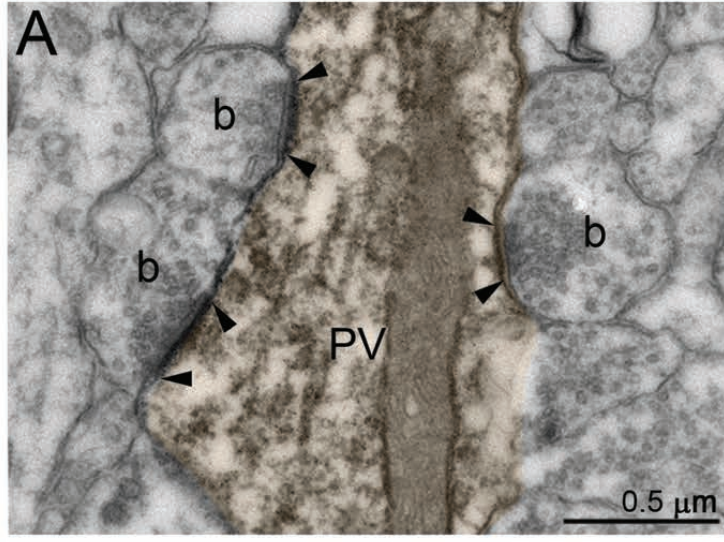
944

945   **Figure 6.** The densities of Cav2.2 subunit in AZs of PC boutons targeting Kv3.1b$^+$ and

946   mGluR1α$^+$ dendrites. *A*, *B*, A Kv3.1b$^+$ dendrite (dendrite$_{PF}$ in *A*) is contacted by boutons

947   whose PF (b$_{PF}$) membranes show strong Cav2.2 subunit reactivity in their AZs (orange in *B*).
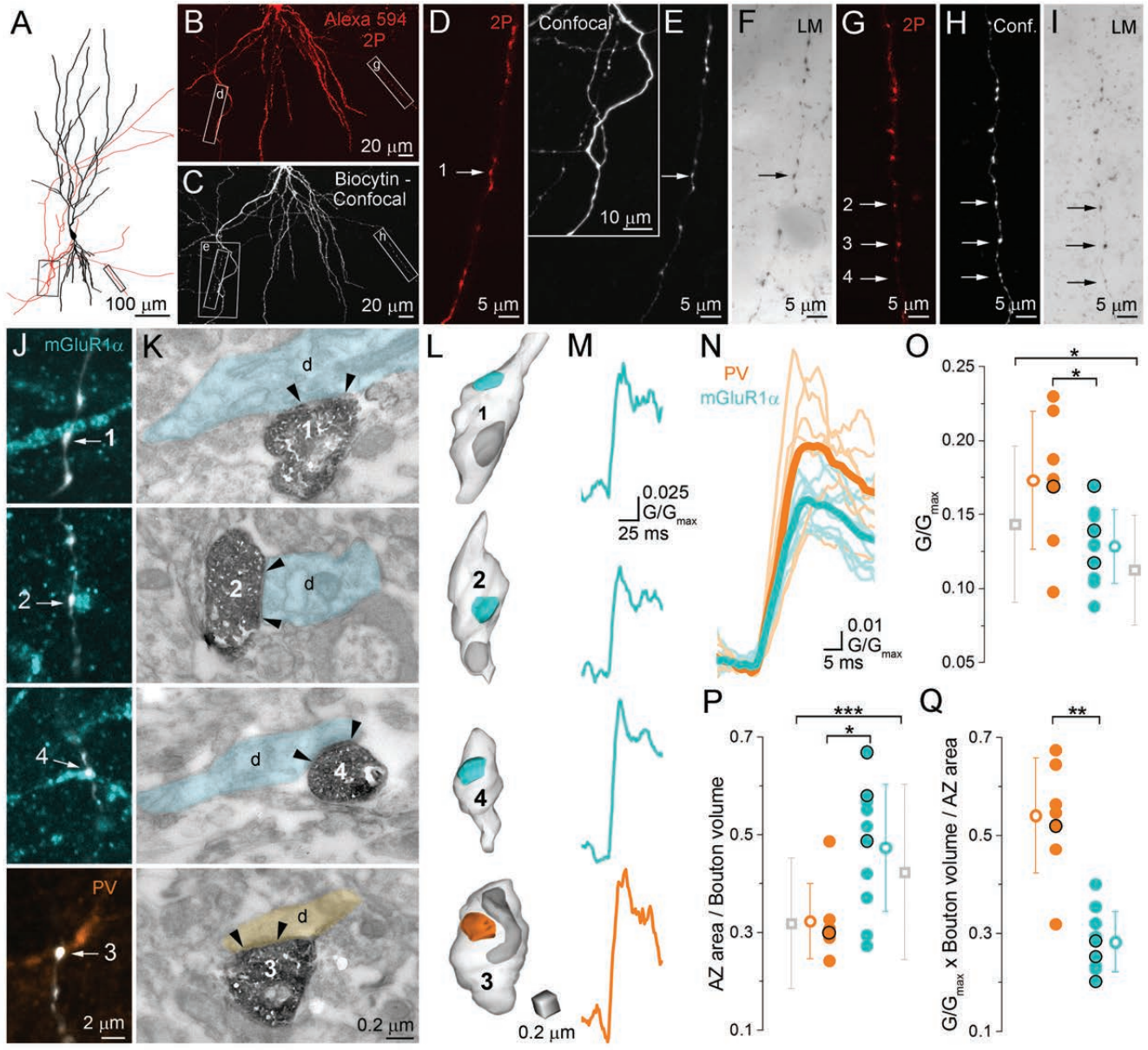
948   *C*, *D*, High magnification images of the boxed areas shown in *A*, *B*. *E*, *F*, PF bouton

