

# Calcium dependence of neurotransmitter release at a high fidelity synapse

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## 1 **Abstract**

2 The  $\text{Ca}^{2+}$ -dependence of the recruitment, priming, and fusion of synaptic vesicles are  
3 fundamental parameters controlling neurotransmitter release and synaptic plasticity.  
4 Despite intense efforts, these important steps in the synaptic vesicles' cycle remain poorly  
5 understood because disentangling recruitment, priming, and fusion of vesicles is  
6 technically challenging. Here, we investigated the  $\text{Ca}^{2+}$ -sensitivity of these steps at  
7 cerebellar mossy fiber synapses, which are characterized by fast vesicle recruitment  
8 mediating high-frequency signaling. We found that the basal free  $\text{Ca}^{2+}$  concentration  
9 ( $<200 \text{ nM}$ ) critically controls action potential-evoked release, indicating a high-affinity  $\text{Ca}^{2+}$   
10 sensor for vesicle priming.  $\text{Ca}^{2+}$  uncaging experiments revealed a surprisingly shallow  
11 and non-saturating relationship between release rate and intracellular  $\text{Ca}^{2+}$  concentration  
12 up to  $50 \mu\text{M}$ . Sustained vesicle recruitment was  $\text{Ca}^{2+}$ -independent. Finally, quantitative  
13 mechanistic release schemes with five  $\text{Ca}^{2+}$  binding steps incorporating rapid vesicle  
14 recruitment via parallel or sequential vesicle pools could explain our data. We thus show  
15 that co-existing high and low-affinity  $\text{Ca}^{2+}$  sensors mediate recruitment, priming, and  
16 fusion of synaptic vesicles at a high-fidelity synapse.

17 **Introduction**

18 During chemical synaptic transmission  $\text{Ca}^{2+}$  ions diffuse through voltage-gated  $\text{Ca}^{2+}$   
19 channels, bind to  $\text{Ca}^{2+}$  sensors, and thereby trigger the fusion of neurotransmitter-filled  
20 vesicles (Südhof, 2012). The  $\text{Ca}^{2+}$ -sensitivity of synaptic release is one of the most  
21 fundamental parameters influencing our understanding of fast neurotransmission.  
22 However, the  $\text{Ca}^{2+}$ -sensitivity of the recruitment, priming, and fusion of synaptic vesicles  
23 is difficult to determine due to the large spatial gradients of the  $\text{Ca}^{2+}$  concentration, which  
24 occurs during  $\text{Ca}^{2+}$  influx through the  $\text{Ca}^{2+}$  channels. While the basal free intracellular  
25  $\text{Ca}^{2+}$  concentration is  $\sim 50 \text{ nM}$ ,  $\text{Ca}^{2+}$  microdomains around the  $\text{Ca}^{2+}$  channels reach  
26 concentrations above  $100 \text{ }\mu\text{M}$  (Llinás et al., 1992). The technical development of caged  
27  $\text{Ca}^{2+}$  compounds (Kaplan and Ellis-Davies, 1988) allows to experimentally elevate the  
28  $\text{Ca}^{2+}$  concentration homogenously by photolysis and thus the direct measurement of the  
29  $\text{Ca}^{2+}$ -sensitivity of vesicle fusion (reviewed by Neher, 1998; Kochubey et al., 2011). First  
30 experiments with this technique at retinal bipolar cells of goldfish found a very low  
31 sensitivity of the release sensors with a half saturation at  $\sim 100 \text{ }\mu\text{M}$   $\text{Ca}^{2+}$  concentration  
32 and a fourth to fifth order relationship between  $\text{Ca}^{2+}$  concentration and neurotransmitter  
33 release (Heidelberger et al., 1994), similar to previous estimates at the squid giant  
34 synapse (Adler et al., 1991; Llinás et al., 1992). Subsequent work at other preparations  
35 showed different dose-response curves. For example, analysis of a central excitatory  
36 synapse, the calyx of Held (Forsythe, 1994) at a young pre-hearing age, found a much  
37 higher affinity with significant release below  $5 \text{ }\mu\text{M}$  intracellular  $\text{Ca}^{2+}$  concentration and  
38 similar slope of the dose-response curve (Bollmann et al., 2000; Lou et al., 2005;  
39 Schneggenburger and Neher, 2000; Sun et al., 2007). Further developmental analysis of  
40 the calyx of Held comparing the  $\text{Ca}^{2+}$ -sensitivity of the release sensors at the age of P9  
41 to P12-P15 (Kochubey et al., 2009) and P9 to P16-P19 (Wang et al., 2008) showed a  
42 developmental decrease in the  $\text{Ca}^{2+}$ -sensitivity of vesicle fusion at the calyx of Held. A  
43 recent study at another excitatory central synapse, the hippocampal mossy fiber bouton,  
44 observed a high  $\text{Ca}^{2+}$ -sensitivity of vesicle fusion in rather mature rats (P18–30; Fukaya  
45 et al., 2021), however the release rates in that study were not tested above  $20 \text{ }\mu\text{M}$   $\text{Ca}^{2+}$   
46 concentration. Analysis at an inhibitory central synapse revealed a high-affinity  $\text{Ca}^{2+}$   
47 sensor and in addition a profoundly  $\text{Ca}^{2+}$ -dependent priming step (Sakaba, 2008).

48 Moreover, analysis of the  $\text{Ca}^{2+}$ -dependence of neurotransmitter release revealed a more  
49 shallow relationship between the rate of exocytosis and  $\text{Ca}^{2+}$  concentration at the sensory  
50 neurons of the rod photoreceptors (Duncan et al., 2010; Thoreson et al., 2004), and an  
51 absence of vesicle fusion below 7  $\mu\text{M}$   $\text{Ca}^{2+}$  concentration at the cochlear inner hair cells  
52 (Beutner et al., 2001).

53 Measuring the  $\text{Ca}^{2+}$ -sensitivity of vesicle fusion is technically challenging and  
54 methodological errors could contribute to the differing  $\text{Ca}^{2+}$ -sensitivity of various types of  
55 synapses. However, synapses show type-specific functional and structural differences  
56 (Atwood and Karunianithi, 2002; Nusser, 2018; Zhai and Bellen, 2004), which may lead to  
57 distinct  $\text{Ca}^{2+}$ -sensitivities. Moreover, the rate at which new vesicles are recruited to empty  
58 release sites seems to be particularly different between synapses. The cerebellar mossy  
59 fiber bouton (cMFB) conveys high-frequency sensory information to the cerebellar cortex  
60 relying on extremely fast vesicle recruitment (Miki et al., 2020; Ritzau-Jost et al., 2014;  
61 Saviane and Silver, 2006). One aim of this study was therefore to determine the  $\text{Ca}^{2+}$ -  
62 sensitivity of vesicle fusion at mature cMFBs synapses at physiological temperature, and  
63 to test whether and how the prominent fast vesicle recruitment affects the  $\text{Ca}^{2+}$ -  
64 dependence of exocytosis at this synapse.

65 Compared with the  $\text{Ca}^{2+}$ -sensitivity of vesicle fusion, the  $\text{Ca}^{2+}$ -sensitivity of the vesicle  
66 recruitment and priming steps preceding fusion is even less well understood. While some  
67 studies at cMFBs proposed  $\text{Ca}^{2+}$ -independent vesicle recruitment (Hallermann et al.,  
68 2010; Saviane and Silver, 2006), evidence for  $\text{Ca}^{2+}$ -dependent steps preceding the fusion  
69 have been observed at several types of synapses (Awatramani et al., 2005; Doussau et  
70 al., 2017; Hosoi et al., 2007; Millar et al., 2005; Pan and Zucker, 2009; Sakaba, 2008).  
71 However, the dissection of vesicle recruitment, priming, and fusion is in general  
72 technically challenging. Therefore, we aimed to quantify the  $\text{Ca}^{2+}$ -dependence of vesicle  
73 recruitment and priming at cMFBs by direct modification of the free intracellular  $\text{Ca}^{2+}$   
74 concentration.

75 Our data revealed a strong dependence of the number of release-ready vesicles on basal  
76  $\text{Ca}^{2+}$  concentrations between 30 and 180 nM, a significant release below 5  $\mu\text{M}$ , and an  
77 apparent shallow dose-response curve in the studied  $\text{Ca}^{2+}$  concentration range of  
78 1-50  $\mu\text{M}$ . Computational simulations incorporating mechanistic release schemes with five  
79  $\text{Ca}^{2+}$  binding steps and fast vesicle recruitment via sequential or parallel pools of vesicles  
80 could explain our data. Our results show the co-existence of  $\text{Ca}^{2+}$  sensors with high- and  
81 low-affinities that cover a large range of intracellular  $\text{Ca}^{2+}$  concentrations and mediate fast  
82 signaling at this synapse.

83 **Materials and Methods**

84 **Preparation**

85 Animals were treated in accordance with the German Protection of Animals Act and with  
86 the guidelines for the welfare of experimental animals issued by the European  
87 Communities Council Directive. Acute cerebellar slices were prepared from mature P35–  
88 P42 C57BL/6 mice of either sex as previously described (Hallermann et al., 2010).  
89 Isoflurane was used to anesthetize the mice which were then sacrificed by decapitation.  
90 The cerebellar vermis was quickly removed and mounted in a chamber filled with chilled  
91 extracellular solution. 300- $\mu\text{m}$ -thick parasagittal slices were cut using a Leica VT1200  
92 microtome (Leica Microsystems), transferred to an incubation chamber at 35 °C for ~30  
93 min, and then stored at room temperature until use. The extracellular solution for slice  
94 cutting and storage contained (in mM) the following: NaCl 125,  $\text{NaHCO}_3$  25, glucose 20,  
95 KCl 2.5, 2,  $\text{NaH}_2\text{PO}_4$  1.25,  $\text{MgCl}_2$  1 (310 mOsm, pH 7.3 when bubbled with Carbogen [5%  
96 (vol/vol)  $\text{O}_2$ /95% (vol/vol)  $\text{CO}_2$ ]). All recordings were restricted to lobules IV–V of the  
97 cerebellar vermis to reduce potential functional heterogeneity among different lobules  
98 (Straub et al., 2020).

99 **Presynaptic recordings and flash photolysis**

100 All recordings were performed at physiological temperature by setting the temperature in  
101 the center of the recording chamber with immersed objective to 36°C using a TC-324B  
102 perfusion heat controller (Warner Instruments, Hamden, CT, United States). Presynaptic  
103 patch-pipettes were from pulled borosilicate glass (2.0/1.0 mm outer/inner diameter;

104 Science Products) to open-tip resistances of 3-5 M $\Omega$  (when filled with intracellular  
105 solution) using a DMZ Puller (Zeitz-Instruments, Munich, Germany). Slices were  
106 superfused with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 105,  
107 NaHCO<sub>3</sub> 25, glucose 25, TEA 20, 4-AP 5, KCl 2.5, CaCl<sub>2</sub> 2, NaH<sub>2</sub>PO<sub>4</sub> 1.25, MgCl<sub>2</sub> 1, and  
108 tetrodotoxin (TTX) 0.001, equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Cerebellar mossy fiber  
109 boutons (cMFBs) were visualized with oblique illumination and infrared optics (Ritzau-  
110 Jost et al., 2014). Whole-cell patch-clamp recordings of cMFBs were performed using a  
111 HEKA EPC10/2 amplifier controlled by Patchmaster software (HEKA Elektronik,  
112 Lambrecht, Germany). The intracellular solution contained (in mM): CsCl 130, MgCl<sub>2</sub> 0.5,  
113 TEA-Cl 20, HEPES 20, Na<sub>2</sub>ATP 5, NaGTP 0.3. For Ca<sup>2+</sup> uncaging experiments, equal  
114 concentrations of DM-nitrophen (DMn) and CaCl<sub>2</sub> were added depending on the aimed  
115 post-flash Ca<sup>2+</sup> concentration, such that either 0.5, 2, or 10 mM was used for low, middle,  
116 or high target range of post-flash Ca<sup>2+</sup> concentration, respectively (Supplementary Table  
117 1). To quantify post-flash Ca<sup>2+</sup> concentration with a previously established dual indicator  
118 method (see below; Delvendahl et al., 2015; Sabatini et al., 2002), Atto594, OGB-5N, and  
119 Fluo-5F were used at concentrations as shown in (Supplementary Table 1).

	weak Ca <sup>2+</sup> elevation	middle Ca <sup>2+</sup> elevation	strong Ca <sup>2+</sup> elevation
<b>UV illumination</b>			
Duration (ms)	0.1 or 1	0.1	0.1 or 0.2
Intensity (%)	10 - 100	20 - 100	100
<b>Concentration in intracellular solution (mM)</b>			
ATTO 594	0.010	0.020	0.020
Fluo 5F	0.050	0	0
OGB 5N	0	0.200	0.200
CaCl <sub>2</sub>	0.500	2.000	10.000
DM-N	0.500	2.000	10.000
<b>Obtained peak post-flash Ca<sup>2+</sup> (μM)</b>			
Min	1.1	2.7	15.7

Max	7.1	36.0	62.6
Median	2.4	8.8	25.1
<b>Simulated uncaging fraction of DMn</b>			
$\alpha$	0.08-0.5	0.15-0.55	0.14-0.25

120 **Supplementary Table 1 Parameters for weak, middle, and strong post-flash  $\text{Ca}^{2+}$  elevations**

121 A 50 mM solution stock of DMn was prepared by neutralizing 50 mM DMn in  $\text{H}_2\text{O}$  with  
122 200 mM CsOH in  $\text{H}_2\text{O}$ . The purity of each DMn batch was determined in the intracellular  
123 solution used for patching through titration with sequential addition of  $\text{Ca}^{2+}$  as previously  
124 described (Schneggenburger, 2005) and by measuring the  $\text{Ca}^{2+}$  concentration using the  
125 dual indicator method with 10  $\mu\text{M}$  Atto594 and 50  $\mu\text{M}$  OGB1 (Delvendahl et al., 2015).

126 After waiting for at least one minute in whole-cell mode to homogenously load the terminal  
127 with intracellular solution, capacitance measurements were performed at a holding  
128 potential of  $-100$  mV with sine-wave stimulation (5 kHz or 10 kHz frequency and  $\pm 50$  mV  
129 amplitude; Hallermann et al., 2003). During the ongoing sine-wave stimulation, a UV laser  
130 source (375 nm, 200 mW, Rapp OptoElectronic) was used to illuminate the whole  
131 presynaptic terminal. According to a critical illumination, the end of the light guide of the  
132 UV laser was imaged into the focal plan resulting in a homogeneous illumination in a  
133 circular area of  $\sim 30$   $\mu\text{m}$  diameter (Fig. 2 – figure supplement 1). The duration of the UV  
134 illumination was 100  $\mu\text{s}$  controlled with sub-microsecond precision by an external  
135 triggering of the laser source. In capacitance measurements with 10 kHz sine wave  
136 frequency, longer pulses of 200  $\mu\text{s}$  were used to reach high  $\text{Ca}^{2+}$  levels. In a subset of  
137 experiments, UV pulses of 1 ms were used to rule out fast undetectable  $\text{Ca}^{2+}$  overshoots  
138 (Bollmann et al., 2000; Fig. 3 – figure supplement 3). The UV flash intensity was set to  
139 100% and reduced in some experiments (10 – 100%) to obtain small elevations in  $\text{Ca}^{2+}$   
140 concentrations (Supplementary Table 1). All chemicals were from Sigma-Aldrich. Atto594  
141 was purchased from Atto-Tec,  $\text{Ca}^{2+}$ -sensitive fluorophores from Life Technologies, and  
142 DMn from Synaptic Systems.

143 **Paired Recordings between cMFBs and GCs**

144 For paired pre- and postsynaptic recordings, granule cells (GCs) were whole-cell voltage-  
145 clamped with intracellular solution containing the following (in mM): K-gluconate 150,  
146 NaCl 10, K-HEPES 10, MgATP 3 and Na-GTP 0.3 (300–305 mOsm, pH adjusted to 7.3  
147 with KOH). 10  $\mu$ M Atto594 was included to visualize the dendrites of the GCs (Ritzau-  
148 Jost et al., 2014). After waiting sufficient time to allow for the loading of the dye, the GC  
149 dendritic claws were visualized through two-photon microscopy, and subsequently,  
150 cMFBs near the dendrites were identified by infrared oblique illumination and were  
151 patched and loaded with caged  $\text{Ca}^{2+}$  and fluorescent indicators as previously described.  
152 The reliable induction of an EPSC in the GC was used to unequivocally confirm a cMFB-  
153 GC synaptic connection. In a subset of the  $\text{Ca}^{2+}$  uncaging experiments, simultaneous  
154 presynaptic capacitance and postsynaptic EPSC recordings were performed from GC  
155 and cMFB, respectively.

156 **Clamping intracellular basal  $\text{Ca}^{2+}$  concentrations**

157 The intracellular solution for presynaptic recordings of the data shown in Fig. 1 contained  
158 the following in mM: K-gluconate 150, NaCl 10, K-HEPES 10, MgATP 3, Na-GTP 0.3.  
159 With a combination of EGTA and  $\text{CaCl}_2$  (5 mM EGTA / 0.412 mM  $\text{CaCl}_2$  or 6.24 mM EGTA  
160 / 1.65 mM  $\text{CaCl}_2$ ), we aimed to clamp the free  $\text{Ca}^{2+}$  concentration to low and high resting  
161  $\text{Ca}^{2+}$  concentrations of ~50 or ~200 nM, respectively, while maintaining a free EGTA  
162 concentration constant at 4.47 mM. The underlying calculations were based on a  $\text{Ca}^{2+}$   
163 affinity of EGTA of 543 nM (Lin et al., 2017). The resulting free  $\text{Ca}^{2+}$  concentration was  
164 quantified with the dual indicator method (see below) and was found to be to ~30 or ~180  
165 nM, respectively (Fig. 1A).

166 **Quantitative two-photon  $\text{Ca}^{2+}$  imaging**

167 For the quantification of  $\text{Ca}^{2+}$  signals elicited through UV flash-induced uncaging, two-  
168 photon  $\text{Ca}^{2+}$  imaging was performed as previously described (Delvendahl et al., 2015)  
169 using a Femto2D laser-scanning microscope (Femtonics) equipped with a pulsed  
170 Ti:Sapphire laser (MaiTai, SpectraPhysics) adjusted to 810 nm, a 60 $\times$ /1.0 NA objective  
171 (Olympus), and a 1.4 NA oil-immersion condenser (Olympus). Data were acquired by

172 doing line-scans through the cMFB. To correct for the flash-evoked luminescence from  
173 the optics, the average of the fluorescence from the line-scan in an area outside of the  
174 bouton was subtracted from the average of the fluorescence within the bouton (Fig. 2B).  
175 Imaging data were acquired and processed using MES software (Femtonics). Upon  
176 releasing  $\text{Ca}^{2+}$  from the cage, we measured the increase in the green fluorescence signal  
177 of the  $\text{Ca}^{2+}$  sensitive indicator (OGB-5N or Fluo-5F) and divided it by the fluorescence of  
178 the  $\text{Ca}^{2+}$  insensitive Atto594 (red signal). The ratio ( $R$ ) of green-over-red fluorescence  
179 was translated into a  $\text{Ca}^{2+}$  concentration through the following calculation (Yasuda et al.,  
180 2004).

