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1 **Analysis of neuronal Ca²⁺ handling properties by combining**
2 **perforated patch clamp recordings and the added buffer approach**
3

4
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11 Running Head: Improved analysis of Ca²⁺ handling

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34 **Keywords**

35 added buffer approach, β -escin, calcium-binding ratio, calcium buffering, cerebellum, fura-2,
36 midbrain, perforated patch clamp
37

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41

42 **Competing Interests**

43 The authors declare that they have no competing interests.
44

45 **Abstract**

46 Ca^{2+} functions as an important intracellular signal for a wide range of cellular processes.
47 These processes are selectively activated by controlled spatiotemporal dynamics of the free
48 cytosolic Ca^{2+} . Intracellular Ca^{2+} dynamics are regulated by numerous cellular parameters.
49 Here, we established a new way to determine neuronal Ca^{2+} handling properties by combining
50 the ‘added buffer’ approach (Neher and Augustine, 1992) with perforated patch-clamp
51 recordings (Horn and Marty, 1988). Since the added buffer approach typically employs the
52 standard whole-cell configuration for concentration-controlled Ca^{2+} indicator loading, it only
53 allows for the reliable estimation of the immobile fraction of intracellular Ca^{2+} buffers.
54 Furthermore, crucial components of intracellular signaling pathways are being washed out
55 during prolonged whole-cell recordings, leading to cellular deterioration. By combining the
56 added buffer approach with perforated patch-clamp recordings, these issues are circumvented,
57 allowing the precise quantification of the cellular Ca^{2+} handling properties, including
58 immobile as well as mobile Ca^{2+} buffers.

59

60 **Introduction**

61 This study combined perforated patch clamp recordings with ratiometric Ca^{2+} imaging
62 and the 'added buffer approach' to achieve significant improvements in the quantitative
63 analysis of neuronal Ca^{2+} handling. In neurons, Ca^{2+} serves as a ubiquitous signal in a wide
64 range of cellular processes, including the synaptic release of neurotransmitters, membrane
65 excitability, enzyme activation, and activity-dependent gene activation (Augustine et al.,
66 2003; Berridge, 1998; Clapham, 2007; Gilibert, 2020). These processes are selectively
67 regulated by controlled spatiotemporal dynamics of the free cytosolic Ca^{2+} (Berridge, 2006;
68 Berridge et al., 2000). Intracellular Ca^{2+} dynamics are regulated by numerous cellular
69 parameters (Ca^{2+} handling parameters) including Ca^{2+} influx through voltage- and ligand-
70 gated Ca^{2+} channels, Ca^{2+} release from intracellular stores, the location of the Ca^{2+} source,
71 Ca^{2+} buffering by mobile and immobile buffers, Ca^{2+} extrusion, locally changing diffusion
72 coefficients as well as the geometry of the cell (Helmchen et al., 1996; Kloppenburg et al.,
73 2000; Lin et al., 2017; Maravall et al., 2000; Neher and Augustine, 1992; Sabatini et al., 2002;
74 Tank et al., 1995). Impairment of this finely tuned system can cause neuronal dysfunction and
75 even neurodegeneration (Berridge, 2011; Chan et al., 2009). Neuronal Ca^{2+} handling
76 properties have been, up to now, analyzed by combining electrophysiological recordings and
77 Ca^{2+} imaging into what is referred to as the 'added buffer' approach (Neher and Augustine,
78 1992).

79 The added buffer approach is based on a single-compartment model with the following
80 rationale. During the measurement of intracellular Ca^{2+} dynamics with Ca^{2+} chelator-based
81 indicators, the amplitude and time course of the Ca^{2+} signals (free cytosolic Ca^{2+}
82 concentration) are influenced by the concentration of the Ca^{2+} indicator (e.g., fura-2), since
83 the indicator acts as an exogenous (added) Ca^{2+} buffer and competes with the endogenous
84 buffer(s). Accordingly, increasing indicator concentrations reduce the amplitude and prolong
85 the decay time constants of free intracellular Ca^{2+} fluctuations (Helmchen et al., 1996; Neher

86 and Augustine, 1992; Pippow et al., 2009). If the buffering capacity (Ca^{2+} binding ratio) of the
87 added buffer is known, the decay time constant can be used to estimate the Ca^{2+} signal
88 kinetics in the absence of exogenous buffer. The model of the original added buffer approach
89 (Neher and Augustine, 1992) provides estimates of Ca^{2+} handling parameters such as the free
90 cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), the Ca^{2+} extrusion rate (γ), the Ca^{2+} binding ratio (κ_B),
91 and the endogenous decay time constant (τ_{endo}).