949   membranes contacting an mGluR1α$^+$ dendrite (dendrite$_{PF}$ in *E*) contain Cav2.2 subunit

950   immunolabeled AZs (cyan in *F*). *G-I*, Enlarged views of the boxed areas in *E*, *F*. *J*,

951   Normalized densities of gold particles labeling the Cav2.2 subunit within presynaptic AZs

952   (syn.) and extrasynaptic membranes (extrasyn.) of boutons contacting Kv3.1b$^+$ or mGluR1α$^+$

953   profiles and in surrounding EF membranes (background: bg., grey). *Post hoc* MW U-tests

954   with Bonferroni correction after Kruskal-Wallis test (p < 0.0001) demonstrated a significant

955   difference between the synaptic and background labeling (p < 0.0001). Circles indicate

956   individual measurements of AZs, the boxes represent interquartile ranges with the horizontal

957   bars showing the medians. *K*, Comparison of $\overline{NNDs}$ measured in 21 Kv3.1b$^+$ and 40

958   mGluR1α$^+$ dendrite-contacting AZs to their random controls (repeated 1000 times). Wilcoxon

959   signed-rank test revealed significant difference (p < 0.0001) between the data and the random

960   distributions for both AZ populations. *L*, The $\overline{g(r)}$ of individual Kv3.1b$^+$ (n = 21) or

961   mGluR1α$^+$ (n = 40) dendrite-targeting AZs is plotted against the $\overline{g(r)}$ of their random

962   controls (repeated 1000 times). Wilcoxon signed-rank test revealed significant differences (p

963   <0.0001) between the data and the random distributions for both AZ populations.

964

965   **Figure 7.** EM tomography of the AZs of PC axon terminals synapsing onto PV$^+$ or mGluR1α$^+$

966   dendrites. *A*, *D*, Electron tomographic subvolumes (0.6 nm thick) of representative boutons

967   (b) establishing asymmetric synaptic contacts (arrowheads demarcate the edges of the

968   synapses) on a PV$^+$ (orange, *A*) and a mGluR1α$^+$ (cyan, *D*) dendrite (labeled with pre-

969   embedding peroxidase reaction). *B-C* and *E-F*, Higher magnification views show docked (*B*

970   and *E*, area boxed on *A* and *D*, respectively) and predocked vesicles (*C*, *F* from the same

971   tomographic subvolumes). *G*, *H*, Docked (*G*, p = 0.01, MW U-test) and membrane proximal

972   (*H*, p = 0.037, MW U-test, docked + pre-docked vesicles) vesicles have significantly different

39

973   densities (PV$^+$: n = 47; mGluR1α^+: n = 49 subvolumes) in the two AZ populations. Circles

974   indicate individual AZ subvolumes; boxes represent interquartile ranges with the horizontal

975   bars showing the medians.