181

$$[\text{Ca}^{2+}] = K_D \frac{(R - R_{min})}{(R_{max} - R)}$$

182 To avoid pipetting irregularities, which might influence the quantification of the  
183 fluorescence signals, pre-stocks of  $\text{Ca}^{2+}$ -sensitive and  $\text{Ca}^{2+}$ -insensitive indicators were  
184 used. For each pre-stock and each intracellular solution, 10 mM EGTA or 10 mM  $\text{CaCl}_2$   
185 were added to measure minimum ( $R_{min}$ ) and maximum ( $R_{max}$ ) fluorescence ratios,  
186 respectively. We performed these measurements in cMFBs and GCs as well as in  
187 cuvettes. Consistent with a previous report (Delvendahl et al., 2015), both  $R_{min}$  and  $R_{max}$   
188 were higher when measured in cells than in cuvettes (by a factor of  $1.73 \pm 0.05$ ;  $n = 83$   
189 and 63 measurements *in situ* and in cuvette; Fig. 3 – figure supplement 2A). The values  
190 in cMFBs and GCs were similar (Fig. 3 – figure supplement 2B). OGB-5N is not sensitive  
191 in detecting  $\text{Ca}^{2+}$  concentrations less than 1  $\mu\text{M}$ . Therefore, we deliberately adjusted  $R_{min}$   
192 of OGB-5N in the recordings where the pre-flash  $\text{Ca}^{2+}$  had negative values, to a value  
193 resulting in a pre-flash  $\text{Ca}^{2+}$  concentration of 60 nM, which corresponds to the average  
194 resting  $\text{Ca}^{2+}$  concentration in these boutons (Delvendahl et al., 2015). This adjustment of  
195  $R_{min}$  resulted in a reduction of post-flash  $\text{Ca}^{2+}$  amplitudes of on average  $7.5 \pm 0.4\%$  ( $n = 37$ ).  
196 Without this adjustment, the estimated  $K_D$  of the  $\text{Ca}^{2+}$  sensors for release would be  
197 even slightly higher.

198 The fluorescence properties of DMn change after flash photolysis, and the  $\text{Ca}^{2+}$  sensitive  
199 and insensitive dyes can differentially bleach during UV flash (Schneggenburger, 2005;

200 Zucker, 1992). We assumed no effect of the UV flash on the  $K_D$  of the  $\text{Ca}^{2+}$  sensitive dyes  
201 (Escobar et al., 1997), and measured  $R_{\min}$  and  $R_{\max}$  before and after the flash for each  
202 used UV flash intensity and duration in each of the three solutions (Supplementary  
203 Table 1; Schneggenburger et al., 2000). The flash-induced change was strongest for  $R_{\max}$   
204 of solutions with OGB-5N, but reached only ~20% with the strongest flashes (Fig. 3 –  
205 figure supplement 2C - 2F).

206 **Deconvolution**

207 Deconvolution of postsynaptic currents was performed essentially as described by  
208 Ritzau-Jost et al. (2014), based on routines developed by Neher and Sakaba (2001b).  
209 The principle of this method is that the EPSC comprises currents induced by synchronous  
210 release and residual glutamate in the synaptic cleft due to delayed glutamate clearance  
211 and glutamate spill-over from neighboring synapses. Kynurenic acid (2 mM) and  
212 Cyclothiazide (100  $\mu\text{M}$ ) were added to the extracellular solution to reduce postsynaptic  
213 receptor saturation and desensitization, respectively. The amplitude of the miniature  
214 EPSC (mEPSC) was set to the mean value of 10.1 pA ( $10.1 \pm 0.2 \text{ pA}$ ;  $n = 8$ ) as measured  
215 in 2 mM kynurenic acid and 100  $\mu\text{M}$  cyclothiazide.

216 The deconvolution kernel had the following free parameters: the mEPSC early slope  $\tau_0$ ,  
217 the fractional amplitude of the slow mEPSC decay phase  $\alpha$ , the time constant of the slow  
218 component of the decay  $\tau_2$  of the mEPSC, the residual current weighting factor  $\beta$ , and the  
219 diffusional coefficient  $d$ . Applying the “fitting protocol” described by Neher and Sakaba  
220 (2001b) before flash experiments might affect the number of vesicles released by  
221 subsequent  $\text{Ca}^{2+}$  uncaging. On the other hand, applying the “fitting protocol” after  $\text{Ca}^{2+}$   
222 uncaging might overestimate the measured number of vesicles due to flash-induced  
223 toxicity and synaptic fatigue especially when applying strong  $\text{Ca}^{2+}$  uncaging. Therefore,  
224 we used the experiments with weak and strong flashes to extract the mini-parameters  
225 and the parameters for the residual current, respectively, as described in the following in  
226 more detail. To obtain the mini parameters (early slope,  $\alpha$ , and  $\tau_2$ ) using weak flashes,  
227 deconvolution was first performed with a set of trial parameters for each cell pair. The  
228 mini-parameters of the deconvolution were optimized in each individual recording to yield

229 low (but non-negative) step-like elevations in the cumulative release corresponding to  
230 small EPSCs measured from the postsynaptic terminal (the parameters for the residual  
231 current had little impact on the early phase of the cumulative release rate within the first  
232 5 ms, therefore, some reasonable default values for the parameters of the residual current  
233 were used while iteratively adjusting the fast mini parameters for each individual  
234 recording). Next, using the average of the mini-parameters obtained from weak flashes,  
235 the deconvolution parameters for the residual current ( $\beta$  and  $d$ ) were optimized in each  
236 recording with strong flashes until no drops occurred in the cumulative release in the  
237 range of 5 – 50 ms after the stimulus (while iteratively readjusting the mini parameters, if  
238 needed, to avoid any drops in the cumulative release in the window of 5 - 10 ms that  
239 might arise when adjusting the slow parameters based on the cumulative release in the  
240 range of 5 – 50 ms). Finally, we averaged the values of each parameter and the  
241 deconvolution analysis of all recordings was re-done using the average parameters  
242 values. To test the validity of this approach, cumulative release from deconvolution of  
243 EPSCs and presynaptic capacitance recordings were compared in a subset of paired  
244 recordings (Ritzau-Jost et al., 2014). Exponential fits to the cumulative release and the  
245 presynaptic capacitance traces provided average time constants of  $2.43 \pm 0.81$  and  $2.65 \pm 0.88$  ms,  
246 respectively ( $n = 9$  pairs). On a paired-wise comparison, the difference in the  
247 time constant was always less than 40%. Therefore, both approaches yielded similar  
248 results.

249 To measure the number of GCs connected by one cMFB, we compared the product of  
250 the amplitude and the inverse of the time constant of the exponential fits of presynaptic  
251 capacitance trace and the simultaneously measured cumulative release trace obtained  
252 by deconvolution analysis of EPSC. Assuming a capacitance of 70 aF per vesicle  
253 (Hallermann 2003), we obtained an average value of 90.1 GCs per MFB in close  
254 agreement with previous estimates using a similar approach (Ritzau-Jost et al., 2014).  
255 This connectivity ratio is larger than previous estimates (~10, Billings et al., 2014; ~50,  
256 Jakab and Hamori, 1988) which could be due to a bias towards larger terminals, ectopic  
257 vesicle release, postsynaptic rundown, or release onto Golgi cells.

258 **Measurement of  $\text{Ca}^{2+}$  concentration using a  $\text{Ca}^{2+}$ -sensitive electrode**

259 A precise estimation of the binding affinity of the  $\text{Ca}^{2+}$  sensitive dyes is critical in  
260 translating the fluorescence signals into  $\text{Ca}^{2+}$  concentration. It has been reported that the  
261  $K_D$  of fluorescent indicators differs significantly depending on the solution in which it is  
262 measured (Tran et al., 2018) due to potential differences in ionic strength, pH, and  
263 concentration of other cations. Accordingly, different studies have reported different  
264 estimates of the  $K_D$  of OGB-5N having an up to 8-fold variability (Delvendahl et al., 2015;  
265 Digregorio and Vergara, 1997; Neef et al., 2018). In these studies, the estimation of the  
266  $K_D$  of the  $\text{Ca}^{2+}$  sensitive dyes depended on the estimated  $K_D$  of the used  $\text{Ca}^{2+}$  chelator,  
267 which differs based on the ionic strength, pH, and temperature of the solution used for  
268 calibration. So, we set out to measure the  $K_D$  of OGB-5N, in the exact solution and  
269 temperature which we used during patching, through direct potentiometry using an ion-  
270 selective electrode combined with two-photon  $\text{Ca}^{2+}$  imaging. An ion-selective electrode  
271 for  $\text{Ca}^{2+}$  ions provides a direct readout of the free  $\text{Ca}^{2+}$  concentration independent of the  
272  $K_D$  of the used  $\text{Ca}^{2+}$  chelator. Using the same intracellular solution and temperature as  
273 used during experiments, the potential difference between the  $\text{Ca}^{2+}$ -sensitive electrode  
274 (ELIT 8041 PVC membrane, NICO 2000) and a single junction silver chloride reference  
275 electrode (ELIT 001n, NICO 2000) was read out with a pH meter in mV mode. A series  
276 of standard solutions, with defined  $\text{Ca}^{2+}$  concentration (Thermo Fisher) covering the  
277 whole range of our samples, were used to plot a calibration curve of the potential (mV)  
278 versus  $\text{Ca}^{2+}$  concentration ( $\mu\text{M}$ ). Then, the potential of several sample solutions  
279 containing the same intracellular solution used for patching, but with different  $\text{Ca}^{2+}$   
280 concentrations buffered with EGTA, was determined. This way, we got a direct measure  
281 of the free  $\text{Ca}^{2+}$  concentration of several sample solutions, which were later used after  
282 the addition of  $\text{Ca}^{2+}$  sensitive fluorometric indicators to plot the fluorescence signal of  
283 each solution versus the corresponding free  $\text{Ca}^{2+}$  concentration verified by the  $\text{Ca}^{2+}$ -  
284 sensitive electrode, and accordingly the  $K_D$  of the  $\text{Ca}^{2+}$  indicators were obtained from fits  
285 with Hill equation. The estimated  $K_D$  was two-fold higher than the estimate obtained using  
286 only the  $\text{Ca}^{2+}$  Calibration Buffer Kit (Thermofischer) without including intracellular  
287 patching solution (Fig. 3 – figure supplement 1). Comparable results were obtained when  
288 estimating the free  $\text{Ca}^{2+}$  concentration using Maxchelator software

289 (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/). Therefore, we  
290 used two independent approaches to confirm the  $K_D$  of OGB-5N. We found that TEA  
291 increased the potential of the solutions measured through the  $\text{Ca}^{2+}$ -sensitive electrode,  
292 which is consistent with a previous report showing a similar effect of quaternary  
293 ammonium ions on potassium sensitive microelectrodes (Neher and Lux, 1973). We  
294 compared the fluorescence signals of our samples with or without TEA, to check if this  
295 effect of TEA is due to an interaction with the electrode or due to an effect on the free  
296  $\text{Ca}^{2+}$  concentration, and found no difference. Therefore, TEA had an effect on the  
297 electrode read-out without affecting the free  $\text{Ca}^{2+}$ , and accordingly, TEA was removed  
298 during the potentiometric measurements (Fig. 3 – figure supplement 1). This resulted in  
299 a good agreement of the estimates of the free  $\text{Ca}^{2+}$  concentration measured using a  
300  $\text{Ca}^{2+}$ -sensitive electrode and those calculated via Maxchelator.

301 **Assessment of the UV energy profile**

302 The homogeneity of the UV laser beam at the specimen plane was assessed *in vitro* by  
303 uncaging fluorescein (CMNB-caged fluorescein, Invitrogen). Caged fluorescein (2 mM)  
304 was mixed with glycerol (5% caged fluorescein/ 95% glycerol) to limit the mobility of the  
305 released dye (Bollmann et al., 2000). We did the measurements at the same plane as  
306 we put the slice during an experiment. The fluorescence profile of the dye after being  
307 released from the cage was measured at different z-positions over a range of 20  $\mu\text{m}$ .  
308 The intensity of fluorescein was homogenous over an area of 10  $\mu\text{m} \times 10 \mu\text{m}$  which  
309 encompasses the cMFB.

310 **Data analysis**

311 The increase in membrane capacitance and in cumulative release based on  
312 deconvolution analysis was fitted with the following single or bi-exponential functions  
313 using Igor Pro (WaveMetrics) including a baseline and a variable onset.

315

$$314 f_{mono}(t) = \begin{cases} 0 & \text{if } t < d, \\ a \left(1 - \exp\left[-\frac{(t-d)}{\tau}\right]\right) & \text{if } t \geq d \end{cases} \quad (\text{eq. 1})$$

322 
$$f_{bi}(t) = \begin{cases} 0 & \text{if } t < d, \\ a \left( 1 - a_1 \exp \left[ -\frac{(t-d)}{\tau_1} \right] - (1 - a_1) \exp \left[ -\frac{(t-d)}{\tau_2} \right] \right) & \text{if } t \geq d \end{cases}$$

323

316 where  $d$  defines the delay,  $a$  the amplitude,  $\tau$  the time constant of the mono-exponential  
317 fit,  $\tau_1$  and  $\tau_2$  the time constant of the fast and slow component of the bi-exponential fit,  
318 respectively, and  $a_1$  the relative contribution of the fast component of the bi-exponential  
319 fit. The fitting of the release traces was always done with a time window of 5 ms before  
320 and 10 ms after flash onset. If the time constant of the mono-exponential fit exceeded 10  
321 ms, a longer fitting duration of 60 ms after flash onset was used.

324 The acceptance of a bi-exponential fit was based on the fulfillment of the following three  
325 criteria: (1) at least 4% decrease in the sum of squared differences between the  
326 experimental trace and the fit compared with a mono-exponential fit ( $\chi^2_{\text{mono}}/\chi^2_{\text{bi}} > 1.04$ ),  
327 (2) the time constants of the fast and the slow components differed by a factor  $>3$ , and  
328 (3) the relative contribution of each component was  $>10\%$  (i.e.  $0.1 < a_1 < 0.9$ ). If any of  
329 these criteria was not met, a mono-exponential function was used instead. In the case of  
330 weak flashes, where we could observe single quantal events within the initial part of the  
331 EPSC, mono-exponential fits were applied. In Fig. 1, bi-exponential functions were used  
332 to fit the decay of the EPSC and the weighted time constants were used.

333 Hill equations were used to fit the release rate versus intracellular  $\text{Ca}^{2+}$  concentration on  
334 a double logarithmic plot according to the following equation:

335 
$$H(x) = \text{Log} \left[ V_{\text{max}} \frac{1}{1 + \left( \frac{K_D}{10^x} \right)^n} \right] \quad (\text{eq. 2})$$

336 where  $\text{Log}$  is the decadic logarithm,  $V_{\text{max}}$  the maximal release rate,  $K_D$  the  $\text{Ca}^{2+}$   
337 concentration at the half-maximal release rate, and  $n$  the Hill coefficient.  $H(x)$  was fit on  
338 the decadic logarithm of the release rates and  $x$  was the decadic logarithm of the  
339 intracellular  $\text{Ca}^{2+}$  concentration.

340 **Modeling of intra-bouton  $\text{Ca}^{2+}$  dynamics**

341 We simulated the intra-bouton  $\text{Ca}^{2+}$  dynamics using a single compartment model. The  
342 kinetic reaction schemes for  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  uncaging and -binding (Fig. 6A) were  
343 converted to a system of ordinary differential equations (ODEs) that was numerically  
344 solved using the NDSolve function in Mathematica 12 (Wolfram) as described previously  
345 (Bornstein et al., 2019). The initial conditions for the uncaging simulation were derived  
346 by first solving the system of ODEs for the steady state using total concentrations of all  
347 species and the experimentally determined  $[\text{Ca}^{2+}]_{\text{rest}}$  as starting values. Subsequently,  
348 the values obtained for all free and bound species were used as initial conditions for the  
349 uncaging simulation. The kinetic properties of DMn were simulated according to Faas et  
350 al. (2005, 2007). The total DMn concentration ( $[\text{DMn}]_T$ ) includes the free form ( $[\text{DMn}]_f$ ),  
351 the  $\text{Ca}^{2+}$  bound form ( $[\text{CaDMn}]_f$ ), and the  $\text{Mg}^{2+}$  bound form ( $[\text{MgDMn}]_f$ ). Each of these  
352 forms is subdivided into an uncaging fraction ( $\alpha$ ) and a non-uncaging fraction ( $1-\alpha$ ). The  
353 uncaging fraction were further subdivided into a fast (af) and a slow (1-af) uncaging  
354 fraction:

355  $[\text{DMn}]_T = [\text{DMn}]_f + [\text{DMn}]_s + [\text{CaDMn}]_f + [\text{CaDMn}]_s + [\text{MgDMn}]_f + [\text{MgDMn}]_s$

356  $[\text{DMn}] = [\text{DMn}]_f + [\text{DMn}]_s$

357  $[\text{DMn}]_f = \alpha \text{ af} [\text{DMn}]$

358  $[\text{DMn}]_s = \alpha (1-\text{af}) [\text{DMn}]$

359  $[\text{CaDMn}] = [\text{CaDMn}]_f + [\text{CaDMn}]_s$

360  $[\text{CaDMn}]_f = \alpha \text{ af} [\text{CaDMn}]$

361  $[\text{CaDMn}]_s = \alpha (1-\text{af}) [\text{CaDMn}]$

362  $[\text{MgDMn}] = [\text{MgDMn}]_f + [\text{MgDMn}]_s$

363  $[\text{MgDMn}]_f = \alpha \text{ af} [\text{MgDMn}]$

364  $[\text{MgDMn}]_s = \alpha (1-\text{af}) [\text{MgDMn}]$

365 The suffixes “T”, “f”, and “s” indicate total, fast or slow, respectively. The transition of fast  
366 and slow uncaging fractions into low-affinity photoproducts (PP) occurred with fast ( $\tau_f$ ) or

367 slow ( $\tau_s$ ) time constants, respectively. Free  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ -bound DMn decomposed into  
368 two photoproducts (PP1, PP2) differing with respect to their binding kinetics. The binding  
369 kinetics of all species were governed by the corresponding forward ( $k_{on}$ ) and backward  
370 ( $k_{off}$ ) rate constants

371

$$372 \frac{d[\text{CaDMn}]}{dt}_x = k_{on}[\text{Ca}][\text{DMn}]_x - k_{off}[\text{CaDMn}]_x - \frac{[\text{CaDMn}]_x}{\tau_x} H(t - t_{\text{flash}}) \quad x = f, s$$

373

$$374 \frac{d[\text{MgDMn}]}{dt}_x = k_{on}[\text{Mg}][\text{DMn}]_x - k_{off}[\text{MgDMn}]_x - \frac{[\text{MgDMn}]_x}{\tau_x} H(t - t_{\text{flash}}) \quad x = f, s$$

375

$$376 \frac{d[\text{DMn}]}{dt}_x = -k_{on}[\text{Ca}][\text{DMn}]_x + k_{off}[\text{CaDMn}]_x - k_{on}[\text{Mg}][\text{DMn}]_x + k_{off}[\text{MgDMn}]_x \\ 377 - \frac{[\text{DMn}]_x}{\tau_x} H(t - t_{\text{flash}}) \quad x = f, s$$

378

$$379 \frac{d[\text{CaPP1}]}{dt} = k_{on}[\text{Ca}][\text{PP1}] - k_{off}[\text{CaPP1}] \\ 380 + \frac{[\text{CaDMn}]_f}{\tau_f} H(t - t_{\text{flash}}) + \frac{[\text{CaDMn}]_s}{\tau_s} H(t - t_{\text{flash}})$$

381

$$382 \frac{d[\text{MgPP1}]}{dt} = k_{on}[\text{Mg}][\text{PP1}] - k_{off}[\text{MgPP1}]$$

383

$$384 \frac{d[\text{PP1}]}{dt} = -k_{on}[\text{Ca}][\text{PP1}] + k_{off}[\text{CaPP1}] - k_{on}[\text{Mg}][\text{PP1}] + k_{off}[\text{MgPP1}] \\ 385 + \frac{[\text{CaDMn}]_f}{\tau_f} H(t - t_{\text{flash}}) + \frac{[\text{CaDMn}]_s}{\tau_s} H(t - t_{\text{flash}})$$