92 The added buffer approach relies on information about the precise exogenous Ca^{2+}
93 buffer concentration in the neuron. Typically, the Ca^{2+} indicator concentration can be
94 determined at any time during the experiment from loading curves. The neurons are loaded
95 *via* the patch pipette, whose solution contains a known Ca^{2+} indicator concentration. During
96 loading, fluorescence is monitored continuously, and it is assumed that when the fluorescence
97 reaches a plateau, the indicator concentrations in the cell and the pipette are equal. Loading is
98 usually performed in the whole-cell patch clamp configuration since this recording
99 configuration provides low access resistance, ensuring that the solution in the patch pipette
100 can freely interchange with the cytoplasm. Thus, the whole-cell configuration is ideally suited
101 to “control” the composition of the intracellular milieu, including the Ca^{2+} indicator (added
102 Ca^{2+} buffer) concentration (what is in the patch pipette ends up being what is in the neuron).
103 However, the free exchange of molecules between cytoplasm and patch pipette disturbs
104 intracellular signaling by washing out components of the cytosolic signaling system,
105 including, for example, mobile cytosolic Ca^{2+} buffers. This not only impairs neuronal function
106 but also causes estimation errors of the Ca^{2+} handling properties (Akaike, 1994; Akaike and
107 Harata, 1994; Delvendahl et al., 2015; Lindau and Fernandez, 1986; Matthews et al., 2013).

108 For pure electrophysiological recordings, this issue can be minimized or even ignored
109 by using the perforated patch clamp configuration originally introduced by Horn and Marty
110 (Horn and Marty, 1988). In this recording configuration, electrical access between the
111 recording pipette and the intracellular space is mediated by perforating agents that form

112 channels or pores in the membrane. The washout of cytosolic components and the disruption
113 of intracellular signaling pathways are then drastically reduced compared to the whole-cell
114 configuration (Korn and Horn, 1989). The original and most commonly used perforating
115 agents have been the antibiotic polyenes nystatin and amphotericin B, and the antibiotic
116 polypeptide gramicidin (Akaike, 1994; Akaike and Harata, 1994; Hess et al., 2013; Horn and
117 Marty, 1988; Klöckener et al., 2011; Könner et al., 2011; Kyrozis and Reichling, 1995). While
118 polyenes and the peptide exhibit differences in their pore-forming mechanisms and ion
119 selectivities (De Kruijff and Demel, 1974; Myers and Haydon, 1972; Russell et al., 1977;
120 Tajima et al., 1996), their pores share key common properties: they are permeable to small
121 molecules with a molecular weight up to ~200 Da, including monovalent ions (Kruijff et al.,
122 1974; Kyrozis and Reichling, 1995; Urry, 1971), but they are neither permeable to divalent
123 ions like Ca^{2+} nor to Ca^{2+} indicators like fura-2 (whose molecular weight is >600 Da). While
124 the combination of perforated patch clamp recordings and the added buffer approach would
125 be ideal, it cannot be implemented with the previous standard small pore-forming perforating
126 agents.

127 To alleviate the “small pore size” problem, we tried β -escin as the perforating agent. β -
128 escin is a saponin (Sirtori, 2001) and is structurally not related to the previous perforating
129 agents. The action of saponins on biological membranes is concentration-dependent and
130 complex. Most models of its action propose that membrane permeabilization is mediated by
131 cholesterol complexation (formation of saponin-cholesterol complexes), which disturbs the
132 normal membrane structure or, at high concentrations, removes the cholesterol from the
133 membrane (Bangham et al., 1962; Böttger and Melzig, 2013). Since the pore size is generally
134 larger and accordingly less selective compared to the ones of nystatin, amphotericin B and
135 gramicidin, molecules of higher molecular weight (e.g., fluo-3, MW 770 Da) can pass through
136 the cell membrane (Fan and Palade, 1998; Orta et al., 2012). Nevertheless, β -escin
137 permeabilized membrane still constitutes an effective diffusion barrier to larger molecules like

138 Ca^{2+} binding proteins (Fan and Palade, 1998; Sarantopoulos et al., 2004). In previous studies,
139 β -escin has been successfully used to permeabilize smooth muscle cells (Kobayashi et al.,
140 1989) and β -escin based perforated patch clamp recordings have been performed on cardiac
141 myocytes (Fan and Palade, 1998) and different neuron types (Inoue et al., 2014;
142 Sarantopoulos et al., 2004).

143 Here, we combined β -escin based perforated patch clamp recordings with the added
144 buffer approach to measure intracellular Ca^{2+} handling properties. A thorough quantitative
145 analysis of the data showed that this approach leads to a significantly improved quantification
146 of the Ca^{2+} handling properties of individual neurons from brain slices of adult mice. The core
147 experiments were performed on *substantia nigra* (SN) dopaminergic (DA) neurons from brain
148 slices of adult mice. SN DA neurons are endogenous pacemakers and rely very heavily on
149 intact, precisely orchestrated intracellular pathways, especially cytosolic Ca^{2+} signaling.
150 Additionally, we performed a set of immunohistochemical experiments in cerebellar Purkinje
151 cells, a cell type which is known for containing very high concentrations of calbindin D-28k,
152 a mobile Ca^{2+} binding protein (Abe et al., 1992).