A basket cell

str. l-m.

str. rad.

str. luc.

str. pyr.

str. ori.

100 μm

B biocytin   PV

10 μm

C

10 mV

50 ms

D

25 pA

10 ms

E oriens-bistratified cell

str. rad.

str. luc.

str. pyr.

str. ori.

100 μm

F biocytin   mGluR1α

10 μm

G

10 mV

50 ms

H

25 pA

10 ms

I PV mGluR1α

Normalized eEPSC

6
5
4
3
2
1
0

1 2 3 4 5

Stimulus No.

J biocytin

mGluR1α

Elfn 1/2

10 μm

K Elfn1/2+ Elfn1/2−

Normalized eEPSC

6
5
4
3
2
1
0

1 2 3 4 5

Stimulus No.

A

b

b

b

PV

0.5 μm

B

b

b

b

mGluR1α

0.5 μm

C

0.5 μm

D

Bouton volume (μm³)

1.0

0.8

0.6

0.4

0.2

0.0

E

Total AZ area (μm²)

***

0.30

0.25

0.20

0.15

0.10

0.05

0.00

	PV+ dendrite-targeting boutons					mGluR1α+ dend.-targeting boutons						
	Mean	SD	Median	n	# animal	Mean	SD	Median	n	# animal	Test	p
Peak amplitude of $[Ca^{2+}]$ transient (300 μM Fluo5F) (G/G_{max})	0.14	0.05	0.13	26*	15	0.11	0.04	0.11	61*	23	MW U-test	0.012
Normalized peak amplitude of $[Ca^{2+}]$ transient (300 μM Fluo5F)[1]	1.20	0.41	1.12	26*	15	1.00	0.28	0.98	61*	23	t-test	0.024
Peak amplitude of $[Ca^{2+}]$ transient (100 μM Fluo5F) (G/G_{max})	0.21	0.10	0.22	14*	9	0.17	0.11	0.14	21*	13	MW U-test	0.100
Normalized peak amplitude of $[Ca^{2+}]$ transient (100 μM Fluo5F)[1]	1.33	0.51	1.36	18*	12	1.04	0.25	1.03	35*	18	t-test	0.037
Bouton volume in perfused tissue (μm^3)	0.20	0.10	0.20	67*	3	0.25	0.18	0.20	87*	3	MW U-test	0.650
Bouton volume *in vitro* slices (μm^3)	0.31	0.12	0.26	7	5	0.20	0.12	0.18	10	4		
Total AZ area in perfused tissue (μm^2)	0.06	0.03	0.06	67*	3	0.09	0.05	0.08	87*	3	MW U-test	0.000
AZ area *in vitro* slices (μm^2)	0.10	0.04	0.09	7	5	0.09	0.03	0.09	10	4		
Cav2.1 density in AZs (gold/μm^2)[2]	373	47	370	112	5	321	46	325	172	5		
Cav2.2 density in AZs (gold/μm^2)[2]	151	30	139	52	4	130	39	130	114	4		
Cav2.1 extrasynaptic density (gold/μm^2)[2]	2.14	2.46	2.21	93	5	2.88	2.64	2.23	174	5		
Cav2.2 extrasynaptic density (gold/μm^2)[2]	2.69	3.15	2.35	48	4	1.02	1.26	0.75	113	4		
Background Cav2.1 density (gold/μm^2)[2]	2.27	1.93	2.94	132	5	2.27	1.93	2.94	132	5		
Background Cav2.2 density (gold/μm^2)[2]	0.66	0.33	0.62	104	4	0.66	0.33	0.62	104	4		
Cav2.1 NND distance (nm)	24.7	4.0	23.3	43	5	24.3	4.6	23.1	72	5		
Cav2.2 NND distance (nm)	32.1	7.2	30.3	21	4	32.0	8.3	28.7	40	4		
Docked vesicle density (vesicle/μm^2)	33.1	21.1	35.5	47*	3	21.9	17.5	22.2	49*	3	MW U-test	0.010
Membrane proximal vesicle density (vesicle/μm^2)	64.9	42.0	59.1	47*	3	47.3	26.6	49.9	49*	3	MW U-test	0.037