386

$$387 \frac{d[\text{CaPP2}]}{dt} = k_{on}[\text{Ca}][\text{PP2}] - k_{off}[\text{CaPP2}]$$

388

$$389 \frac{d[\text{MgPP2}]}{dt} = k_{on}[\text{Mg}][\text{PP2}] - k_{off}[\text{MgPP2}] \\ 390 + \frac{[\text{MgDMn}]_f}{\tau_f} H(t - t_{\text{flash}}) + \frac{[\text{MgDMn}]_s}{\tau_s} H(t - t_{\text{flash}})$$

391

$$392 \frac{d[\text{PP2}]}{dt} = -k_{on}[\text{Ca}][\text{PP2}] + k_{off}[\text{CaPP2}] - k_{on}[\text{Mg}][\text{PP2}] + k_{off}[\text{MgPP2}]$$

$$393 \quad +2 \frac{[DMn]_f}{\tau_f} H(t - t_{\text{flash}}) + \frac{[DMn]_s}{\tau_s} H(t - t_{\text{flash}})$$

$$394 \quad + \frac{[MgDMn]_f}{\tau_f} H(t - t_{\text{flash}}) + \frac{[MgDMn]_s}{\tau_s} H(t - t_{\text{flash}})$$

395 where  $H$  is the Heaviside step function and  $t_{\text{flash}}$  the time of the UV flash.  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$   
 396 binding to the dye, ATP, and an endogenous buffer (EB) were simulated by second order  
 397 kinetics:

398

$$399 \quad \frac{d[Ca]}{dt}_{\text{buffer}} = -k_{on,j}[Ca][B] + k_{off,j}[CaB] \quad j = \text{dye, ATP, EB}$$

$$400 \quad \frac{d[Mg]}{dt} = -k_{on,j}[Mg][B] + k_{off,j}[MgB] \quad j = \text{ATP}$$

$$401 \quad \frac{d[B]}{dt} = -\frac{d[CaB]}{dt} - \frac{d[MgB]}{dt} \quad B = \text{dye, ATP, EB}$$

402 The time course of the total change  $\text{Ca}^{2+}$  concentration or  $\text{Mg}^{2+}$  concentration is given by  
 403 the sum of all the above equations involving changes in  $\text{Ca}^{2+}$  concentration or  $\text{Mg}^{2+}$   
 404 concentration, respectively.  $\text{Ca}^{2+}$  concentration as reported by the dye was calculated  
 405 from the concentration of the  $\text{Ca}^{2+}$ -dye complex assuming equilibrium conditions  
 406 (Markram et al., 1998). The clearing of  $\text{Ca}^{2+}$  from the cytosol was not implemented in  
 407 these simulations. Instead, the  $\text{Ca}^{2+}$  concentration was simulated only for 10 ms after the  
 408 flash. The experimentally observed subsequent decay of the  $\text{Ca}^{2+}$  concentration was  
 409 implemented by an exponential decay to the resting  $\text{Ca}^{2+}$  concentration with a time  
 410 constant of 400 ms. The parameters of the model are given in Supplementary Table 2.

Parameters		Values	References number / Notes
Resting $\text{Ca}^{2+}$	$[\text{Ca}^{2+}]_{\text{rest}}$	$227 * 10^{-9} \text{ M}$	Measured
Total magnesium	$[\text{Mg}^{2+}]_T$	$0.5 * 10^{-3} \text{ M}$	Pipette concentration
Fluo-5F	$[\text{Fluo}]$	0 or $50 * 10^{-6} \text{ M}$ (see Supplementary Table 1)	Pipette concentration
	$K_D$	$0.83 * 10^{-6} \text{ M}$	(Delvendahl et al., 2015)

	$k_{off}$	249 s <sup>-1</sup>	ibid
	$k_{on}$	$3*10^8$ M <sup>-1</sup> s <sup>-1</sup>	(Yasuda et al., 2004)
OGB-5N	[OGB]	0 or $200*10^{-6}$ M (see Supplementary Table 1)	Pipette concentration
	$K_D$	$24*10^{-6}$ M	(Delvendahl et al., 2015)
	$k_{off}$	6000 s <sup>-1</sup>	ibid.
	$k_{on}$	$2.5*10^8$ M <sup>-1</sup> s <sup>-1</sup>	(Digregorio and Vergara, 1997)
ATP	[ATP]	$5 *10^{-3}$ M	Pipette concentration
Ca <sup>2+</sup> binding	$K_D$	$2*10^{-4}$ M	(Meinrenken et al., 2002)
	$k_{off}$	100 000 s <sup>-1</sup>	ibid.
	$k_{on}$	$5*10^8$ M <sup>-1</sup> s <sup>-1</sup>	ibid.
Mg <sup>2+</sup> binding	$K_D$	$100*10^{-6}$ M	(Bollmann et al., 2000); MaxC
	$k_{off}$	1000 s <sup>-1</sup>	ibid.
	$k_{on}$	$1*10^7$ M <sup>-1</sup> s <sup>-1</sup>	ibid.
Endogenous buffer	[EB]	$480 *10^{-6}$ M	(Delvendahl et al., 2015)
	$K_D$	$32*10^{-6}$ M	ibid
	$k_{off}$	16 000 s <sup>-1</sup>	ibid.
	$k_{on}$	$5*10^8$ M <sup>-1</sup> s <sup>-1</sup>	ibid.
Total DM nitrophen	[DMn] <sub>T</sub>	$500*10^{-6} - 10*10^{-3}$ M (see Supplementary Table 1)	Pipette concentration
Ca <sup>2+</sup> binding	$K_D$	$6.5*10^{-9}$ M	(Faas et al., 2005)
	$k_{off}$	0.19 s <sup>-1</sup>	ibid.
	$k_{on}$	$2.9*10^7$ M <sup>-1</sup> s <sup>-1</sup>	ibid.
Mg <sup>2+</sup> binding	$K_D$	$1.5*10^{-6}$ M	ibid.
	$k_{off}$	0.2 s <sup>-1</sup>	ibid.
Uncaging fraction	$\alpha$	See Supplementary Table 1	
Fast uncaging fraction	af	0.67	(Faas et al., 2005)
Photoproduct 1	[PP1]		
Ca <sup>2+</sup> binding	$K_D$	$2.38*10^{-3}$ M	(Faas et al., 2005)
	$k_{off}$	69 000 s <sup>-1</sup>	ibid.
	$k_{on}$	$2.9*10^7$ M <sup>-1</sup> s <sup>-1</sup>	ibid.
Mg <sup>2+</sup> binding	$K_D$	$1.5*10^{-6}$ M	ibid.
	$k_{off}$	300 s <sup>-1</sup>	ibid.
	$k_{on}$	$1.3*10^5$ M <sup>-1</sup> s <sup>-1</sup>	ibid.
Photoproduct 2	[PP2]		

Ca <sup>2+</sup> binding	$K_D$	124.1*10 <sup>-6</sup> M	Ibid.
	$k_{off}$	3600 s <sup>-1</sup>	ibid.
	$k_{on}$	2.9*10 <sup>7</sup> M <sup>-1</sup> s <sup>-1</sup>	ibid.
Mg <sup>2+</sup> binding	$K_D$	1.5*10 <sup>-6</sup> M	ibid.
	$k_{off}$	300 s <sup>-1</sup>	ibid.
	$k_{on}$	1.3*10 <sup>5</sup> M <sup>-1</sup> s <sup>-1</sup>	ibid.

411 **Supplementary Table 2 Parameters for simulations of Ca<sup>2+</sup> release from DMN cage**

412 These simulations were used to obtain Ca<sup>2+</sup> transients with peak amplitudes covering the  
 413 entire range of post-flash Ca<sup>2+</sup> concentrations. To this end, the uncaging efficiency  $\alpha$  was  
 414 varied in each of the three experimentally used combinations of concentrations of DMN  
 415 and Ca<sup>2+</sup> indicators (see Supplementary Table 1 for details).

416 **Modeling of release schemes**

417 Model 1 with two Ca<sup>2+</sup> binding steps mediating fusion and one Ca<sup>2+</sup>-dependent priming  
 418 step was defined according to the following differential equation

$$419 \begin{pmatrix} dV_{0Ca}(t)/dt \\ dV_{1Ca}(t)/dt \\ dV_{2Ca}(t)/dt \\ dV_{fused}(t)/dt \end{pmatrix} = M \begin{pmatrix} V_{0Ca}(t) \\ V_{1Ca}(t) \\ V_{2Ca}(t) \\ V_{fused}(t) \end{pmatrix}$$

420  $V_{0Ca}$ ,  $V_{1Ca}$ , and  $V_{2Ca}$  denote the fraction of vesicles with a fusion sensor with 0 to 2 bound  
 421 Ca<sup>2+</sup> ions, respectively, and  $V_{fused}$  denotes the fused vesicles as illustrated in Fig. 6D.  
 422 The reserve pool  $V_R$  is considered to be infinite.  $M$  denotes the following 4x4 matrix:

$-2k_{on} - k_{unprim} + k_{prim}/V_{0Ca}(t)$	$k_{off}$	0	0
$2k_{on}$	$-k_{off} - k_{on}$	$2k_{off}b$	0
0	$k_{on}$	$-\gamma - 2k_{off}b$	0
0	0	$\gamma$	0

423  
 424 See Supplementary Table 3 for the values and Ca<sup>2+</sup>-dependence of the rate constants  
 425 in the matrix.

426 The initial condition was defined as  $V_{0Ca}(0) = k_{prim}/k_{unprim}$  and  $V_{1Ca}(0)$ ,  $V_{2Ca}(0)$ , and  
 427  $V_{fused}(0)$  was zero.  $k_{prim}$  was the sum of a Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent rate

428 constants. The  $\text{Ca}^{2+}$ -dependence was implemented as a Michaelis-Menten kinetic with  
429 a maximum rate constant of  $30 \text{ s}^{-1}$  (Ritzau-Jost et al., 2014) and a  $K_D$  of  $2 \mu\text{M}$  (Miki et al.,  
430 2018). The  $\text{Ca}^{2+}$ -independent rate constant was  $0.6 \text{ s}^{-1}$ , adjusted to reproduce the factor  
431 of 3 upon elevating  $\text{Ca}^{2+}$  from 30 to 180 nM (cf. Fig. 1D and 7D).  $k_{unprim}$  was defined such  
432 that the occupancy  $V_{0Ca}(0) = 1$  for the default pre-flash resting  $\text{Ca}^{2+}$  concentration of 227  
433 nM (Supplementary Tables 2 and 3).

434 The differential equations were solved with the NDSolve function of Mathematica. The  
435  $\text{Ca}^{2+}$  concentration,  $\text{Ca}^{2+}(t)$ , was obtained from the simulations as described in the  
436 previous paragraph.  $V_{fused}(t)$  represents the cumulative release normalized to the pool  
437 of release-ready vesicles per cMFB to GC connection. To reproduce the absolute  
438 sustained release rate (Figs. 5 and 6D),  $V_{fused}(t)$  was multiplied by a pool of release-  
439 ready vesicles per connection of 10 vesicles. The cumulative release,  $V_{fused}(t)$ , including  
440 a pre-flash baseline was sampled with 5 or 10 kHz. Realistic noise for 5- or 10 kHz-  
441 capacitance or deconvolution measurements was added and the data, in the 10 ms-  
442 window after the flash, were fit with mono- and bi- exponential functions (eq. 1). The  
443 selection of a bi- over a mono-exponential fit was based on identical criteria as in the  
444 analysis of the experimental data including the prolongation of the fitting duration from  
445 10 to 60 ms if the time constant of the mono-exponential fit was  $>10$  ms (see section  
446 Data analysis). For each peak post-flash  $\text{Ca}^{2+}$  concentration (i.e. simulated  $\text{Ca}^{2+}(t)$   
447 transient) the sampling, addition of noise, and exponential fitting were repeated 50 times.  
448 The median of these values represents the prediction of the model for each peak post  
449 flash  $\text{Ca}^{2+}$  concentration. The parameters of the models were manually adjusted to obtain  
450 best-fit results.

451 Model 2 was a sequential two-pool model based on Miki et al. (2018) with five  $\text{Ca}^{2+}$   
452 binding steps mediating fusion and two  $\text{Ca}^{2+}$ -dependent priming steps defined according  
453 to the following differential equations

454

$$\begin{pmatrix} dV_{2,0Ca}(t)/dt \\ dV_{2,1Ca}(t)/dt \\ dV_{2,2Ca}(t)/dt \\ dV_{2,3Ca}(t)/dt \\ dV_{2,4Ca}(t)/dt \\ dV_{2,5Ca}(t)/dt \\ dV_{2,fused}(t)/dt \end{pmatrix} = M \begin{pmatrix} V_{2,0Ca}(t) \\ V_{2,1Ca}(t) \\ V_{2,2Ca}(t) \\ V_{2,3Ca}(t) \\ V_{2,4Ca}(t) \\ V_{2,5Ca}(t) \\ V_{2,fused}(t) \end{pmatrix}$$

455

456  $V_{2,0Ca}$ ,  $V_{2,1Ca}$ , ..., and  $V_{2,5Ca}$  denote the fraction of vesicles with a fusion sensor with 0 to  
 457 5 bound  $\text{Ca}^{2+}$  ions, respectively, and  $V_{2,fused}$  denotes fused vesicles as illustrated in Fig.  
 458 6D. The fraction of vesicles in state  $V_1$  is calculated according to the following differential  
 459 equation

460

$$\frac{dV_1(t)}{dt} = k_{prim1} - k_{unprim1} V_1(t) - k_{prim2} V_1(t) + k_{unprim2} V_{2,0Ca}(t)$$

461  $M$  denotes the following 7x7 matrix:

$-5k_{on} - k_{unprim2}$ $+k_{prim2}V_1(t)/V_{2,0Ca}(t)$	$k_{off}$	0	0	0	0	0
$5k_{on}$	$-k_{off} - 4k_{on}$	$2k_{off}b$	0	0	0	0
0	$4k_{on}$	$-2k_{off}b - 3k_{on}$	$3k_{off}b^2$	0	0	0
0	0	$3k_{on}$	$-3k_{off}b^2 - 2k_{on}$	$4k_{off}b^3$	0	0
0	0	0	$2k_{on}$	$-4k_{off}b^3 - k_{on}$	$5k_{off}b^4$	0
0	0	0	0	$k_{on}$	$-\gamma - 5k_{off}b^4$	0
0	0	0	0	0	$\gamma$	0

462

463 To implement the use-dependent slowing of the release rate constants of this model (Miki  
 464 et al., 2018) in a deterministic way, a site-plugging state,  $P(t)$ , was defined according to

465 
$$\frac{dP(t)}{dt} = (1 - P(t)) \frac{dV_{2,fused}}{dt}(t) - 40\text{ms} P(t) \quad (\text{eq.3})$$

466  $P(t)$  is approaching 1 during strong release and decays with a time constant of 40 ms  
 467 back to zero. Similar to the implementation by Miki et al. (2018), the rate constants  $k_{on}$   
 468 and  $k_{off}$  were linearly interpolated between two values depending on  $P(t)$  as

469 
$$k_{on}(t) = k_{on,init} + (k_{on,plugged} - k_{on,init}) P(t) \quad (\text{eq. 4})$$

470  $k_{off}(t) = k_{off,init} + (k_{off,plugged} - k_{off,init}) P(t)$

471 The reserve pool  $V_R$  is considered to be infinite. See Supplementary Table 3 for the  
472 values and  $\text{Ca}^{2+}$ -dependence of the rate constants in these differential equations.

473 The initial condition is defined as  $V_1(0) = k_{prim1}/k_{unprim1}$  and  $V_{2,0Ca}(0) =$   
474  $(k_{prim1}/k_{unprim1}) * (k_{prim2}/k_{unprim2})$ . The initial condition of the other state  $V_{2,1Ca}(0)$  to  $V_{5,0Ca}(0)$ ,  
475  $V_{fused}(0)$ , and  $P(0)$  were zero.  $k_{prim1}$  and  $k_{prim2}$  were the sum of a  $\text{Ca}^{2+}$ -dependent and  
476  $\text{Ca}^{2+}$ -independent rate constant defined similarly as described in Miki et al. (2018) and  
477 adjusted as described for model 1.  $k_{unprim1}$  and  $k_{unprim2}$  were defined such that the  
478 occupancy  $V_1(0) = 1$  and  $V_{2,0Ca}(0) = 1$  for the default pre-flash resting  $\text{Ca}^{2+}$  concentration  
479 of 227 nM (Supplementary Tables 2 and 3).

480 Model 3 was a parallel two-pool model similar as described by Voets (2000) and Walter  
481 et al (2013) but with five  $\text{Ca}^{2+}$  binding steps mediating fusion of both types of vesicles  
482 and a  $\text{Ca}^{2+}$ -independent priming step for  $V_1$  vesicles and a  $\text{Ca}^{2+}$ -dependent transition  
483 step from  $V_1$  to  $V_2$  vesicles defined according to the following differential equations

484 
$$\begin{pmatrix} dV_{1,0Ca}(t)/dt \\ dV_{1,1Ca}(t)/dt \\ dV_{1,2Ca}(t)/dt \\ dV_{1,3Ca}(t)/dt \\ dV_{1,4Ca}(t)/dt \\ dV_{1,5Ca}(t)/dt \\ dV_{1,fused}(t)/dt \end{pmatrix} = M_1 \begin{pmatrix} V_{1,0Ca}(t) \\ V_{1,1Ca}(t) \\ V_{1,2Ca}(t) \\ V_{1,3Ca}(t) \\ V_{1,4Ca}(t) \\ V_{1,5Ca}(t) \\ V_{1,fused}(t) \end{pmatrix}$$

485 
$$\begin{pmatrix} dV_{2,0Ca}(t)/dt \\ dV_{2,1Ca}(t)/dt \\ dV_{2,2Ca}(t)/dt \\ dV_{2,3Ca}(t)/dt \\ dV_{2,4Ca}(t)/dt \\ dV_{2,5Ca}(t)/dt \\ dV_{2,fused}(t)/dt \end{pmatrix} = M_2 \begin{pmatrix} V_{2,0Ca}(t) \\ V_{2,1Ca}(t) \\ V_{2,2Ca}(t) \\ V_{2,3Ca}(t) \\ V_{2,4Ca}(t) \\ V_{2,5Ca}(t) \\ V_{2,fused}(t) \end{pmatrix}$$

486  $V_{1,0Ca}$ ,  $V_{1,1Ca}$ , ..., and  $V_{1,5Ca}$  denote the fraction of vesicles with a low-affinity fusion sensor  
487 with 0 to 5 bound  $\text{Ca}^{2+}$  ions, respectively, and  $V_{2,0Ca}$ ,  $V_{2,1Ca}$ , ..., and  $V_{2,5Ca}$  denote the

488 fraction of vesicles with a high-affinity fusion sensor with 0 to 5 bound  $\text{Ca}^{2+}$  ions,  
 489 respectively.  $V_{1,fused}$  and  $V_{2,fused}$  denote fused vesicles as illustrated in Fig. 6D.