153

154 **Results**

155 The goal of this study was to implement the ‘added buffer approach’, a powerful
156 method to determine neuronal Ca^{2+} handling properties, in combination with perforated patch
157 clamp recordings, which allow for electrophysiological recordings without compromising
158 intracellular signaling. Since the typical perforating agents, nystatin, amphotericin B, and
159 gramicidin, do not allow controlled loading of Ca^{2+} indicators, we explored the suitability of
160 β -escin for this purpose. This study was performed in brain slices of mice, mainly on DA SN
161 neurons. These neurons are particularly well suited to test this experimental approach since
162 they are endogenous pacemakers (Fig. 1) that strongly rely on intact, precisely controlled Ca^{2+}
163 signaling (Bean, 2007; Lacey et al., 1989; Richards et al., 1997).

164 The first part of the study shows that β -escin enables long-lasting, high quality
165 perforated, patch clamp recordings with minimal rundown. The quality of these recordings is
166 comparable to amphotericin B perforated recordings. In the second part, we show that β -escin
167 perforated patch clamp recordings allow controlled Ca^{2+} indicator loading and can
168 significantly improve the added buffer approach to analyze the Ca^{2+} handling properties of
169 individual neurons. We achieved controlled fura-2 loading with a minimized impact on the
170 integrity of the cells' cytoplasmic components. A thorough quantitative analysis of the data
171 shows that this approach significantly improves the quantification of Ca^{2+} handling properties
172 in single neurons of adult animals compared to conventional whole-cell recordings. The
173 versatility of this new approach is complemented by immunohistochemical stainings of
174 cerebellar Purkinje cells, which possess very high concentrations of the mobile Ca^{2+} -binding
175 protein calbindin D-28k. Here we could show that calbindin D-28k is retained during β -escin
176 perforated patch clamp recordings, which is in stark contrast to results obtained during whole-
177 cell patch clamp recordings.

178

179 *β -escin as perforating agent for perforated patch clamp recordings*

180 After GΩ-seal formation, the perforation of the cell membrane was monitored by
181 continuously measuring the membrane potential and the amplitude of the action potentials.
182 Initially, ‘on-cell’ action potentials were observed in the cell-attached configuration (Fig. 2A).
183 Diffusion of β-escin into the membrane gradually changed the recording to the perforated-
184 patch configuration, decreasing the series resistance and increasing the action potential
185 amplitude (Fig. 2A from left to right). The perforation process was considered fully
186 established when action potential amplitude and series resistance (R_S) had stabilized. Under
187 our experimental conditions, we observed that indicator loading started within ~5 min of seal
188 formation, and perforation was fully established after ~20 min (Fig. 2B).

189

190 *Comparison with other patch clamp configurations*

191 To compare the β-escin mediated recordings with other configurations of patch clamp
192 recordings, we analyzed whether and how important intrinsic neurophysiological parameters
193 such as spontaneous or evoked action potential firing, membrane potential, and resting Ca^{2+}
194 levels ($[Ca^{2+}]_i$) are affected by the different recording configuration (whole-cell recordings,
195 and amphotericin B- and β-escin perforated patch recordings). After establishing recordings
196 with stable access (as judged by the series resistance), spontaneous and evoked action
197 potential firing was monitored for at least 30 min. During whole-cell recordings, we observed
198 a dramatic rundown of these parameters. As expected, they did not change during
199 amphotericin B perforated-patch recordings. β-escin perforated-patch recordings gave results
200 similar to amphotericin B mediated recordings, and only a small decline of the spontaneous
201 firing was observed (Fig. 3A-D).

202 More specifically, during whole-cell recordings, more than 80% of the cells stopped
203 spontaneous firing within 10 min after break-in (Fig. 3A, C). This loss of activity was
204 accompanied by a ~4 fold increase in the resting $[Ca^{2+}]_i$ levels after 30 min of recording time

205 (Fig. 4). This substantial rise in $[Ca^{2+}]_i$ was a clear and direct indicator for the deteriorating
206 physiological state of the neurons (Kuchibhotla et al., 2008; Mattson, 2007) and showed
207 directly that the Ca^{2+} handling properties change during whole-cell recordings. In contrast,
208 $[Ca^{2+}]_i$ remained stable during β -escin perforated patch recordings over the same time (Fig 4).
209 Taken together, β -escin perforated patch clamp recordings significantly outperformed whole-
210 cell recordings, and β -escin perforated patch clamp recordings were of similar quality to
211 amphotericin B mediated recordings. Overall, the data are in line with many earlier studies
212 (e.g. (Horn and Marty, 1988; Husch et al., 2011; Lindau and Fernandez, 1986)) which clearly
213 show the impact of whole-cell recordings on the cytosolic integrity and to what extend
214 perforated patch clamp can minimize this impact (rundown), and thus are suitable to analyze
215 ‘true’ intrinsic neurophysiological parameters over long periods of time.