490  $M_1$  denotes the following 7x7 matrix:

$-5k_{on1} - k_{unprim1} - k_{prim2}$ $+k_{prim1}/V_{1,0Ca}(t)$ $+k_{unprim2}V_{2,0Ca}(t)/$ $V_{1,0Ca}(t)$	$k_{off1}$	0	0	0	0	0
$5k_{on1}$	$-k_{off1} - 4k_{on1}$	$2k_{off1}b$	0	0	0	0
0	$4k_{on1}$	$-2k_{off1}b - 3k_{on1}$	$3k_{off1}b^2$	0	0	0
0	0	$3k_{on1}$	$-3k_{off1}b^2 - 2k_{on1}$	$4k_{off1}b^3$	0	0
0	0	0	$2k_{on1}$	$-4k_{off1}b^3 - k_{on1}$	$5k_{off1}b^4$	0
0	0	0	0	$k_{on1}$	$-\gamma - 5k_{off1}b^4$	0
0	0	0	0	0	$\gamma$	0

491  $M_2$  denotes the following 7x7 matrix:

$-5k_{on2} - k_{unprim2}$ $+k_{prim2}V_{1,0Ca}(t)/$ $V_{2,0Ca}(t)$	$k_{off2}$	0	0	0	0	0
$5k_{on2}$	$-k_{off2} - 4k_{on2}$	$2k_{off2}b$	0	0	0	0
0	$4k_{on2}$	$-2k_{off2}b - 3k_{on2}$	$3k_{off2}b^2$	0	0	0
0	0	$3k_{on2}$	$-3k_{off2}b^2 - 2k_{on2}$	$4k_{off2}b^3$	0	0
0	0	0	$2k_{on2}$	$-4k_{off2}b^3 - k_{on2}$	$5k_{off2}b^4$	0
0	0	0	0	$k_{on2}$	$-\gamma - 5k_{off2}b^4$	0
0	0	0	0	0	$\gamma$	0

492 The initial condition is defined as  $V_{2,0Ca}(0) = k_{prim1}/k_{unprim1}$  and  $V_{2,0Ca}(0) =$   
 493  $(k_{prim1}/k_{unprim1}) * (k_{prim2}/k_{unprim2})$ . The initial condition of the other state  $V_{1,1Ca}(0)$  to  $V_{1,0Ca}(0)$ ,  
 494  $V_{1,fused}(0)$ , and  $V_{2,1Ca}(0)$  to  $V_{2,0Ca}(0)$ ,  $V_{2,fused}(0)$  were zero.  $k_{prim1}$  was a  $\text{Ca}^{2+}$ -independent  
 495 rate constant and  $k_{prim2}$  was the sum of a  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent rate  
 496 constants defined similarly as described in Hallermann et al. (2010) and adjusted as  
 497 described for model 1.  $k_{unprim1}$  and  $k_{unprim2}$  were defined such that the occupancy  $V_{1,0Ca}(0)$

498 = 1 and  $V_{2,0Ca}(0) = 1$  for the default pre-flash resting  $\text{Ca}^{2+}$  concentration of 227 nM  
499 (Supplementary Tables 2 and 3).

500

Model1		Model2		Model3	
$k_{\text{on}}$	$2.95 \times 10^9 \text{ Ca}^{2+}(t) \text{ M}^{-1} \text{ s}^{-1}$	$k_{\text{on,init}}$	$5.10 \times 10^8 \text{ Ca}^{2+}(t) \text{ M}^{-1} \text{ s}^{-1}$	$k_{\text{on1}}$	$0.5 k_{\text{on2}}$
		$k_{\text{on,plug}}$	$0.1 k_{\text{on,init}}$	$k_{\text{on2}}$	$5.10 \times 10^8 \text{ Ca}^{2+}(t) \text{ M}^{-1} \text{ s}^{-1}$
$k_{\text{off}}$	$4.42 \times 10^5 \text{ s}^{-1}$	$k_{\text{off,init}}$	$2.55 \times 10^4 \text{ s}^{-1}$	$k_{\text{off1}}$	$10 k_{\text{off2}}$
		$k_{\text{off,plug}}$	$0.4 k_{\text{off,init}}$	$k_{\text{off2}}$	$2.55 \times 10^4 \text{ s}^{-1}$
$b$	0.25	$b$	0.25	$b$	0.25
$\gamma$	$1.77 \times 10^4 \text{ s}^{-1}$	$\gamma$	$1.77 \times 10^4 \text{ s}^{-1}$	$\gamma$	$1.77 \times 10^4 \text{ s}^{-1}$
$k_{\text{prim}}$	$0.6 + 30 * (\text{Ca}^{2+}(t) / (K_{d,\text{prim}} + \text{Ca}^{2+}(t))) \text{ s}^{-1}$	$k_{\text{prim1}}$	$2.5 + 60 * (\text{Ca}^{2+}(t) / (K_{d,\text{prim1}} + \text{Ca}^{2+}(t))) \text{ s}^{-1}$	$k_{\text{prim1}}$	$30 \text{ s}^{-1}$
$k_{\text{unprim}}$	$0.6 + 30 * (\text{Ca}^{2+}_{\text{Rest}} / (K_{d,\text{prim}} + \text{Ca}^{2+}_{\text{Rest}})) \text{ s}^{-1}$	$k_{\text{unprim1}}$	$2.5 + 60 * (\text{Ca}^{2+}_{\text{Rest}} / (K_{d,\text{prim1}} + \text{Ca}^{2+}_{\text{Rest}})) \text{ s}^{-1}$	$k_{\text{unprim1}}$	$30 \text{ s}^{-1}$
$K_{d,\text{prim}}$	2 $\mu\text{M}$	$K_{d,\text{prim1}}$	2 $\mu\text{M}$		
		$k_{\text{prim2}}$	$100 + 800 * (\text{Ca}^{2+}(t) / (K_{d,\text{prim2}} + \text{Ca}^{2+}(t))) \text{ s}^{-1}$	$k_{\text{prim2}}$	$0.5 + 30 * (\text{Ca}^{2+}(t) / (K_{d,\text{prim2}} + \text{Ca}^{2+}(t))) \text{ s}^{-1}$
		$k_{\text{unprim2}}$	$100 + 800 * (\text{Ca}^{2+}_{\text{Rest}} / (K_{d,\text{prim2}} + \text{Ca}^{2+}_{\text{Rest}})) \text{ s}^{-1}$	$k_{\text{unprim2}}$	$0.5 + 30 * (\text{Ca}^{2+}_{\text{Rest}} / (K_{d,\text{prim2}} + \text{Ca}^{2+}_{\text{Rest}})) \text{ s}^{-1}$
		$K_{d,\text{prim2}}$	2 $\mu\text{M}$	$K_{d,\text{prim2}}$	2 $\mu\text{M}$

501 **Supplementary Table 3 Parameters for release scheme models**

502 **Statistical analysis**

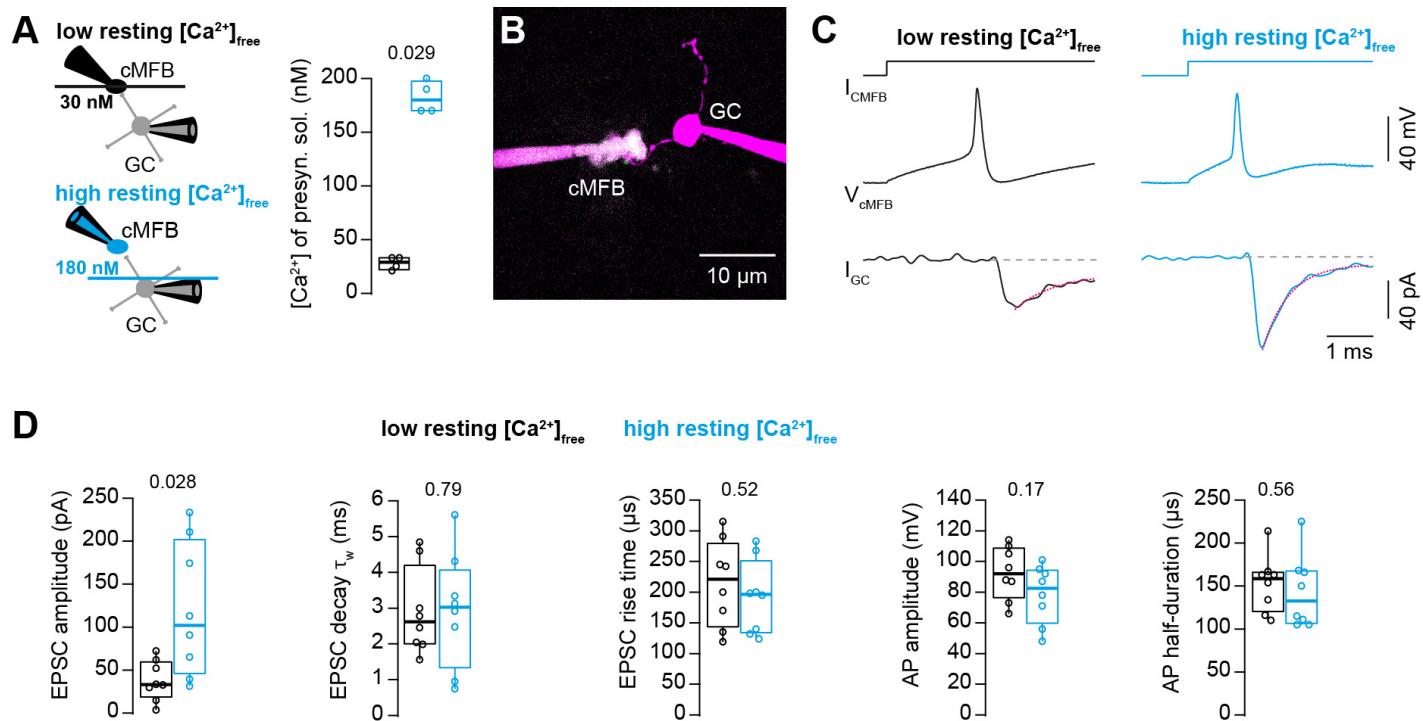
503 Boxplots show median and 1st/3rd quartiles with whiskers indicating the whole data  
504 range (Figs. 1 and 7). For statistical comparison, Mann-Whitney U tests were used, and  
505 the P-values are indicated above the boxplots.

506 **Results**

507 **Action potential-evoked synaptic release critically depends on basal intracellular**

508 **Ca<sup>2+</sup> concentration**

509 To investigate the impact of the basal intracellular Ca<sup>2+</sup> concentration on synaptic  
510 release, we performed simultaneous patch-clamp recordings from presynaptic cerebellar  
511 mossy fiber boutons (cMFB) and postsynaptic granule cells (GC) of 5- to 6-weeks old  
512 mice at physiological temperatures (Fig. 1A and B). We aimed at clamping the free Ca<sup>2+</sup>  
513 concentration in the presynaptic patch solution to either low or high basal Ca<sup>2+</sup>  
514 concentrations by adding different concentrations of Ca<sup>2+</sup> and the Ca<sup>2+</sup> chelator EGTA  
515 (see methods). Two-photon quantitative Ca<sup>2+</sup> imaging with the dual-indicator method  
516 using Fluo-5F as the Ca<sup>2+</sup> indicator (Delvendahl et al., 2015; Sabatini et al., 2002)  
517 revealed the free Ca<sup>2+</sup> concentration of the presynaptic intracellular solution to be 28 ± 3  
518 and 183 ± 8 nM, for the low and high basal Ca<sup>2+</sup> conditions (n = 4 and 4), respectively  
519 (Fig. 1A). In both solutions, the free EGTA concentration was 4.47 mM (see methods).  
520 In response to triggering a single action potential in the presynaptic terminal, the  
521 recorded excitatory postsynaptic current (EPSC) depended strongly on the presynaptic  
522 resting Ca<sup>2+</sup> concentration (Fig. 1C). We found an almost three-fold increase in the EPSC  
523 amplitude when elevating the resting Ca<sup>2+</sup> concentration in the presynaptic terminals  
524 from 30 to 180 nM. On average, the EPSC amplitudes were 39 ± 8 and 117 ± 27 pA for  
525 the low and high basal Ca<sup>2+</sup> conditions, respectively (n = 8 and 8; P<sub>Mann-Whitney</sub> = 0.028;  
526 Fig. 1D). The EPSC rise and decay kinetics were not significantly different (Fig. 1D). No  
527 significant differences were observed in the action potential waveform including  
528 amplitude and half duration (Fig. 1D) indicating that the altered synaptic strength was not  
529 caused by changes in the shape of the presynaptic action potential. These data indicate  
530 that moderate changes in the presynaptic basal Ca<sup>2+</sup> concentration can alter synaptic  
531 strength up to three-fold.



532 **Figure 1 Action potential-evoked synaptic release critically depends on basal intracellular  $\text{Ca}^{2+}$   
533 concentration**

534 A. *Left:* Illustration of the cellular connectivity of the cMFB to GC synapse during simultaneous pre- and  
535 postsynaptic patch-clamp recording. The presynaptic terminal was loaded with an intracellular solution  
536 having either low or high free basal  $\text{Ca}^{2+}$  concentration (top and bottom, respectively). *Right:* Comparison  
537 of the average free  $\text{Ca}^{2+}$  concentration in the presynaptic patch pipette (quantified by two-photon  $\text{Ca}^{2+}$   
538 imaging) for the intracellular solutions with low and high basal  $\text{Ca}^{2+}$  ( $n = 4$  each).

539 B. Example two-photon microscopic image of a cMFB and a GC in the paired whole-cell configuration.

540 C. Example traces of a paired cMFB-GC recording with current injection ( $I_{\text{cMFB}}$ ) (*top*) eliciting an action  
541 potential in the cMFB (*middle*) and an EPSC in the postsynaptic GC (*bottom*). Black and blue color code  
542 corresponds to low and high free basal  $\text{Ca}^{2+}$  concentration in the presynaptic solution, respectively. The  
543 decay of the EPSC was fitted with a bi-exponential function (magenta line).

544 D. Comparison of the properties of presynaptic action potentials and EPSCs evoked after eliciting an  
545 action potential in the presynaptic terminal using solutions having either low (black) or high (blue) free  
546  $\text{Ca}^{2+}$  concentration. From left to right: peak amplitude of the EPSC, weighted decay time constant of the  
547 EPSC, 10-to-90% rise time of the EPSC, amplitude of the presynaptic action potential, and action  
548 potential half-duration ( $n = 8$  and 8 pairs for the conditions with low and high resting  $\text{Ca}^{2+}$  concentration,  
549 respectively).

550 Boxplots show median and 1<sup>st</sup>/3<sup>rd</sup> quartiles with whiskers indicating the whole data range. Values of  
551 individual experiments are superimposed as circles. The numbers above the boxplots represent P-values  
552 of Mann-Whitney U tests.

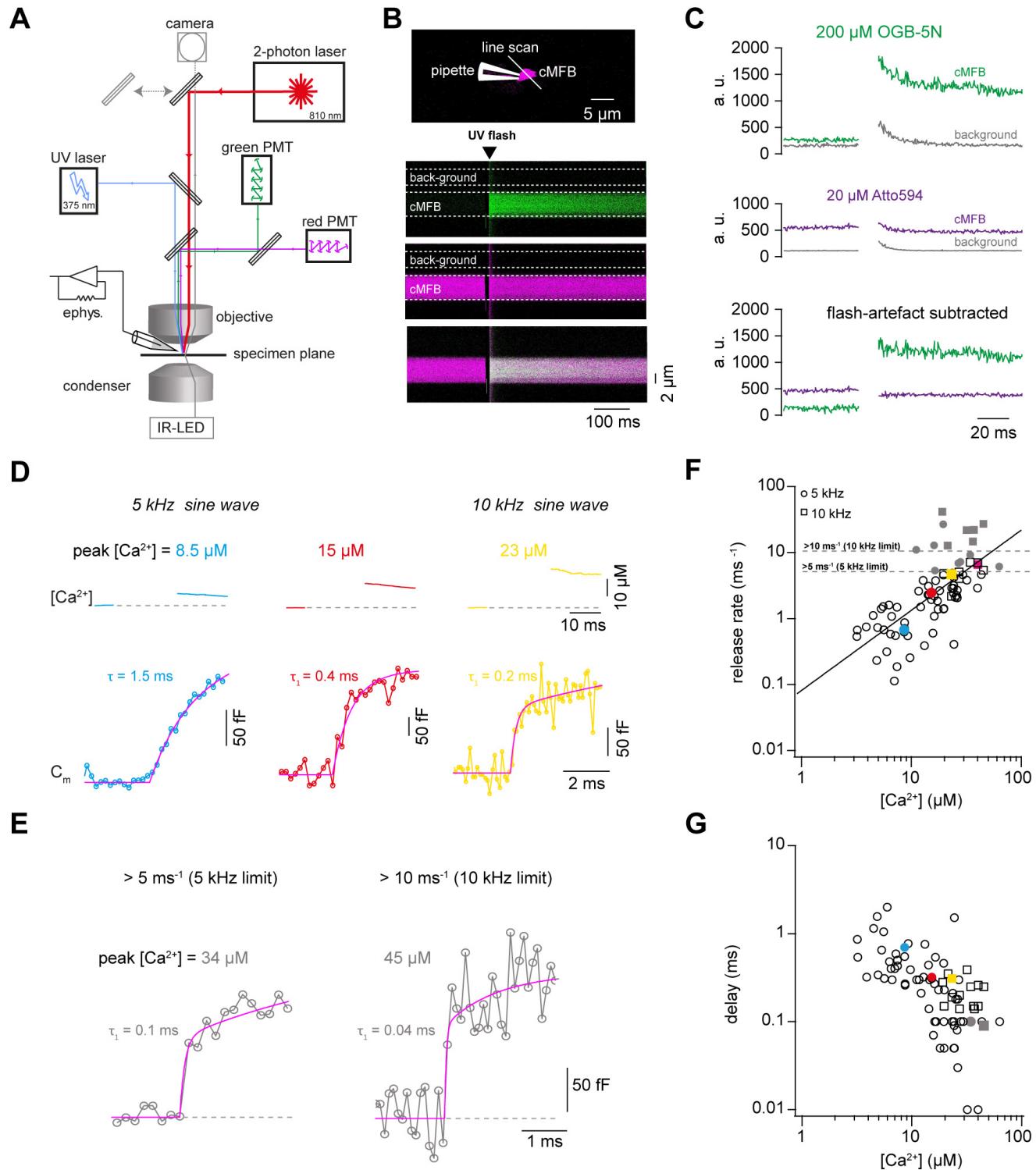
553 **Ca<sup>2+</sup> uncaging dose-response curve measured with presynaptic capacitance  
554 measurements**

555 To gain a better understanding of the profound sensitivity of AP-evoked release on  
556 presynaptic basal Ca<sup>2+</sup> concentration, we established presynaptic Ca<sup>2+</sup> uncaging and  
557 measured the release kinetics upon step-wise elevation of Ca<sup>2+</sup> concentration. We  
558 combined wide-field illumination using a high-power UV laser with previously established  
559 quantitative two-photon Ca<sup>2+</sup> imaging (Delvendahl et al., 2015) to quantify the post-flash  
560 Ca<sup>2+</sup> concentration (Fig. 2A). This approach offers sub-millisecond control of the UV  
561 flashes and a high signal to noise ratio of the two-photon Ca<sup>2+</sup> imaging deep within the  
562 brain slice. The flash-evoked artefacts in the two-photon signals, presumably due to  
563 luminescence in the light path, could be reduced to a minimum with an optimal set of  
564 spectral filters and gate-able photomultipliers (PMTs). Subtraction of the remaining  
565 artefact in the background region of the two-photon line scan resulted in artefact-free  
566 fluorescence signals (Fig. 2B and C).

567 To obtain a large range of post-flash Ca<sup>2+</sup> concentrations within the bouton, we varied the  
568 concentration of the Ca<sup>2+</sup>-cage DMn (1-10 mM) and the intensity (10 - 100%) and the  
569 duration (100 or 200  $\mu$ s) of the UV laser pulse. The spatial homogeneity of the Ca<sup>2+</sup>  
570 elevation was assessed by UV illumination of caged fluorescein mixed with glycerol (Fig.  
571 2 – figure supplement 1; Schneggenburger et al., 2000; Bollmann et al., 2000). The  
572 resulting post-flash Ca<sup>2+</sup> concentration was quantified with either high- or low-affinity Ca<sup>2+</sup>  
573 indicator (Fluo-5F or OGB-5N). To measure the kinetics of neurotransmitter release  
574 independent of dendritic filtering or postsynaptic receptor saturation, vesicular fusion was  
575 quantified by measuring the presynaptic capacitance with a 5 kHz-sinusoidal stimulation  
576 (Hallermann et al., 2003). The first 10 ms of the flash-evoked capacitance increase was  
577 fitted with functions containing a baseline and mono- or bi-exponential components  
578 (magenta line in Fig. 2D and E; see eq. 1 in the methods section). With increasing post-  
579 flash Ca<sup>2+</sup> concentration the fast time constant decreased ( $\tau$  in case of mono- and  $\tau_1$  in  
580 case of bi-exponential fits; Fig. 2D). The inverse of the fast time constant represents a  
581 direct readout of the fusion kinetics of the release-ready vesicles. The observed scatter  
582 could be due to the invasiveness of presynaptic recordings and/or heterogeneity among

583 boutons (Chabrol et al., 2015; Fekete et al., 2019; Grande and Wang, 2011). When  
584 plotting the inverse of the time constant as a function of post-flash  $\text{Ca}^{2+}$  concentration, we  
585 obtained a shallow dose-response curve that showed a continuous increase in the  
586 release rate with increasing post-flash  $\text{Ca}^{2+}$  concentration up to 50  $\mu\text{M}$  (Fig. 2F). In some  
587 experiments with high  $\text{Ca}^{2+}$  concentrations, the release was too fast to be resolved with 5  
588 kHz capacitance sampling (i.e. time constants were smaller than 200  $\mu\text{s}$ ; Fig. 2E). We  
589 therefore increased the frequency of the sinusoidal stimulation in a subset of experiments  
590 to 10 kHz (15 out of 80 experiments). Such high-frequency capacitance sampling is to  
591 our knowledge unprecedented at central synapses and technically challenging because  
592 exceptionally low access resistances are required ( $\sim 15 \text{ M}\Omega$ ) to obtain an acceptable  
593 signal-to-noise ratio (Gillis, 1995; Hallermann et al., 2003). Despite these efforts, the time  
594 constants were sometimes faster than 100  $\mu\text{s}$ , representing the resolution limit of 10 kHz  
595 capacitance sampling (Fig. 2E). These results indicate that the entire pool of release-  
596 ready vesicles can fuse within less than 100  $\mu\text{s}$ . Fitting a Hill equation on both 5- and 10  
597 kHz data resulted in a best-fit  $K_D$  of  $> 50 \mu\text{M}$  with a best-fit Hill coefficient,  $n$ , of 1.2 (Fig.  
598 2F).

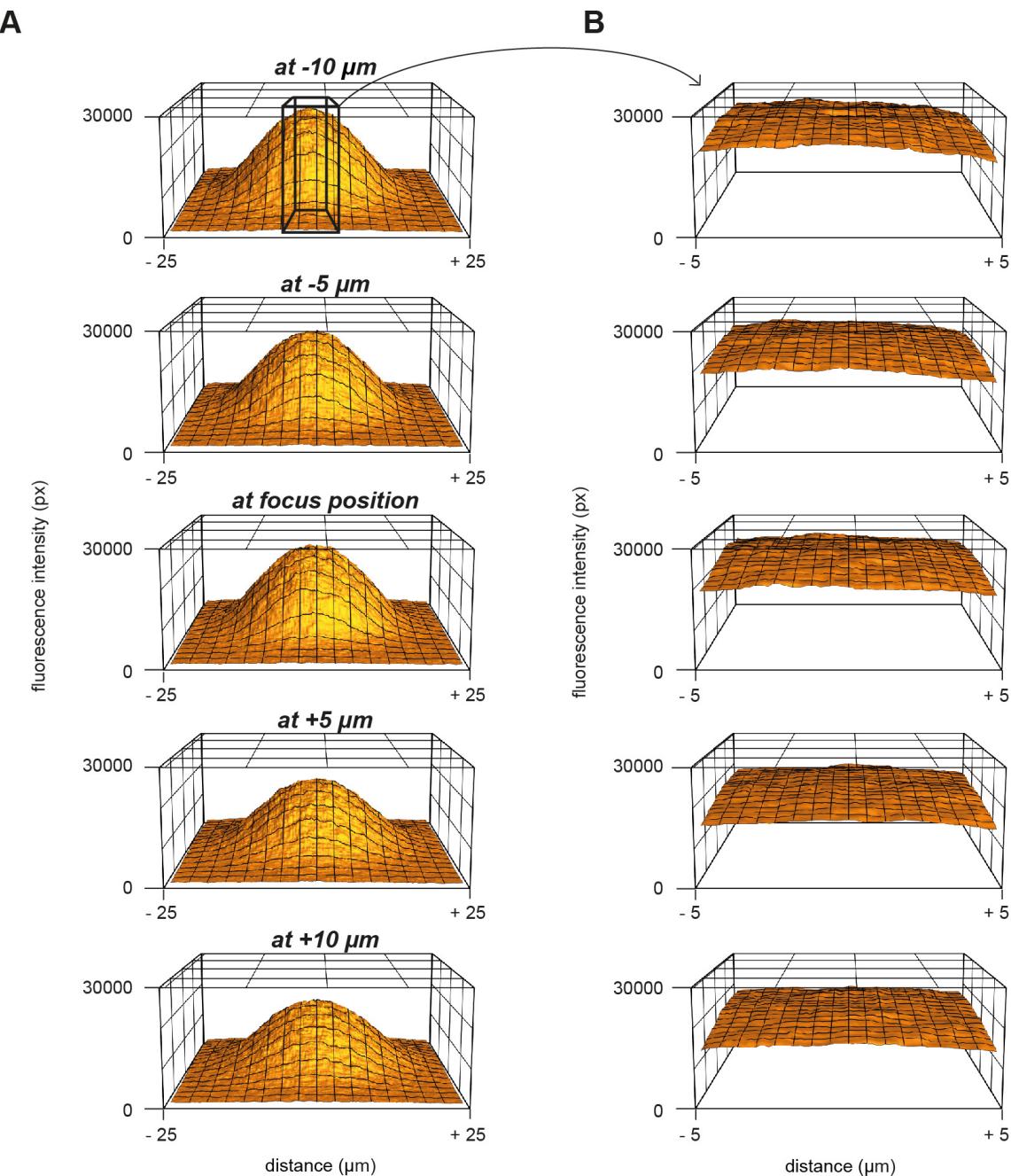
599 In addition to the speed of vesicle fusion, we analyzed the delay from the onset of the UV-  
600 illumination to the onset of the rise of membrane capacitance, which was a free parameter  
601 in our fitting functions (see eq. 1). The delay was strongly dependent on the post-flash  
602  $\text{Ca}^{2+}$  concentration and the dose-response curve showed no signs of saturation at high  
603  $\text{Ca}^{2+}$  concentrations (Fig. 2G), which is consistent with the non-saturating release rates.  
604 These data reveal that the fusion kinetics of synaptic vesicles increased up to a  $\text{Ca}^{2+}$   
605 concentration of 50  $\mu\text{M}$  without signs of saturation, suggesting a surprisingly low apparent  
606 affinity of the fusion sensor at mature cMFBs under physiological temperature conditions  
607 ( $K_D > 50 \mu\text{M}$ ).



608 **Figure 2**  $\text{Ca}^{2+}$  uncaging dose-response curve measured with presynaptic capacitance  
609 measurements

610 A. Illustration of the experimental setup showing the light path of the two-photon laser illumination (red  
611 line), the UV laser illumination (blue line), the electrophysiology amplifier ('ephys.'), the red and green  
612 gate-able photomultiplier tubes (PMTs), and infrared LED illumination with oblique illumination via the

613 condenser for visualization of the cells at the specimen plane by the camera (grey line) when the upper  
614 mirror is moved out of the light path (grey arrow).  
615 B. *Top*: Two-photon microscopic image of a cMFB in the whole-cell configuration loaded with OGB-5N,  
616 Atto594, and DMn/ Ca<sup>2+</sup>. Positions of the patch pipette and line scan are indicated. *Bottom*: Two-photon  
617 line scan showing the fluorescence signal as measured through the green PMT, red PMT, and an overlay  
618 of the green and red channels. Arrow indicates the onset of the UV flash and dashed lines represent the  
619 flash-induced luminescence artefact as detected outside the cMFB. The lookup tables for the green and  
620 red channel were arbitrarily but linearly adjusted independent of the absolute values in C.  
621 C. *Top*: change in fluorescence intensity within the cMFB for the green channel along with the  
622 corresponding flash-induced green artefact measured in the background. *Middle*: change in fluorescence  
623 intensity within the cMFB for the red channel along with the corresponding flash-induced red artefact.  
624 *Bottom*: green and red fluorescence signal after subtracting the flash-induced artefacts.  
625 D. *Top*: Ca<sup>2+</sup> signals of different concentrations elicited through Ca<sup>2+</sup> uncaging in three different cells, the  
626 flash was blanked. *Bottom*: corresponding traces of capacitance recordings measured using a 5 kHz  
627 sinusoidal stimulation (left and middle) or 10 kHz sinusoidal stimulation (right).  $\tau$  represents the time  
628 constant from a mono-exponential fit,  $\tau_1$  represents the time constant of the fast component of a bi-  
629 exponential fit.  
630 E. Traces of capacitance recordings showing the resolution limit in detecting fast release rates of  $>5\text{ ms}^{-1}$   
631 using 5 kHz sinusoidal stimulation or  $>10\text{ ms}^{-1}$  using 10 kHz sinusoidal stimulation.  
632 F. Plot of release rate versus post-flash Ca<sup>2+</sup> concentration. The line represents a fit with a Hill equation  
633 (eq. 2) with best-fit values  $V_{max} = 1.7 \times 10^7\text{ ms}^{-1}$ ,  $K_D = 7.2 \times 10^6\text{ }\mu\text{M}$ , and  $n = 1.2$ . Color coded symbols  
634 correspond to traces in D – E. Grey symbols represent values above the resolution limit.  
635 G. Plot of synaptic delay versus post-flash Ca<sup>2+</sup> concentration. Color coded symbols correspond to traces  
636 in D – E.



637 **Figure 2 – figure supplement 1 Measurement of the UV energy profile with caged fluorescein**  
638 A. 3D plot of the fluorescence profile in response to UV uncaging of caged-fluorescein at different z-  
639 positions.  
640 B. Magnification of the middle part in panel (A) over a range of 10  $\mu\text{m}$ .

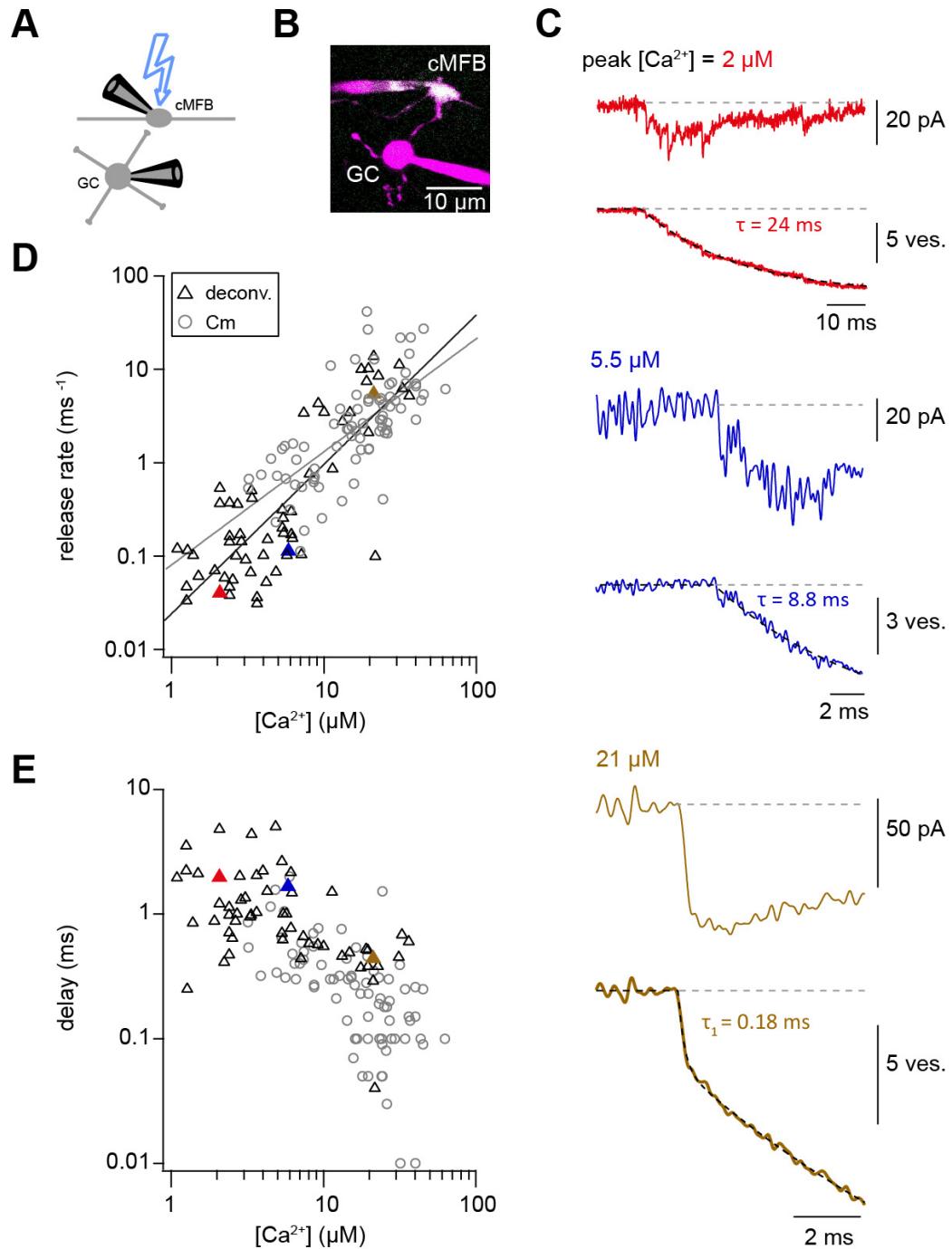
641 **Ca<sup>2+</sup> uncaging dose-response curve measured with deconvolution of EPSCs**

642 Capacitance recordings are not very sensitive in detecting low release rates. We therefore  
643 performed simultaneous pre- and postsynaptic recordings and used established  
644 deconvolution techniques to calculate the presynaptic release rate by analyzing the EPSC  
645 as previously applied at this synapse (Fig. 3A, B; Ritzau-Jost et al., 2014). Kynurenic acid  
646 (2 mM) and cyclothiazide (100  $\mu$ M) were added to the extracellular solution in order to  
647 prevent the saturation and desensitization of postsynaptic AMPA receptors, respectively.  
648 Ca<sup>2+</sup> uncaging in the presynaptic terminal evoked EPSCs with kinetics which strongly  
649 depended on the post-flash Ca<sup>2+</sup> concentration. The cumulative release obtained from  
650 deconvolution analysis of the recorded EPSCs was fitted as the capacitance traces (eq.  
651 1). At low Ca<sup>2+</sup> concentrations (<5  $\mu$ M), a significant amount of neurotransmitter release  
652 could be measured, which is consistent with previous reports from central synapses  
653 (Bollmann et al., 2000; Fukaya et al., 2021; Sakaba, 2008; Schneggenburger and Neher,  
654 2000). The presynaptic release rates increased with increasing post-flash Ca<sup>2+</sup>  
655 concentration and no saturation in the release rate occurred in the dose-response curve  
656 (Fig. 3D). The dose-response curve for the delay from the onset of the UV illumination to  
657 the onset of the rise of the cumulative release trace (eq. 1) did not show signs of saturation  
658 of the release kinetics in the investigated range. Thus, consistent with capacitance  
659 measurements, deconvolution analysis of postsynaptic currents revealed a shallow Ca<sup>2+</sup>-  
660 dependence of neurotransmitter release kinetics (Fig. 3D and E). Fitting a Hill equation  
661 to the deconvolution data resulted in a best-fit  $K_D > 50$   $\mu$ M and a Hill coefficient of 1.6 (Fig.  
662 3D). Therefore, two independent measures of synaptic release (presynaptic capacitance  
663 measurements and postsynaptic deconvolution analysis) indicate a non-saturating  
664 shallow dose-response curve up to ~50  $\mu$ M.

665 To rule out methodical errors that might influence the dose-response curve, we carefully  
666 determined the  $K_D$  of the Ca<sup>2+</sup> indicator OGB-5N using several independent approaches  
667 including direct potentiometry (Fig. 3 – figure supplement 1), because this value  
668 influences the estimate of the Ca<sup>2+</sup> affinity of the fusion sensors linearly. We estimated a  
669  $K_D$  of OGB-5N of ~30  $\mu$ M being at the lower range of previous estimates ranging from 20  
670 to 180  $\mu$ M (Delvendahl et al., 2015; Digregorio and Vergara, 1997; Neef et al., 2018),

671 arguing against an erroneously high  $K_D$  of the  $\text{Ca}^{2+}$  indicator as a cause for the non-  
672 saturation.