216

217 *Permeability of β -escin mediated pores*

218 Next, we tested if the β -escin perforated membranes are permeable to fura-2 and to
219 molecules with a MW in the range of Ca^{2+} buffering proteins, like calbindin D-28k. To clarify
220 this question, tetramethylrhodamine-dextran (TRD-40kDa, MW 40 kDa) or fura-2 was added
221 to the pipette solution, and the fluorescence in the cell body was monitored during the
222 recording. During β -escin perforated-patch recordings, the TRD-40kDa remained in the
223 recording pipette, while it diffused into the neuron during whole-cell recordings (Fig 5).
224 Furthermore, immunohistochemical staining showed that the endogenous calbindin-D28k
225 remains in the cell during β -escin perforated patch clamp recordings but is washed out during
226 whole-cell recordings (Supplement Fig. 1). In contrast, the loading curves for fura-2 (Fig. 6A)
227 confirmed that fura-2 could diffuse readily through the β -escin perforated membrane into the
228 cell. These results suggested that β -escin is well suited to implement the added buffer
229 approach.

230

231 *Ca²⁺ handling properties*

232 Given the stable and reliable recording conditions provided by β -escin, we combined β -
233 escin perforated patch clamp recordings with ratiometric fura-2 Ca^{2+} imaging to analyze Ca^{2+}
234 handling properties. Using the added buffer approach, we determined the resting Ca^{2+}
235 concentration ($[\text{Ca}^{2+}]_i$), the Ca^{2+} binding ratio κ_S , and the extrusion rate γ of SN DA neurons,
236 which display Ca^{2+} dependent endogenous pacemaking properties (Liu et al., 2014; Neuhoff
237 et al., 2002; Smits et al., 2013). Neurons were held at -70 mV to avoid spontaneous spiking.
238 Clear and reproducible Ca^{2+} influx could be induced by trains of action potentials elicited by
239 current pulses (500 pA, 500 ms) (Fig. 6B). The resulting intracellular Ca^{2+} signals were
240 monitored by ratiometric Ca^{2+} imaging (Fig 6C), and the data quality was compared with data
241 acquired with conventional whole-cell patch clamp recordings.

242 Fura-2 loading: The intracellular concentration of the exogenous buffer at any time
243 during the experiment was determined from the loading curves of the calcium indicator (Fig.
244 6A). DA SN neurons were loaded *via* the patch pipette with solutions containing 100 – 200
245 μM fura-2. During dye loading, the fluorescence of the cell body was monitored in 30 s
246 intervals using excitation at 360 nm (isosbestic point) to determine the dye concentration.
247 After the β -escin perforated-patch configuration was fully established, fluorescence appeared
248 in the cell bodies, and fluorescence intensity increased until it reached a stable plateau. The
249 intracellular fura-2 concentration was estimated for different times during the loading curve,
250 assuming that cells were fully loaded when the fluorescence reached a plateau and the fura-2
251 concentration in the cell and the pipette is equal at this point (for details see Methods).

252 Ca^{2+} handling properties: If the buffer capacity of the added buffer is known, the time
253 constant of the transient decay ($\tau_{\text{transient}}$) can be used to estimate by extrapolation the Ca^{2+}
254 signal to conditions, with only endogenous buffers present ($-\kappa_B = 1 + \kappa_S$). The model used for
255 this study (Eq. (14), (Neher and Augustine, 1992) assumes that the decay time constants
256 $\tau_{\text{transient}}$ are a linear function of the Ca^{2+} binding ratios (κ_B and κ_S). κ_S was determined from the

257 negative x-axis intercept of a $\tau_{\text{transient}}$ over κ_{S} plot (Fig. 6D). The slope of the straight-line fit is
258 the inverse of the extrusion rate (γ). The point of intersection of the linear fit with the y-axis
259 denotes the endogenous decay time constant τ_{endo} (no exogenous Ca^{2+} buffer in the cell). This
260 is demonstrated in Fig. 6 with an example of a DA SN neuron with following Ca^{2+} handling
261 parameters: τ_{endo} : 0.7 ± 0.3 s, γ : 91.5 ± 10.4 , s^{-1} , κ_{S} : 66.4 ± 24.7 .

262

263 *Comparison of data quality between whole-cell and β -escin perforated patch clamp*
264 *recordings*

265 The data quality of the Ca^{2+} imaging experiments used to determine the Ca^{2+} handling
266 properties was markedly better for the β -escin perforated-patch configuration compared to
267 whole-cell recordings. Obvious evidence of "low-quality" data were implausible results such
268 as negative Ca^{2+} binding coefficients or Ca^{2+} transients that did not return to baseline as
269 frequently observed by us and previous studies (Fierro and Llano, 1996; Pippow et al., 2009).
270 For data sets that did not show such obvious flaws, an analysis on a finer scale confirmed the
271 significantly higher data quality of the β -escin perforated records. For this purpose,
272 parameters, which characterize the quality of data sets of individual neurons, were quantified.
273 This included the goodness of fit for each Ca^{2+} transient (a mono-exponential fit) and the
274 linear regression that was used to fit the $\tau_{\text{transient}} - \kappa_{\text{B}}$ relation.

275 First, we tested the goodness of the mono-exponential fit for each Ca^{2+} transient. Since
276 our analysis method yields a meaningful standard error for the estimated $[\text{Ca}^{2+}]_{\text{i}}$ (see
277 Methods), a fit obtained with a weighted nonlinear least-square produces standard errors for
278 the estimated parameters (basal $[\text{Ca}^{2+}]_{\text{i}}$, $\Delta[\text{Ca}^{2+}]_{\text{i}}$ and, most importantly here, $\tau_{\text{transient}}$) as well
279 as residuals following a known distribution if the data do indeed follow the model; namely the
280 residual sum of squares (RSS) should be the realization of a draw from a χ^2 distribution with a
281 known number of degrees of freedom, and the residuals should not be correlated. We decided

282 to keep a transient for further analysis if both the p-values of the RSS and the auto-correlation
283 at lag 1 were larger than 0.01 (giving a probability of false-negative of 0.02). Since the decay
284 fits were started once the transient had lost 50% of the peak increase (see Materials and
285 Methods), the number of data points used for each fit is not fixed, meaning that the number of
286 degrees of freedom of the χ^2 distribution associated with each RSS is also changing. A proxy
287 of the RSS that is easy to compare from transient to transient is the RSS per degree of
288 freedom (that should be close to 1 for a good fit) and is illustrated in Fig. 7C. This goodness
289 of fit criterium was fulfilled by 75% (24/32) of the transients measured in the whole-cell
290 configuration compared to 92% (67/73) of the transient measured in the perforated
291 configuration (Fig. 7D; using an exact test, based on the hypergeometric distribution, the
292 probability that these two samples arise from the same underlying distribution is found to be
293 smaller than 0.02). We then used the data sets with at least three ‘good’ transients to perform
294 the linear regression ($\tau_{\text{transient}} - \kappa_B$ relation) and found that 15/15 (100 %) data sets with
295 perforated recordings lead to an acceptable regression but only 2/6 (33 %) of those with
296 whole-cell recordings (Fig. 7G). Thus, while experiments in the whole-cell configuration can
297 yield results comparable to β -escin experiments (Fig. 8A [*right panel*]; κ_s : 19.9 ± 25.1 , τ_{endo} :
298 0.4 ± 0.5 s, γ : 55.2 ± 10.4 s⁻¹), it is evident that in many cases the whole-cell results were
299 implausible (Fig. 8A, *middle and left panel*). In these cases, the linear regression fit of the
300 data intercepts the x-axis at positive value leading to a negative Ca²⁺ binding ratio κ_s and a
301 negative endogenous decay time constant τ_{endo} , respectively (see (Delvendahl et al., 2015))(κ_s :
302 -72.9 ± 14.3 , τ_{endo} : -17.9 ± 2.7 s, γ : 4.0 ± 0.5 s⁻¹, *middle panel*; κ_s : -3.1 ± 10.3 , τ_{endo} : $-0.04 \pm$
303 0.2 s, γ : 58.0 ± 4.4 s⁻¹, *left panel*). It is important to note that the implausibility of the results
304 from individual cells can be masked if pooled data sets from multiple neurons are analyzed
305 (Fig. 8C and D).

306 In summary, the overall quality of the data obtained in β -escin perforated patch clamp
307 experiments is tremendously improved compared to experiments in the whole-cell

308 configuration. This improvement results from two factors. The perforated configuration
309 preserves the general integrity and functionality of the cytoplasmic pathways, including the
310 Ca^{2+} handling machinery. In addition, it optimizes the measurements directly by preventing an
311 uncontrolled change in mobile Ca^{2+} buffer during the recording. Ultimately, this resulted in a
312 far more reliable and better-defined estimation of the Ca^{2+} handling properties of single
313 neurons.
314

315 **Discussion**

316 Using β -escin as a perforating agent, we showed that it is possible to combine
317 longlasting perforated-patch recordings with controlled Ca^{2+} indicator loading and the ‘added
318 buffer approach’ to analyze neuronal Ca^{2+} handling properties. We consider this a substantial
319 progress, and of particular importance for the analysis of intracellular Ca^{2+} handling since the
320 perforated-patch clamp configuration allows stable recordings with minimized impact on the
321 intracellular signaling system, including mobile components of the Ca^{2+} handling machinery,
322 i.e., mobile Ca^{2+} buffers. β -escin perforated recordings performed similarly well as
323 amphotericin B, gramicidin, or nystatin perforated patch recordings, which are the ‘gold
324 standards’ for stable long-term recordings (Horn and Marty, 1988; Husch et al., 2011; Rae et
325 al., 1991). β -escin perforated patch recordings clearly outperformed conventional whole-cell
326 recordings, which becomes especially apparent by monitoring the Ca^{2+} levels. In contrast to
327 the whole-cell configuration, the resting Ca^{2+} concentration remained stable, indicating
328 healthy recording conditions.