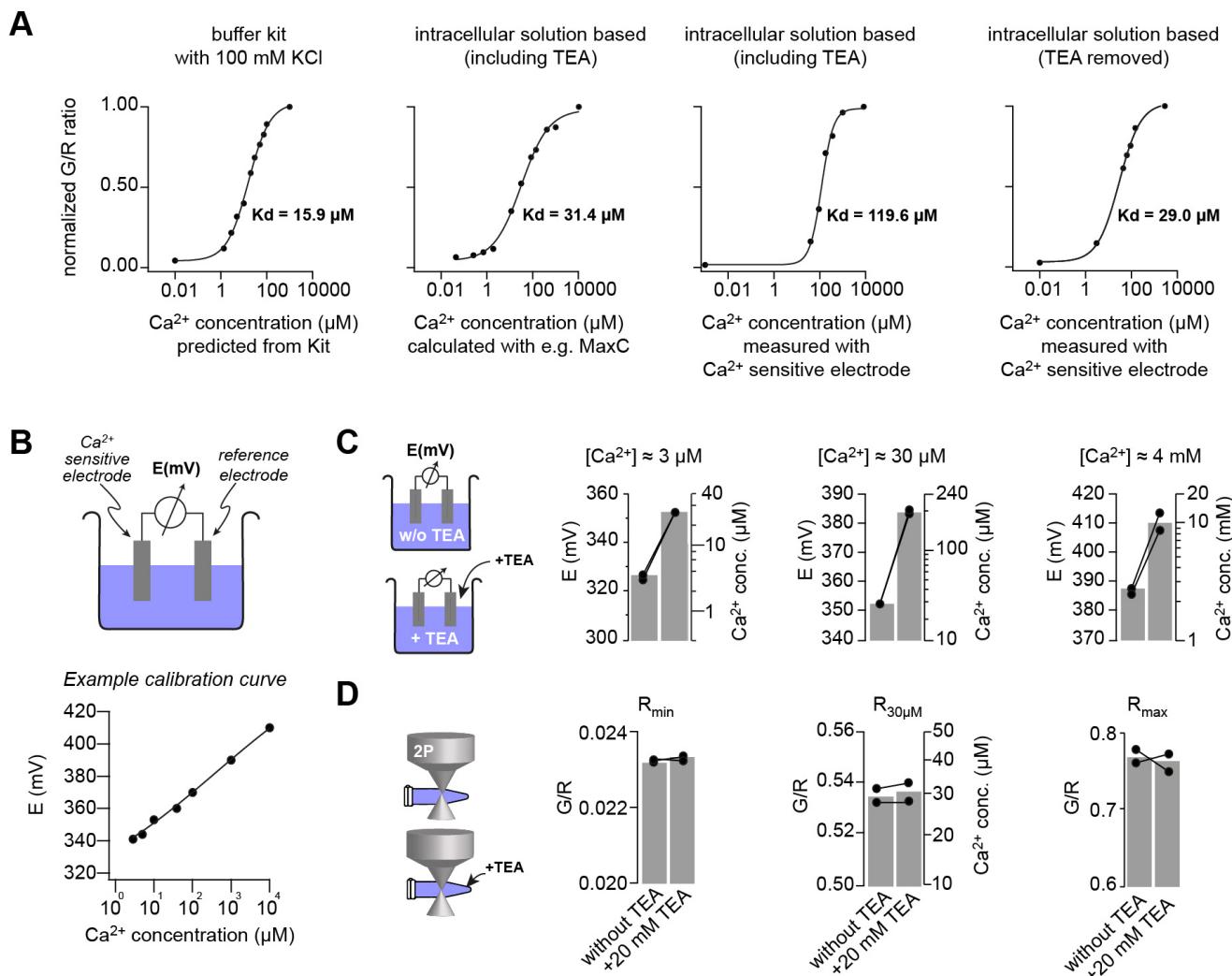
673 In addition, we used the two following independent approaches to rule out a previously  
674 described  $\text{Ca}^{2+}$  overshoot immediately following the UV illumination. Such  $\text{Ca}^{2+}$  overshoot  
675 would be too fast to be detected by the  $\text{Ca}^{2+}$  indicators (Bollmann et al., 2000) but could  
676 trigger strong release with weak UV illumination which, would predict a shallow dose-  
677 response curve. First, the time course of  $\text{Ca}^{2+}$  release from DMn was simulated (see  
678 below; Fig. 6A) and no significant overshoots were observed (see below; Fig. 6A).  
679 Secondly, we experimentally compared strong and short UV illumination (100% intensity;  
680 0.1 ms) with weak and long UV illumination (10% intensity; 1 ms), because a  $\text{Ca}^{2+}$   
681 overshoot is expected to primarily occur with strong and short UV illumination.  
682 Comparison of these two groups of UV illumination resulted in similar post-flash  
683 concentrations but did not reveal a significant difference in the corresponding release rate  
684 indicating that undetectable  $\text{Ca}^{2+}$  overshoots did not affect the measured release rate  
685 (Fig. 3 – figure supplement 3). Therefore, both approaches argue against a  $\text{Ca}^{2+}$   
686 overshoot as an explanation for the shallow dose-response curve.



687 **Figure 3**  $\text{Ca}^{2+}$  uncaging dose-response curve measured with deconvolution of EPSCs

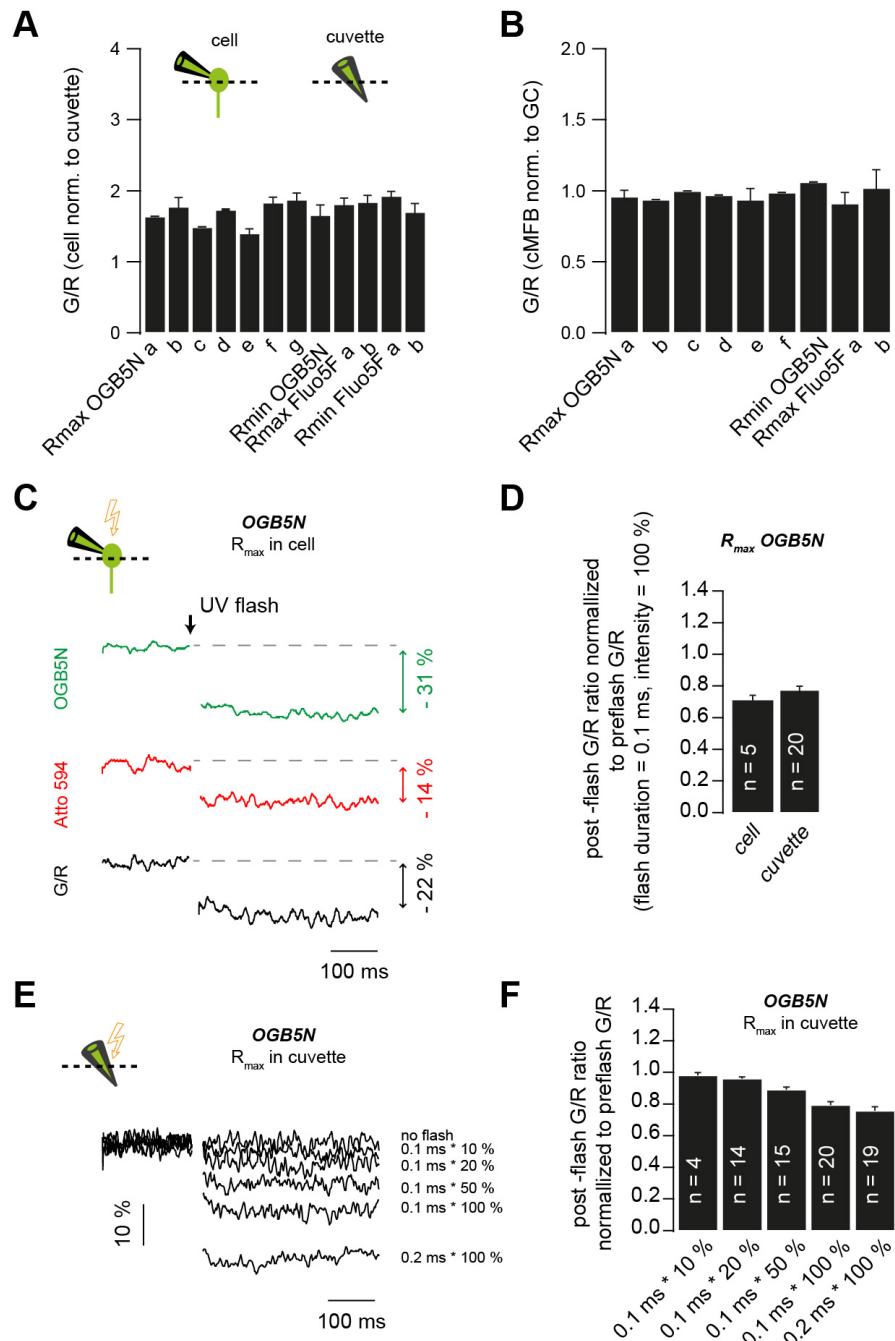
688 A. Illustration of the cellular connectivity in the cerebellar cortex showing the pre- and postsynaptic  
 689 compartments during paired whole-cell patch-clamp recordings and  $\text{Ca}^{2+}$  uncaging with UV-illumination.  
 690 B. Two-photon microscopic image of a cMFB and a GC in the paired whole-cell patch-clamp configuration  
 691 C. Three different recordings showing UV-flash evoked EPSC (top trace) and cumulative release rate  
 692 measured by deconvolution analysis of the EPSCs (bottom trace). The peak  $\text{Ca}^{2+}$  concentration,  
 693 quantified with two-photon  $\text{Ca}^{2+}$  imaging, is indicated in each panel.  $\tau$  represents the time constant from  
 694 mono-exponential fit,  $\tau_1$  represents the time constant of the fast component of bi-exponential fit. Note the  
 695 different lengths of the baselines in the three recordings.

696 D. Plot of release rate versus post-flash  $\text{Ca}^{2+}$  concentration. Grey open circles represent data from  
 697 capacitance measurements (cf. Fig. 2) and black triangles represent data from deconvolution analysis of  
 698 EPSC. Grey and black lines represent fits with a Hill equation of the capacitance (as shown in Fig. 1F)  
 699 and the deconvolution data, respectively. The best-fit parameters for the fit on the deconvolution data  
 700 were  $V_{max} = 6*10^7 \text{ ms}^{-1}$ ,  $K_D = 7.6*10^5 \mu\text{M}$ , and  $n = 1.6$ . Red, blue and brown symbols correspond to the  
 701 traces in (C).  
 702 E. Plot of synaptic delay versus post-flash  $\text{Ca}^{2+}$  concentration. Grey open circles represent data from  
 703 capacitance measurements, and black triangles represent data from deconvolution analysis of EPSC.  
 704 Red, blue and brown symbols correspond to the traces in (C).



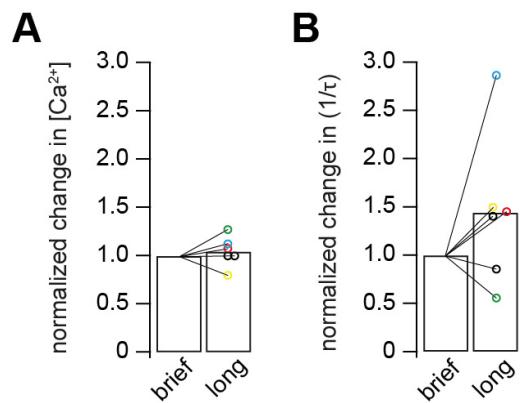
705 **Figure 3 – figure supplement 1 Measuring the  $K_D$  of the  $\text{Ca}^{2+}$  sensitive dyes**  
 706 A. Green (OGB-5N) over red (Atto594) fluorescence ratio for different  $\text{Ca}^{2+}$  concentrations, measured  
 707 using either a  $\text{Ca}^{2+}$  calibration buffered kit or by clamping the free  $\text{Ca}^{2+}$  using EGTA in the intracellular  
 708 patching solution. The free  $\text{Ca}^{2+}$  concentration was predicted from the kit, calculated with software like  
 709 Maxchelator (MaxC) or measured by potentiometry using a  $\text{Ca}^{2+}$ -sensitive electrode. The indicated  $K_D$   
 710 values were obtained from superimposed fits with Hill equations.  
 711 B. Top: illustration of the  $\text{Ca}^{2+}$ -sensitive electrode. Bottom: Example of a calibration curve of the  $\text{Ca}^{2+}$ -  
 712 sensitive electrode fitted with a straight line.

713 C. Effect of Tetraethylammonium (TEA) on the  $\text{Ca}^{2+}$  sensitive electrode at different  $\text{Ca}^{2+}$  concentrations.  
714 20 mM TEA induced ~10-fold increase in the potential (left axis) and thus the read-out  $\text{Ca}^{2+}$  concentration  
715 (right axis) of intracellular solutions which had free  $\text{Ca}^{2+}$  concentrations clamped by EGTA to 3  $\mu\text{M}$ , 30  
716  $\mu\text{M}$ , or 4 mM (pH was kept constant; bar graphs represent the mean; line-connected circles represent two  
717 independent repetitions).  
718 D. Effect of TEA on G/R fluorescence ratio. The ratio of the intracellular solution containing only 10 mM  
719 EGTA (Rmin), free  $\text{Ca}^{2+}$  clamped with EGTA to 30  $\mu\text{M}$  (R30 $\mu\text{M}$ ), or 10 mM  $\text{Ca}^{2+}$  (Rmax) did not change  
720 upon adding 20 mM TEA indicating that TEA is not contaminated with  $\text{Ca}^{2+}$  but instead TEA specifically  
721 interferes with the  $\text{Ca}^{2+}$ -sensitive electrode.



722 **Figure 3 – figure supplement 2 Correction for the post-flash changes in the fluorescent properties**  
723 **of the intracellular solution**

724 A. Green over red fluorescence (G/R) ratios measured *in situ* normalized to G/R ratios measured in  
725 cuvettes. Data represent the different solutions used throughout the study. (a-g) represent measurements  
726 obtained from different solutions prepared using different pre-stocks of the fluorescent indicators or a  
727 different DMn/Ca<sup>2+</sup> concentration.  
728 B. Green over red fluorescence (G/R) ratios measured in cMFBs normalized to G/R ratios measured in  
729 GCs. Data represent different solutions used throughout the study. (a-f) represent measurements  
730 obtained from different solutions prepared using different pre-stocks of the fluorescent indicators or a  
731 different DMn/Ca<sup>2+</sup> concentration.  
732 C. Example traces of *in situ* post-flash alterations in the green fluorescence, in the red fluorescence, and  
733 the overall drop in the G/R ratio (in black) in response to a UV flash of 0.1 ms duration and 100 %  
734 intensity.  
735 D. Comparison of the UV-flash-induced bleaching of fluorescent indicators measured in cells to the UV-  
736 flash-induced bleaching of fluorescent indicators measured in cuvettes, in response to a UV flash of 0.1  
737 ms duration and 100 % intensity.  
738 E. Example traces of UV-flash-induced changes occurring in cuvettes in response to UV flashes of  
739 different intensities or duration.  
740 F. Average UV-flash-induced changes occurring in cuvettes in response to UV flashes of different  
741 intensities or duration.

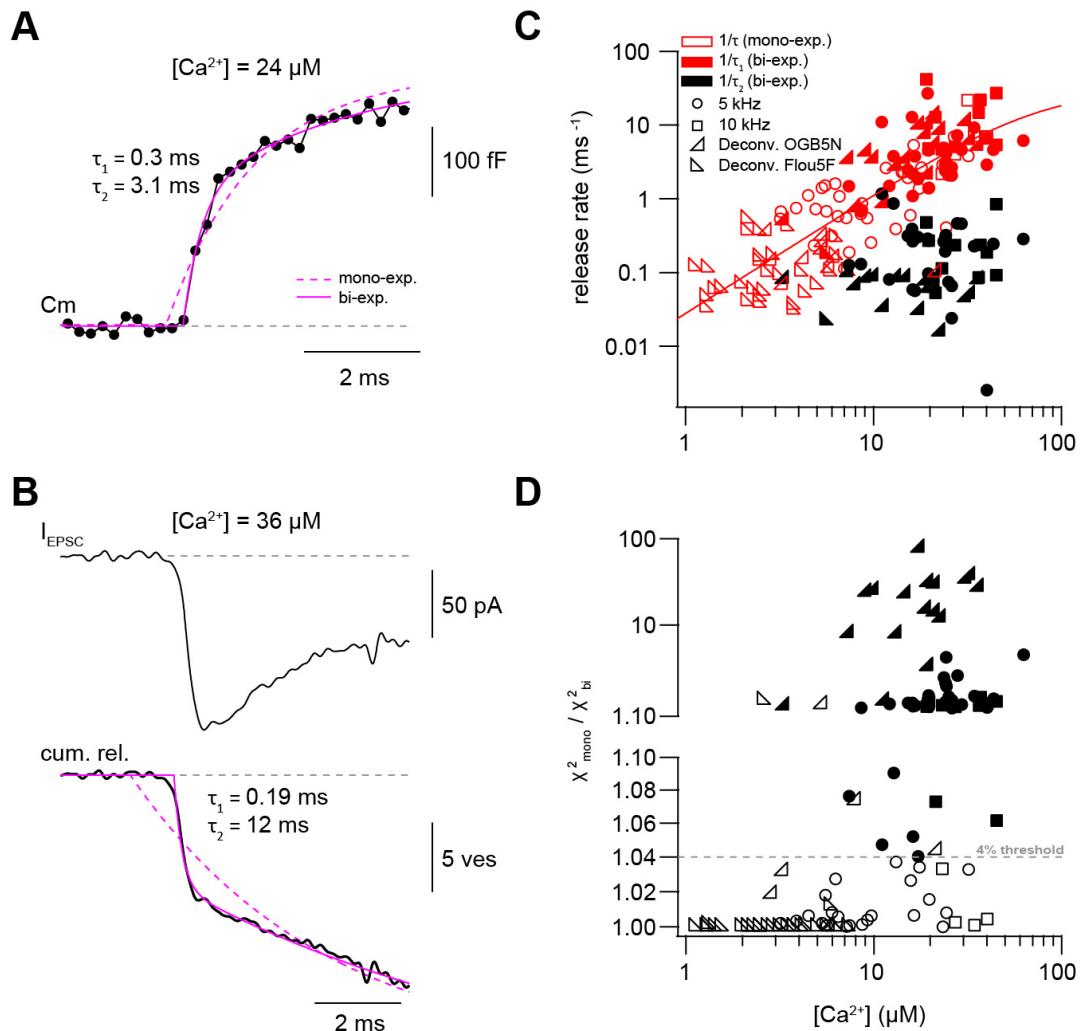


742 **Figure 3 – figure supplement 3 Comparison of brief versus long UV illumination to rule out fast**  
743 **Ca<sup>2+</sup> overshoots**

744 A. Post-flash Ca<sup>2+</sup> concentration obtained from long flashes of 1 ms duration and 10% UV intensity,  
745 normalized to post-flash Ca<sup>2+</sup> concentration obtained from brief flashes of 0.1 ms duration and 100% UV  
746 intensity.  
747 B. Release rates obtained from long flashes of 1 ms duration and 10% UV intensity, normalized to  
748 release rates obtained from brief flashes of 0.1 ms duration and 100% UV intensity. Color code matches  
749 the data in A and B.

750 **Presynaptic and postsynaptic measurements reveal two kinetic processes of**  
751 **neurotransmitter release**

752 In some  $\text{Ca}^{2+}$  uncaging experiments, synaptic release appeared to have two components,  
753 which could be due to heterogeneity amongst release-ready vesicles. We therefore  
754 systematically compared mono- and bi-exponential fits to the capacitance and  
755 deconvolution data (Fig. 4 A and B). Several criteria were used to justify a bi-exponential  
756 fit (see methods). One criterion was at least a 4% increase in the quality of bi- compared  
757 with mono-exponential fits as measured by the sum of squared differences between the  
758 fit and the experimental data ( $\chi^2$ ; Fig. 4D). Consistent with a visual impression, this  
759 standardized procedure resulted in the classification of ~40% of all recordings as bi-  
760 exponential (38 out of 80 capacitance measurements and 17 out of 59 deconvolution  
761 experiments; Fig. 4C and D). The release rate of the fast component ( $1/\tau_1$ ) of the merged  
762 capacitance and deconvolution data showed no signs of saturation consistent with our  
763 previous analyses of each data set separately. Fitting a Hill equation to the merged data  
764 indicated a  $K_D > 50 \mu\text{M}$  and a Hill coefficient of 1.6 (Fig. 4C). The release rate of the slow  
765 component ( $1/\tau_2$ ; if existing) was on average more than 10 times smaller (black symbols,  
766 Fig. 4C). These data indicate that there are at least two distinct kinetic steps contributing  
767 to release within the first 10 ms.