329 Typically, the ‘added buffer’ approach has been used in combination with conventional
330 whole-cell patch clamp recordings, which allows the controlled loading of cells with Ca^{2+}
331 indicator dyes (e.g., fura-2) via the patch pipette (Delvendahl et al., 2015; Lee et al., 2000a;
332 Lin et al., 2017; Neher and Augustine, 1992). In this context, the whole-cell configuration has
333 the obvious advantage that it allows for easy and reliable loading of the cell with Ca^{2+}
334 indicators. However, this advantage is offset by the disadvantage that intracellular
335 components are being washed out, leading to a gradual change of cellular properties over
336 time. This causes not only general damaging effects on the physiology and Ca^{2+} handling
337 machinery of the cell, but it also introduces a systematic error to the analysis, since in the
338 whole-cell configuration endogenous mobile Ca^{2+} buffer is leaving the neuron at an unknown
339 rate during loading of the ‘added’ buffer (here fura-2). Although this is not a new finding,
340 since Neher and Augustine had already explicitly mentioned this matter in their original paper

341 (Neher and Augustine, 1992), and although many studies have applied the added buffer
342 approach in combination with whole-cell patch clamp recordings, this issue has not been
343 addressed directly. In a strict sense, the added buffer approach in combination with the whole-
344 cell configuration can only be used to analyze the immobile fraction of Ca^{2+} buffers and only
345 if the measurements start *after* the mobile Ca^{2+} buffers have diffused out of the cell and do not
346 interfere with the analysis. Accordingly, this approach can be explicitly used to analyze the
347 immobile Ca^{2+} buffers as convincingly shown by Müller et al. (2005) and Matthews et al.
348 (2013) in hippocampal neurons.

349 Employing β -escin as perforating agent addressed this issue and allowed to successfully
350 combine perforated patch clamp recordings with controlled Ca^{2+} indicator loading, and thus
351 the added buffer approach. While our and other's whole-cell experiments (Delvendahl et al.,
352 2015; Matthews et al., 2013; Müller et al., 2005) showed that molecules with a molecular
353 weight comparable to Ca^{2+} binding proteins started being washed out immediately after
354 establishing the whole-cell configuration, our permeability measurements showed that the β -
355 escin perforated membranes retained TRD-40kDa in the recording pipette and calbindin D-
356 28k in the neuron, indicating that the perforated membrane is impermeable for the relative
357 large endogenous mobile Ca^{2+} buffers. Fura-2, on the other hand, with its low molecular
358 weight, could easily permeate through the perforated membrane.

359 For practical purposes, it is important to note that the permeability of the perforated
360 membrane is concentration-dependent. Thus, the β -escin concentration might have to be
361 adjusted to different experimental conditions and cell types. Here, with β -escin concentrations
362 of 10–15 μM , the perforated membrane was permeable to fura-2 but not to TRD-40kDa and
363 calbindin D-28k. In permeabilized muscle cells, β -escin concentrations of 50 – 100 μM render
364 cell membranes permeable to molecules ranging from 10 to 150 kDa MW (Iizuka et al., 1997,
365 1994; Konishi and Watanabe, 1995). In recordings of cardiac myocytes with 50 μM β -escin,
366 the membrane was permeable for fluorescent markers with a MW up to 10 kDa after 20 – 25

367 min recording time (Fan and Palade, 1998).

368 Taken together, this study shows that the newly established approach is suitable to
369 improve the quantitative analysis of intracellular Ca^{2+} handling parameters markedly. The
370 combination of perforated patch clamp recordings, which preserve the integrity and
371 functionality of the intracellular milieu, with the ‘added buffer approach’, is a powerful tool to
372 analyze mobile and immobile components of the intracellular Ca^{2+} handling machinery.

373

374 **Materials and Methods**

375 *Animals and brain slice preparation*

376 All animal procedures were conducted in compliance with protocols that were approved
377 by local government authorities (LANUV NRW, Recklinghausen, Germany). Mice were
378 housed at 22 – 24 °C with a 12-hour light/12 hour dark cycle. Animals had access to water and
379 chow *ad libitum*. Experiments were performed on brain slices from 9 – 11 weeks old male
380 C57BL/6 mice. Preparation of brain slices, recordings from DA SN and Purkinje neurons,
381 labeling, and identification of neurons was performed as previously described (Hess et al.,
382 2013; Könnner et al., 2011; Murru et al., 2019). The mice were lightly anesthetized with
383 isoflurane (B506; AbbVie Deutschland GmbH & Co KG, Ludwigshafen, Germany) and
384 subsequently decapitated. Coronal slices (250 – 300 µm) containing the SN or sagittal slices
385 containing the cerebellum (300 µm) were cut with a vibration microtome (HM-650 V;
386 Thermo Scientific, Walldorf, Germany) under cold (4°C), carbogenated (95% O₂ and 5%
387 CO₂), glycerol-based modified artificial cerebrospinal fluid (GaCSF; (Ye et al., 2006)).
388 GaCSF contained (in mM): 250 Glycerol, 2.5 KCl, 2 MgCl₂, 2 CaCl₂, 1.2 NaH₂PO₄, 10
389 HEPES, 21 NaHCO₃, 5 Glucose, adjusted to pH 7.2 with NaOH. Brain slices were transferred
390 into carbogenated artificial cerebrospinal fluid (aCSF). aCSF contained (in mM): 125 NaCl,
391 2.5 KCl, 2 MgCl₂, 2 CaCl₂, 1.2 NaH₂PO₄, 21 NaHCO₃, 10 HEPES, 5 glucose, adjusted to pH
392 7.2 with NaOH. First, they were kept for 20 min in a 35°C 'recovery bath' and then stored at
393 room temperature (~24°C) for at least 30 min prior to recording. During the recordings, the
394 slices were continuously superfused with carbogenated aCSF. To block synaptic currents,
395 aCSF contained 10⁻⁴ M PTX, 5 x 10⁻⁵ M D-AP₅, and 10⁻⁵ M CNQX. All recordings were
396 carried out at 24°C.

397

398 *Electrophysiological recordings*

399 DA SN neurons and the cerebellar Purkinje cells were recorded using whole-cell and
400 perforated patch clamp recordings. Neurons were visualized and imaged with a fixed stage
401 upright microscope (BX51WI, Olympus, Hamburg, Germany) using a 20× water-immersion
402 objective (XLUMPLFL, 0.95 numerical aperture, 2 mm working distance, Olympus) with a
403 4× magnification changer (U-TVAC, Olympus), infrared differential interference contrast
404 optics (Dodt and Zieglgänsberger, 1990) and fluorescence optics. Neurons were pre-identified
405 by the size and location of their somata. This pre-identification was verified in each case by a
406 physiological characterization during the recording and *post hoc* by immunohistochemistry
407 for cell type-specific markers. DA SN neurons were recorded in the ventral tier SN. They
408 have a low frequency, regular firing patterns with broad action potentials, and generate a clear
409 I_h -dependent “sag”-potential upon hyperpolarization (Fig. 1) (Lacey et al., 1989; Richards et
410 al., 1997; Ungless et al., 2001). Purkinje cells were identified by their distinct morphology
411 and localization within the cerebellar cortex and by their high-frequency spontaneous firing
412 and very narrow action potentials (~200 μ s; Supplement Fig. 1A) (Bean, 2007; McKay and
413 Turner, 2005, 2004)). During the experiments, biocytin (0.1%; B4261; Sigma) diffused
414 through the broken or perforated membrane into the recorded cell. Post recording, the identity
415 of DA SN neurons and cerebellar Purkinje cells was confirmed by their immunoreactivity for
416 the dopamine transporter (Fig. 1) (Ciliax et al., 1995) or the calcium-binding protein calbindin
417 D-28k (Supplement Fig. 1) (Abe et al., 1992; Fournet et al., 1986), respectively. Biocytin-
418 streptavidin labeling combined with dopamine transporter or calbindin D-28k
419 immunohistochemistry was performed as previously described (Hess et al., 2013; Murru et
420 al., 2019).

421

422 *Whole-cell patch and perforated patch-clamp recordings*

423 Electrodes with tip resistances between 3 and 5 M Ω were fashioned from borosilicate

424 glass (0.86 mm inner diameter; 1.5 mm outer diameter; GB150-8P; Science Products,
425 Hofheim, Germany) with a vertical pipette puller (PP-830; Narishige, London, UK).
426 Recordings were performed with an EPC10 patch-clamp amplifier (HEKA, Lambrecht,
427 Germany) controlled by the program PatchMaster (version 2.32; HEKA) running under
428 Windows. Data were sampled at 10 kHz and low-pass filtered at 2 kHz with a four-pole
429 Bessel filter. The calculated liquid junction potential between intracellular and extracellular
430 solution was also compensated (14.6 mV for normal aCSF, calculated with Patcher's Power
431 Tools plug-in from <http://www.mpibpc.mpg.de/groups/neher/index.php?page=software> for
432 IGOR Pro 6 [Wavemetrics, Lake Oswego, OR, USA]).