768 **Figure 4 Presynaptic and postsynaptic measurements reveal two kinetic processes of**  
769 **neurotransmitter release**

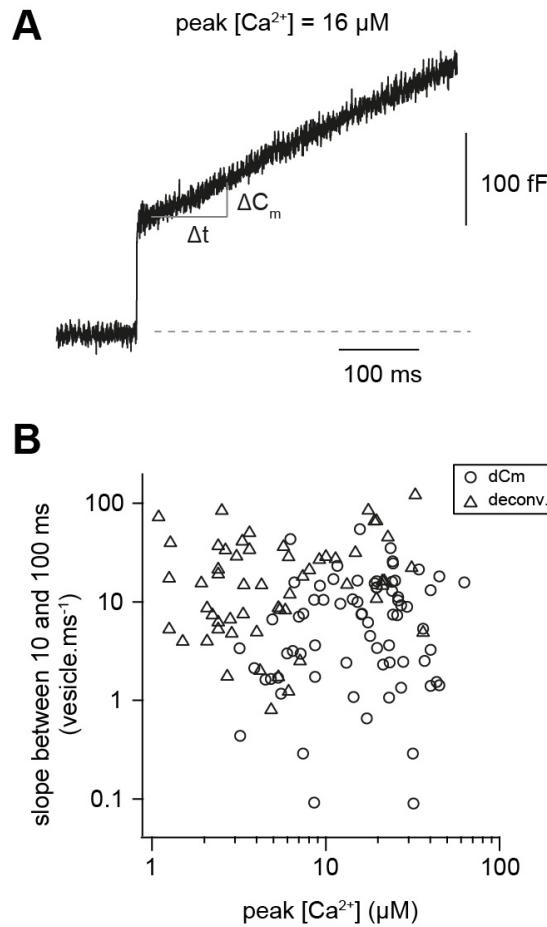
770 A. Example of a capacitance trace showing the two components of release observed within the first 10  
771 ms in response to UV-flash-evoked increase in  $\text{Ca}^{2+}$  concentration to  $24 \mu\text{M}$ . The solid magenta line  
772 represents the bi-exponential fit and the dashed magenta line represents mono-exponential fit (see eq. 1).  
773 B. Top: example trace of an EPSC recording in response to UV-flash evoked increase in  $\text{Ca}^{2+}$   
774 concentration to  $36 \mu\text{M}$ . Bottom: the corresponding cumulative release trace obtained from deconvolution  
775 analysis, showing the two components of release observed within the first 10 ms. The solid magenta line  
776 represents the bi-exponential fit and the dashed magenta line represents mono-exponential fit (see eq. 1).  
777 C. Top: plot of neurotransmitter release rates as a function of peak  $\text{Ca}^{2+}$  concentration. Data obtained  
778 from capacitance measurements with sinusoidal frequency of 5 kHz are shown as circles, data from 10  
779 kHz capacitance measurements are shown as squares, and cumulative release data (obtained from  
780 deconvolution analysis) are shown as lower left- and lower right- triangles for recordings with OGB5N and  
781 Fluo5F, respectively. Open symbols correspond to data from the mono-exponential fits and filled symbols  
782 correspond to data from the bi-exponential fits. Red symbols represent merged data of the release rates  
783 obtained from mono-exponential fit and the fast component of the bi-exponential fit, and black symbols

784 represent the second component of the bi-exponential fit. The line represents a fit with a Hill equation with  
785 best-fit parameters  $V_{max} = 29.9 \text{ ms}^{-1}$ ,  $K_D = 75.5 \mu\text{M}$ , and  $n = 1.61$ .

786 D.  $\chi^2$  ratio for the mono-exponential compared to the bi-exponential fits. Dashed line represents the  
787 threshold of the  $\chi^2$  ratio used to judge the fit quality of double compared to mono-exponential fits (as one  
788 criterion for selection). 5 kHz capacitance data are shown as circles, 10 kHz capacitance data are shown  
789 as squares, and cumulative release data (obtained from deconvolution analysis) are shown as lower left-  
790 and lower right- triangles for recordings with OGB5N and Fluo5F, respectively. Open symbols correspond  
791 to data points judged as mono-exponential and filled symbols correspond to data points judged as bi-  
792 exponential.

### 793 **Fast and $\text{Ca}^{2+}$ -independent sustained release**

794 To gain more insights into the mechanisms of sustained vesicle release, we focused on  
795 the synaptic release within the first 100 ms after  $\text{Ca}^{2+}$  uncaging. To investigate the  $\text{Ca}^{2+}$ -  
796 dependence of sustained release, we estimated the number of vesicles ( $N_v$ ) released  
797 between 10 and 100 ms after flash onset, assuming a single vesicle capacitance of 70  
798 aF and 90 granule cells-contacts per mossy fiber rosette (see methods; Ritzau-Jost et al.,  
799 2014). There was considerable variability in the release rate between 10 and 100 ms,  
800 which could be due to differences in bouton size and wash-out of proteins during whole-  
801 cell recordings. However, the release rate showed no obvious dependence on the post-  
802 flash  $\text{Ca}^{2+}$  concentration (Fig. 5B). These data indicate that the slope of the sustained  
803 component of release is  $\text{Ca}^{2+}$ -independent in the investigated  $\text{Ca}^{2+}$  concentration range  
804 of 1-50  $\mu\text{M}$ , consistent with previously observed  $\text{Ca}^{2+}$ -independent vesicle recruitment as  
805 assessed by depolarizing cMFBs to 0mV in the presence of EGTA (Ritzau-Jost et al.,  
806 2014).



807 **Figure 5 Fast and  $\text{Ca}^{2+}$ -independent sustained release**

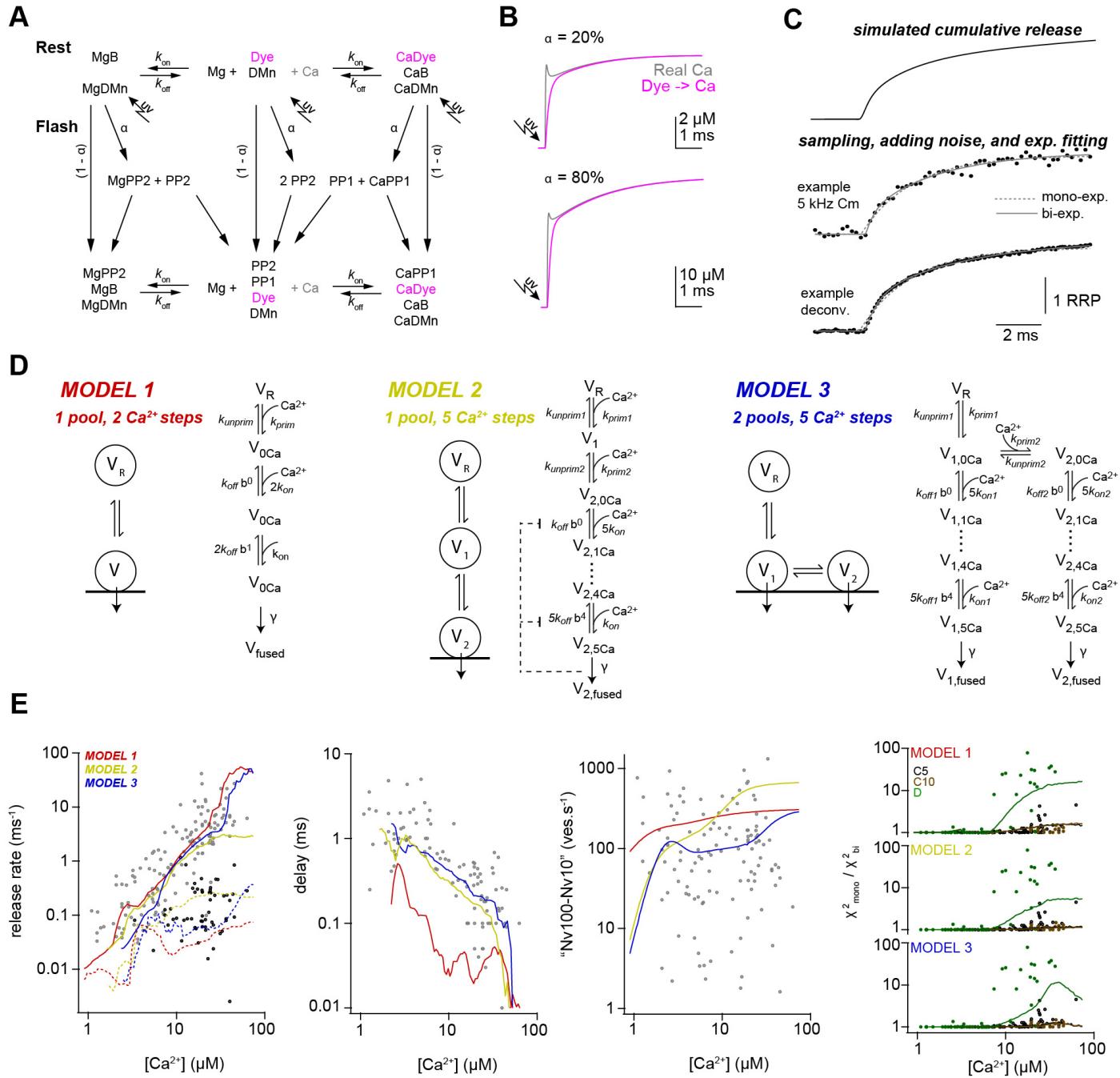
808 A. Examples of capacitance traces showing the sustained component of release.  
809 B. Plot of the number of vesicles released between 10 and 100 ms divided by the time interval (90 ms)  
810 versus the post-flash  $\text{Ca}^{2+}$  concentration. Open circles represent data from capacitance measurements  
811 and triangles represent cumulative release data (obtained from deconvolution analysis).

812 **Release schemes with five  $\text{Ca}^{2+}$  steps and fast recruitment via parallel or sequential  
813 models can explain  $\text{Ca}^{2+}$ -dependence of release**

814 To investigate mechanisms that could explain a non-saturating and shallow dose-  
815 response curve and rapid sustained release, we performed modeling with various release  
816 schemes. First, we simulated the exact time course of the concentration of free  $\text{Ca}^{2+}$ . The  
817  $\text{Ca}^{2+}$  release from DMn and subsequent binding to other buffers and the  $\text{Ca}^{2+}$  indicator  
818 were simulated based on previously described binding and unbinding rates (Faas et al.,  
819 2005; Faas et al., 2007; Fig. 6A; see methods). In contrast to previous results, which  
820 predicted a significant overshoot of  $\text{Ca}^{2+}$  following UV illumination with short laser pulses  
821 (Bollmann et al., 2000), our simulations predict little overshoot compared to the  $\text{Ca}^{2+}$

822 concentration measured by the  $\text{Ca}^{2+}$  indicator (Fig. 6B). The discrepancy is readily  
823 described by recent improvements in the quantification of  $\text{Ca}^{2+}$  binding and unbinding  
824 kinetics (Faas et al., 2005; Faas et al., 2007). The calculations predict an almost step-like  
825 increase in the free  $\text{Ca}^{2+}$  concentration with a 10-90% rise time below 50  $\mu\text{s}$ . These  
826 simulated UV illumination-induced transients of free  $\text{Ca}^{2+}$  concentrations were  
827 subsequently used to drive the release schemes. Realistic noise was added to the  
828 resulting simulated cumulative release rate and the analysis using exponential fits (eq. 1)  
829 was performed as with the experimental data (Fig. 6C).

830 We compared three different release schemes in their ability to reproduce our  
831 experimental data. In model 1, a single pool of vesicles with two  $\text{Ca}^{2+}$  binding steps was  
832 used as previously established, e.g., for chromaffin cells and rod photoreceptors (Duncan  
833 et al., 2010; Voets, 2000). Such an assumption would readily explain the shallow dose-  
834 response curve (Bornschein and Schmidt, 2018). The two components of release could  
835 be replicated by assuming rapid vesicle recruitment from a reserve pool ( $V_R$ ; Fig. 6D).  
836 However, adjusting the free parameters did not allow reproducing the synaptic delay (Fig.  
837 6E). We therefore tested two more sophisticated models in which vesicle fusion is  
838 triggered via five  $\text{Ca}^{2+}$  binding steps (Schneggenburger and Neher, 2000). In model 2,  
839 the first vesicle pool represents the docked vesicles and the second pool represents a  
840 replacement pool, which can undergo rapid docking and fusion (Miki et al., 2016; Miki et  
841 al., 2018), therefore representing two kinetic steps occurring in sequence. In model 3, two  
842 pools of vesicles with different  $\text{Ca}^{2+}$ -sensitivity exist, where both types of vesicles can  
843 fuse with different  $\text{Ca}^{2+}$  affinity (Voets, 2000; Walter et al., 2013; Wölfel et al., 2007),  
844 therefore representing two kinetic steps occurring in parallel. Model 3 reproduced the data  
845 as good as model 2, however the non-saturation up to 50  $\mu\text{M}$  could be reproduced  
846 somewhat better in model 3. Interestingly, models 2 and 3 both replicated the observed  
847 shallow dose-response curve despite the presence of five  $\text{Ca}^{2+}$  binding steps. These  
848 results indicate that established models with five  $\text{Ca}^{2+}$ -steps incorporating fast vesicle  
849 recruitment via sequential or parallel vesicle pools can replicate our data fairly well.



850 **Figure 6 Release schemes with five  $\text{Ca}^{2+}$  steps and fast recruitment via parallel or sequential models**  
851 **can explain  $\text{Ca}^{2+}$ -dependence of release**

852 A. Scheme of the modeling of the intra-bouton  $\text{Ca}^{2+}$  dynamics showing the chemical reaction kinetics that  
853 were implemented in the model. The model covered  $\text{Ca}^{2+}$  (Ca) and  $\text{Mg}^{2+}$  (Mg) binding to the indicator dye  
854 (OGB-5N or Fluo-5F), to DM-nitrophen (DMn), and to buffers (ATP and/or an endogenous buffer). The  
855 forward ( $k_{\text{on}}$ ) and backward ( $k_{\text{off}}$ ) rate constants differ between chemical species. Upon simulated UV flash  
856 photolysis, a fraction  $\alpha$  of metal bound and free DMn made a transition to different photoproducts (PP1  
857 and PP2; cf. Faas et al., 2005). For model parameters see Supplementary Table 2.

858 B. The scheme in (A) was converted to a system of differential equations and the time courses of the  
859 “real” free  $\text{Ca}^{2+}$  (magenta) and the free  $\text{Ca}^{2+}$  reported by OGB-5N (200  $\mu\text{M}$ , green) were simulated for the

860 indicated uncaging fractions  $\alpha$ . Note that already after less than 1 ms the dye reliably reflects the time  
861 course of  $\text{Ca}^{2+}$ .

862 C. Traces showing the steps used in the simulation of the kinetic model of release.

863 D. Graphical illustration of the three models used during the simulations. For model parameters see  
864 Supplementary Table 3.

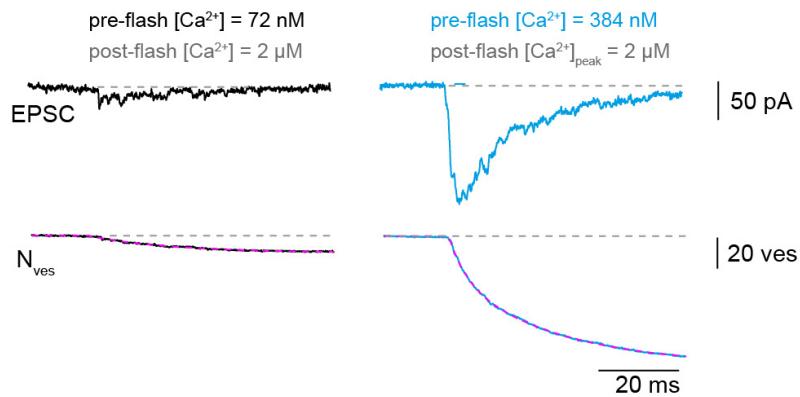
865 E. From left to right, predictions of each model and the experimental data for the inverse of  $\tau_1$  (grey  
866 symbols, solid lines) and inverse of  $\tau_2$  (black symbols, dashed lines), delay, vesicle recruitment speed  
867 between 10 and 100 ms, and the increase in the  $\chi^2$  ratio for the single- compared to the bi-exponential  
868 fits. Red, yellow, and blue lines correspond to simulations of models 1, 2, and 3, respectively. For the  $\chi^2$   
869 ratio (*right plot*), the experimental data and the simulations are shown separately for 5-kHz and 10-kHz  
870 capacitance data (C5 and C10; black and brown, respectively) and the deconvolution data (D; green).

871 **Ca<sup>2+</sup> uncaging with different pre-flash Ca<sup>2+</sup> concentrations indicates Ca<sup>2+</sup>-  
872 dependent vesicle priming**

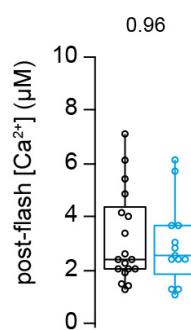
873 Finally, we aimed to obtain a mechanistic understanding that could explain both the strong  
874 dependence of action potential-evoked release on basal  $\text{Ca}^{2+}$  concentration (cf. Fig. 1)  
875 and the  $\text{Ca}^{2+}$ -dependence of vesicle fusion (cf. Figs. 2-6). In principle, the action  
876 potential-evoked data in Fig. 1 could be explained by an acceleration of vesicle fusion  
877 kinetics or, alternatively, an increase in the number of release-ready vesicles upon  
878 elevated basal  $\text{Ca}^{2+}$ . To differentiate between these two mechanistic possibilities, we  
879 investigated the effect of basal  $\text{Ca}^{2+}$  concentration preceding the UV illumination (pre-  
880 flash  $\text{Ca}^{2+}$ ) on flash-evoked release. The pre-flash  $\text{Ca}^{2+}$  concentration can only be reliably  
881 determined with the  $\text{Ca}^{2+}$  indicator Flou5F used in the experiments with weak flashes (see  
882 Supplementary Table 1). We therefore grouped the deconvolution experiments with weak  
883 flashes, which elevated the  $\text{Ca}^{2+}$  concentration to less than 5  $\mu\text{M}$ , into two equally sized  
884 groups of low and high pre-flash  $\text{Ca}^{2+}$  (below and above a value of 200 nM, respectively).  
885 Due to the presence of the  $\text{Ca}^{2+}$  loaded DMn cage, the pre-flash  $\text{Ca}^{2+}$  concentrations  
886 were on average higher than the resting  $\text{Ca}^{2+}$  concentration in physiological conditions of  
887 around 50 nM (Delvendahl et al., 2015). In both groups, the post-flash  $\text{Ca}^{2+}$  concentration  
888 was on average similar ( $\sim 3 \mu\text{M}$ ; Fig. 7B). The peak EPSC amplitude of postsynaptic  
889 current was significantly larger with high compared to low pre-flash  $\text{Ca}^{2+}$  concentration  
890 ( $38 \pm 10$  and  $91 \pm 16$  pA,  $n = 18$  and 13, respectively,  $P_{\text{Mann-Whitney}} = 0.001$ ; Fig. 7A and  
891 C). Correspondingly, the amplitude of the fast component of release as measured from  
892 deconvolution analysis was larger with high compared to low pre-flash  $\text{Ca}^{2+}$  ( $18 \pm 5$  and  
893  $49 \pm 10$ ,  $n = 18$  and 13, respectively,  $P_{\text{Mann-Whitney}} = 0.005$ ; Fig. 7C). However, the kinetics

894 of vesicle fusion, measured as the inverse of the time constant of the fast component of  
895 release, were not significantly different for both conditions ( $0.15 \pm 0.04$  and  $0.12 \pm 0.03$   
896  $\text{ms}^{-1}$  for the low and high pre-flash  $\text{Ca}^{2+}$  conditions,  $n = 18$  and  $13$ , respectively,  $P_{\text{Mann-}}$   
897  $\text{Whitney} = 0.74$ ; Fig. 7C). The delay was also not significantly different ( $P_{\text{Mann-Whitney}} = 0.54$ ;  
898 Fig. 7C). These data indicate that the number of release-ready vesicles were increased  
899 upon elevated basal  $\text{Ca}^{2+}$  concentration but the fusion kinetics were unaltered. We  
900 therefore added an additional  $\text{Ca}^{2+}$ -dependent maturation step to the initial vesicle  
901 priming of the release schemes (see methods; note that this was already present in the  
902 above-described simulations of Fig. 6 but it has little impact on these data). This allowed  
903 replicating the threefold increase in the action potential-evoked release when driving the  
904 release scheme with a previously estimated local  $\text{Ca}^{2+}$  concentration during an action  
905 potential (Fig. 7D; Delvendahl et al., 2015). Thus, the release schemes can explain the  
906  $\text{Ca}^{2+}$ -dependence of the recruitment, priming, and fusion of vesicles at mature cMFBs at  
907 physiological temperature.

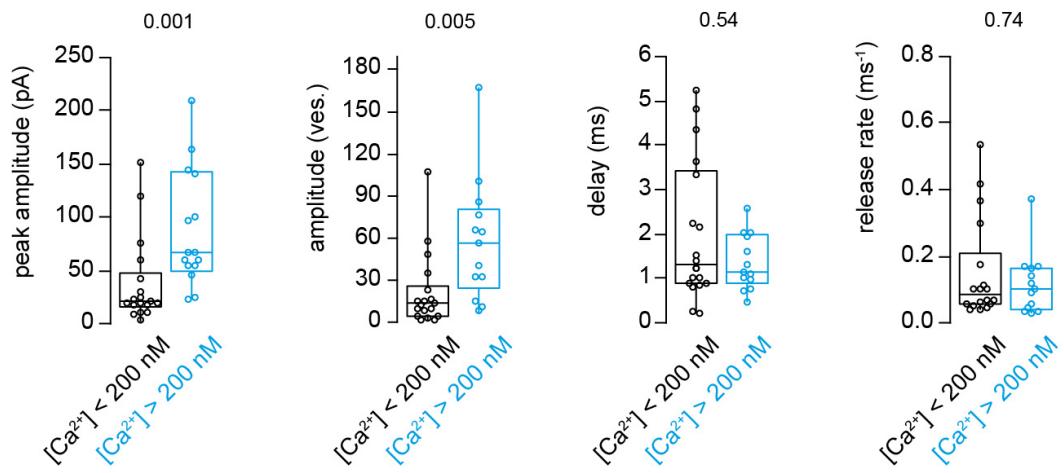
**A**



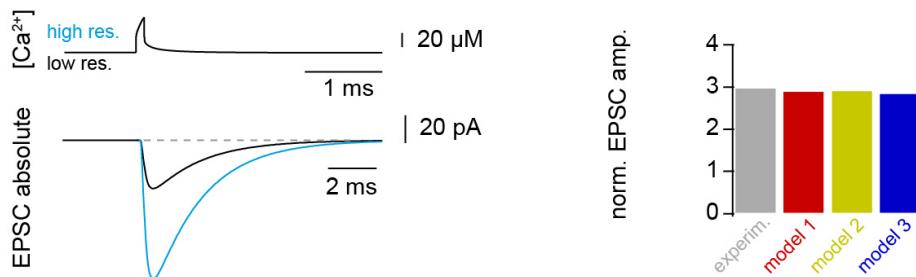
**B**



**C**



**D**



908 **Figure 7**  $Ca^{2+}$  uncaging with different pre-flash  $Ca^{2+}$  concentrations indicates  $Ca^{2+}$ -dependent  
909 vesicle priming

910 A. Two consecutive recordings from the same cell pair, with the same post-flash  $Ca^{2+}$  concentration but  
911 different pre-flash  $Ca^{2+}$  concentration in the presynaptic terminal. *Top*: postsynaptic current. *Bottom*:  
912 cumulative release of synaptic vesicles measured by deconvolution analysis of EPSCs superposed with a  
913 mono-exponential fit (magenta). Black and blue color represent low and high pre-flash  $Ca^{2+}$   
914 concentration, respectively. The pre- and post-flash  $Ca^{2+}$  concentrations are indicated in each panel.  
915 B. Comparison of the average post-flash  $Ca^{2+}$  concentration between both groups of either low or high  
916 pre-flash  $Ca^{2+}$  concentration (black and blue bars, respectively).

917 C. From left to right: comparisons of the peak amplitude, the number of released vesicles measured as  
918 obtained from deconvolution analysis of EPSC, the delay of the release onset, and the release rate.  
919 Boxplots show median and 1<sup>st</sup>/ 3<sup>rd</sup> quartiles with whiskers indicating the whole data range. The values  
920 above the boxplots represent P-values of Mann-Whitney U test.  
921 D. *Top left*: simulated local  $\text{Ca}^{2+}$  signal at 20 nm from the  $\text{Ca}^{2+}$  channel taken from Delvendahl et al.,  
922 2015. Note the almost complete overlap of the two  $\text{Ca}^{2+}$  concentration traces with low and high basal pre-  
923 flash  $\text{Ca}^{2+}$  concentration. *Bottom left*: prediction of the increase in the amplitude of action potential-evoked  
924 EPSC, upon elevating the basal  $\text{Ca}^{2+}$  concentration in the presynaptic terminal. *Right*: comparison  
925 between experimental data and the models' predictions of the effect of basal  $\text{Ca}^{2+}$  on the amplitude of the  
926 action potential-evoked release.

## 927 **Discussion**

928 Here, we provided insights into the  $\text{Ca}^{2+}$ -dependence of vesicle recruitment, priming, and  
929 fusion at cMFBs. The results obtained at this synapse show prominent  $\text{Ca}^{2+}$ -dependent  
930 priming steps, a shallow non-saturating dose-response curve up to 50  $\mu\text{M}$ , and  $\text{Ca}^{2+}$ -  
931 independent sustained vesicle recruitment. Our computational analysis indicates that the  
932 peculiar dose-response curve can be explained by well-established release schemes  
933 having five  $\text{Ca}^{2+}$  steps and rapid vesicle recruitment via sequential or parallel vesicle  
934 pools. Thus, we established quantitative scheme of synaptic release for a mature high-  
935 fidelity synapse, exhibiting both high- and low-affinity  $\text{Ca}^{2+}$  sensors.

## 936 **$\text{Ca}^{2+}$ affinity of the vesicle fusion sensor**

937 The  $\text{Ca}^{2+}$ -sensitivity of vesicle fusion seems to be synapse-specific. In contrast to the  
938 estimated  $\text{Ca}^{2+}$  affinity for vesicle fusion of  $\sim 100 \mu\text{M}$  at the bipolar cell of goldfish  
939 (Heidelberger et al., 1994) and the squid giant synapse (Adler et al., 1991; Llinás et al.,  
940 1992), recent studies showed that the affinity is much higher at three types of mammalian  
941 central synapses: the calyx of Held (Bollmann et al., 2000; Lou et al., 2005;  
942 Schneggenburger and Neher, 2000; Sun et al., 2007; Wang et al., 2008), the inhibitory  
943 cerebellar basket cell to Purkinje cell synapse (Sakaba, 2008), and the hippocampal  
944 mossy fiber boutons (Fukaya et al., 2021). Consistent with reports from mammalian  
945 central synapses, our data revealed prominent vesicle fusion at concentrations below  
946 5  $\mu\text{M}$  arguing for a high-affinity fusion sensor (Figs. 2-4). However, the non-saturation of  
947 the dose-response curve (Figs. 2-4) argues for the presence of a rather low-affinity fusion  
948 sensor at cMFBs. In our simulations, both model 2 and 3 exhibit vesicles with a  $\text{Ca}^{2+}$ -  
949 affinity similar to the calyx of Held. Nevertheless, with high intracellular  $\text{Ca}^{2+}$

950 concentrations ( $>20 \mu\text{M}$ ) these vesicles will fuse very rapidly and the further increase in  
951 the release kinetics (causing the non-saturating dose-response curve) can be explained  
952 by rapid vesicle recruitment from a sequential pool of vesicles exhibiting use-dependent  
953 lowering of the  $\text{Ca}^{2+}$ -affinity ( $V_1$  in model 2; Miki et al., 2018) or from a parallel pool of  
954 vesicles with lower  $\text{Ca}^{2+}$  affinity ( $V_1$  in model 3; Hallermann et al., 2010). Our data  
955 therefore indicate that the shallow and non-saturating dose-response curve is the  
956 consequence of rapid recruitment of vesicles that still exhibit a lower  $\text{Ca}^{2+}$ -affinity  
957 compared to fully recovered vesicles. Consistent with this interpretation, a lowering in the  
958  $\text{Ca}^{2+}$ -affinity of the vesicle fusion sensor has been observed at the calyx of Held with  $\text{Ca}^{2+}$   
959 uncaging following vesicle depletion (Müller et al., 2010; Wadel et al., 2007). These newly  
960 recruited vesicles might contribute particularly to the dose-response curve at the cMFB  
961 because the cMFB has a much faster rate of vesicle recruitment compared with the calyx  
962 of Held synapse (Miki et al., 2020) providing a possible explanation why the here-reported  
963 dose-response curve differs from previous results at the calyx of Held. Furthermore,  
964 cMFBs seem to have functional similarities with ribbon-type synapses because it has  
965 recently been shown that the vesicle mobility in cMFBs is comparable to ribbon-type  
966 synapses (Rothman et al., 2016). The hallmark of ribbon-type synapses is their rapid  
967 vesicle recruitment (Lenzi and von Gersdorff, 2001; Matthews, 2000) and indeed more  
968 shallow dose-response curves were obtained at the ribbon photoreceptors and inner hair  
969 cell synapses (Duncan et al., 2010; Heil and Neubauer, 2010; Johnson et al., 2010;  
970 Thoreson et al., 2004), but see (Beutner et al., 2001). Therefore, these results predict  
971 similar shallow non-saturating dose-response at other central synapses with rapid vesicle  
972 recruitment (Doussau et al., 2017; Miki et al., 2016; Pulido and Marty, 2017).

973  **$\text{Ca}^{2+}$ -sensitivity of vesicle priming**

974 The steps preceding the fusion of synaptic vesicles are in general still poorly understood  
975 (Südhof, 2013). There is evidence that some steps preceding the fusion are strongly  $\text{Ca}^{2+}$ -  
976 dependent (Neher and Sakaba, 2008), as has been demonstrated at chromaffin cells  
977 (Voets, 2000; Walter et al., 2013) and at several types of synapses such as the calyx of  
978 Held (Awatramani et al., 2005; Hosoi et al., 2007), the crayfish neuromuscular junctions  
979 (Pan and Zucker, 2009), parallel fiber to molecular layer interneuron synapses (Malagon

980 et al., 2020), and cultured hippocampal neurons (Chang et al., 2018; Stevens and  
981 Wesseling, 1998). In previous reports, the  $\text{Ca}^{2+}$ -dependence of vesicle priming at cMFBs  
982 was analyzed more indirectly with the  $\text{Ca}^{2+}$  chelator EGTA (Ritzau-Jost et al., 2014;  
983 Ritzau-Jost et al., 2018) and the obtained results could be explained by  $\text{Ca}^{2+}$ -dependent  
984 models but surprisingly also by  $\text{Ca}^{2+}$ -independent models (Hallermann et al., 2010;  
985 Ritzau-Jost et al., 2018). Furthermore, the analysis of molecular pathways showed that  
986 the recovery from depression is independent of the  $\text{Ca}^{2+}$ /calmodulin/Munc13 pathway at  
987 cMFBs (Ritzau-Jost et al., 2018). Our paired recordings and uncaging experiments (Figs.  
988 1 and 7) clearly demonstrate pronounced  $\text{Ca}^{2+}$ -dependence of vesicle priming at cMFBs.  
989 Taken together, these data indicate that some priming steps are mediated by  $\text{Ca}^{2+}$ -  
990 dependent mechanisms, which do not involve the  $\text{Ca}^{2+}$ /calmodulin/Munc13 pathway. A  
991 potential candidate for such a  $\text{Ca}^{2+}$ -dependent mechanism are the interaction of  
992 diacylglycerol/phospholipase C or  $\text{Ca}^{2+}$ /phospholipids with Munc13s (Lee et al., 2013;  
993 Lou et al., 2008; Rhee et al., 2002; Shin et al., 2010).

994 Here, we used single action potentials (Fig. 1) and weak uncaging stimuli (post-flash  $\text{Ca}^{2+}$   
995 concentration of  $\sim 3 \mu\text{M}$ ; Fig. 7) to investigate the impact of the basal  $\text{Ca}^{2+}$  concentration.  
996 Synaptic vesicles that fuse upon single action potentials and weak uncaging stimuli are  
997 particularly fusogenic and thus might represent the superprimed vesicles with a particular  
998 high release probability (Hanse and Gustafsson, 2001; Ishiyama et al., 2014; Kusch et  
999 al., 2018; Lee et al., 2013; Schlüter et al., 2006; Taschenberger et al., 2016) suggesting  
1000 that the process of superpriming is  $\text{Ca}^{2+}$ -dependent. This interpretation would also  
1001 provide an explanation why in a recent report, triggering an action potential in the range  
1002 of 10–50 ms time before another action potential (which elevates basal  $\text{Ca}^{2+}$   
1003 concentrations) restored the synchronicity of synaptic vesicle fusion in mutant synapses  
1004 which has a phenotype of synchronous-release-impairment (Chang et al., 2018). It would  
1005 be furthermore consistent with a proposed rapid, dynamic, and  $\text{Ca}^{2+}$ -dependent  
1006 equilibrium between primed and superprimed vesicles (Neher and Brose, 2018).  
1007 However, further investigations are needed for the dissection between the  $\text{Ca}^{2+}$ -  
1008 dependence of priming and superpriming. Yet, our data show that some priming steps

1009 are strongly  $\text{Ca}^{2+}$ -dependent with a high-affinity  $\text{Ca}^{2+}$  sensor that allow detecting changes  
1010 between 30 and 180 nM at cMFBs.

1011  **$\text{Ca}^{2+}$ -sensitivity of vesicle recruitment**

1012 The upstream steps of vesicle priming, referred to as recruitment, refilling, or reloading,  
1013 remain controversial in particular with respect to their speed. The slow component of  
1014 release (during prolonged depolarizations or  $\text{Ca}^{2+}$  elevations with uncaging) was initially  
1015 interpreted as a sub-pool of release-ready vesicles that fuse with slower kinetics (see e.g.  
1016 Sakaba and Neher, 2001). However, recent studies indicate very fast vesicle recruitment  
1017 steps (Blanchard et al., 2020; Chang et al., 2018; Doussau et al., 2017; Hallermann et  
1018 al., 2010; Lee et al., 2012; Malagon et al., 2020; Miki et al., 2016; Miki et al., 2018; Saviane  
1019 and Silver, 2006; Valera et al., 2012). These findings further complicate the dissection  
1020 between fusion, priming, and recruitment steps. Therefore, the differentiation between  
1021 ‘parallel’ release schemes with fast and slowly fusing vesicles and ‘sequential’ release  
1022 schemes with fast vesicle recruitment and subsequent fusion is technically challenging at  
1023 central synapses. Our data could be described by both sequential and parallel release  
1024 schemes (model 2 and 3; Fig. 6). The non-saturation of the release rate could be  
1025 described somewhat better by the parallel model 3. However, further adjustment of the  
1026 use-dependent slowing of the rates in model 2 (see  $k_{\text{on,plug}}$ ,  $k_{\text{off,plug}}$ , and eq. 3 and 4; Miki  
1027 et al., 2018) can result in a sequential model exhibiting both fast and slowly fusing vesicles  
1028 with different  $\text{Ca}^{2+}$ -sensitivity (see Mahfooz et al., 2016, for an alternative description of  
1029 use-dependence of vesicle fusion). Such use-dependent sequential models ultimately  
1030 complicate the semantic definitions of ‘sequential’ and ‘parallel’, because the newly  
1031 recruited vesicles will fuse in a molecularly different state, which could also be viewed as  
1032 a parallel pathway to reach fusion. Independent of the difficulty to differentiate between  
1033 sequential and parallel release schemes, the sustained component of release exhibited  
1034 little calcium dependence in the here-tested range between 1 and 50  $\mu\text{M}$  (Fig. 5). The  
1035  $\text{Ca}^{2+}$ -independence of vesicle recruitment in the investigated range is consistent with the  
1036 previously observed EGTA-independent slope of the sustained release during prolonged  
1037 depolarizations (Ritzau-Jost et al., 2014). Our data cannot differentiate if recruitment is  
1038 mediated by a fully saturated  $\text{Ca}^{2+}$  sensor for priming (mode 2; assumed  $K_d$  of 2  $\mu\text{M}$ ; Miki

1039 et al., 2018) or a parallel  $\text{Ca}^{2+}$ -independent step (mode 3). Thus, during sustained activity  
1040 at cMFBs vesicle recruitment is either mediated by fully  $\text{Ca}^{2+}$ -independent processes or  
1041 by an apparently  $\text{Ca}^{2+}$ -independent processes in the relevant  $\text{Ca}^{2+}$  concentration range  
1042 because of a saturated high-affinity  $\text{Ca}^{2+}$  sensor.

1043 **Mechanistic and functional implications**

1044 The  $\text{Ca}^{2+}$ -sensitivity of vesicle fusion critically impacts the estimates of the coupling  
1045 distance between  $\text{Ca}^{2+}$  channels and synaptic vesicles, mainly those obtained based on  
1046 functional approaches (Neher, 1998; Eggermann et al., 2011; but not on structural  
1047 approaches, see e.g. Éltes et al., 2017; Rebola et al., 2019). Our previous estimate of the  
1048 coupling distance at the cMFB of 20 nm (Delvendahl et al., 2015) was based on the  
1049 release scheme of Wang et al. (2008) obtained at the calyx of Held synapse at an age of  
1050 (P16-P19) at room temperature and assuming a  $Q_{10}$  factor of 2.5. The now estimated  $k_{on}$   
1051 and  $k_{off}$  rates at mature cMFBs at physiological temperature were slightly larger and  
1052 smaller than the temperature-corrected values from the calyx, respectively, resulting in a  
1053 slightly higher affinity of the fast releasing vesicles ( $V_2$  in model 2 and 3). Therefore, at  
1054 the cMFB, the coupling distance of the vesicles released by a single action potential is if  
1055 anything even smaller than the previous estimate of 20 nm.

1056 In addition, our data might provide a link between  $\text{Ca}^{2+}$ -dependent priming and facilitation.  
1057 Synaptotagmin-7 is a high-affinity  $\text{Ca}^{2+}$  sensor (Sugita et al., 2002) that could mediate the  
1058 here-reported three-fold increase in synaptic strength (Figs. 1 and 7). Synaptotagmin-7  
1059 has been proposed to play a role in synaptic facilitation at different synapses supporting  
1060 a molecularly distinct mechanism of facilitation (Jackman and Regehr, 2017). An increase  
1061 in the size of the fusogenic sub-pool of release-ready vesicles mediated by basal  $\text{Ca}^{2+}$   
1062 might provide the underlying mechanism where Synaptotagmin-7 could be a sensor for  
1063 the changes in basal  $\text{Ca}^{2+}$  levels and therefore affect synaptic strength (Liu et al., 2014).

1064 Finally, synaptic fidelity has been shown to increase with age at cMFBs (Cathala et al.,  
1065 2003), neocortical synapses (Bornschein et al., 2019), and the calyx of Held (Fedchyshyn  
1066 and Wang, 2005; Nakamura et al., 2015; Taschenberger and von Gersdorff, 2000).

1067 During high-frequency transmission, the residual  $\text{Ca}^{2+}$  concentration increases up to a  
1068 few  $\mu\text{M}$  at cMFBs (Delvendahl et al., 2015) but mature cMFBs can still sustain  
1069 synchronous release (Hallermann et al., 2010; Saviane and Silver, 2006). The  
1070 developmental decrease in the affinity of the release sensors observed at the calyx of  
1071 Held (Wang et al., 2008) and the here-reported shallow-dose-response curve at mature  
1072 cMFBs could be an evolutionary adaption of synapses to prevent the depletion of the  
1073 release-ready vesicles at medium  $\text{Ca}^{2+}$  concentrations and therefore allow maintaining  
1074 sustained synchronous neurotransmission with high fidelity (Matthews, 2000).

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