433 Whole-cell recordings were performed with pipette solution containing (in mM): 141 K-
434 gluconate, 10 KCl, 10 HEPES, 0.1 EGTA, 2 MgCl₂, 3 K-ATP, 0.3 Na-ATP and adjusted to pH
435 7.2 with KOH. For perforated patch clamp recordings and Ca²⁺ imaging, ATP and GTP were
436 omitted from the pipette solution since ATP might distort the measurement of Ca²⁺ handling
437 properties (Matthews et al., 2013) and to prevent uncontrolled permeabilization of the cell
438 membrane during perforated patch clamp recordings (Lindau and Fernandez, 1986). For Ca²⁺
439 imaging, EGTA was replaced by 100 or 200 μM fura-2 (pentapotassium salt, F1200,
440 Molecular Probes, OR, USA).

441 Perforated patch recordings were performed using protocols modified from Fan and
442 Palade (Fan and Palade, 1998) and Sarantopoulos (Sarantopoulos et al., 2004). The patch
443 pipette was tip filled with internal solution and backfilled with β-escin (~30 - 60 μg·ml⁻¹;
444 E1378; Sigma) or amphotericin B (~200 μg·ml⁻¹; A4888, Sigma) containing internal solution
445 to achieve perforated patch recordings. β-escin was prepared as a stock solution (25 mM in
446 internal solution) and stored up to 1 week at -20°C protected from light. Shortly before the
447 experiments, the stock solution was diluted in intracellular saline to a final concentration.
448 Amphotericin B was dissolved in dimethyl sulfoxide (DMSO; final concentration 0.4 – 0.5%;
449 D8418, Sigma) as described previously (Hess et al., 2013; Kyrozis and Reichling, 1995; Rae

450 et al., 1991) and added to the modified pipette solution shortly before use. The amphotericin
451 B solution was freshly prepared every day. Ionophore containing solutions were stored on ice
452 and replaced every 4 hours as needed. After obtaining a $G\Omega$ seal, the perforation process was
453 monitored by continually measuring the access resistance (R_a). Experiments were started after
454 R_a had reached steady-state, and the action potential amplitude was stable (20 – 30 min). A
455 rupture of the membrane and the resulting conversion to the whole-cell configuration was
456 indicated by abrupt changes in R_a or action potential amplitude. Such experiments were
457 rejected.

458

459 *Imaging set up for fluorometric measurements*

460 The imaging setup for fluorometric measurements consisted of an Imago/SensiCam
461 CCD camera with a 640x480 chip (Till Photonics, Gräfelfing, Germany) and a Polychromator
462 IV (Till Photonics) that was coupled *via* an optical fiber into the upright microscope
463 (microscope details are provided above). The camera and the polychromator were controlled
464 by the software Vision (version 4.0, Till Photonics) running under Windows. The excitation
465 light from the polychromator was reflected onto the cells with a dichroic mirror, and emitted
466 fluorescence was detected through an emission filter. The specific filter and mirror settings for
467 the different experiments are given below. Data were acquired as 80x60 frames using 8x8 on-
468 chip binning. Images were recorded in arbitrary units (AU) and stored and analyzed as 12-bit
469 grayscale images. For analyzing the fluorescence dynamics, the mean AU values of regions of
470 interest (ROIs) from the middle of the cell bodies were used. 'Background subtraction' for the
471 time series was performed with equivalent time series of neighboring ROIs.

472

473 *Rhodamine-Dextran loading.*

474 Loading experiments with fluorescent tetramethylrhodamine-dextran (TRD-40kDa,
475 MW 40 kDa, D1842, Life Technologies) were performed in the standard whole-cell or the

476 perforated patch configuration. The neurons were loaded with TRD-40kDa *via* the patch
477 pipette (0.08%) and excited at 557 nm (bandpass: 562 nm, 562/40 BrightLine HC, Art-no.
478 F39-562, AHF Analysentechnik AG, Tübingen, Germany; beamsplitter: 593 nm, HC BS 593,
479 Art-no. F38-593, AHF). Emission was detected through a 593 nm long-pass filter (593/LP
480 BrightLine HC, Art-no. F37-594, AHF). TRD-40kDa -fluorescence is reported as F/F_0 , where
481 F is the measured fluorescence, and F_0 is the averaged fluorescence of 5 consecutive images
482 in on-cell mode (before break-in or perforation of the cell membrane). Frames were taken in
483 intervals of 30 s.

484

485 *Fluorimetric Ca^{2+} measurements with fura-2*

486 Intracellular Ca^{2+} concentrations were measured with the Ca^{2+} indicator fura-2, a
487 ratiometric dye suitable for absolute Ca^{2+} concentration determination once calibrated
488 (Grynkiewicz et al., 1985; Poenie, 1990). DA SN neurons were loaded with fura-2
489 (pentapotassium salt, F1200, Molecular Probes) *via* the patch pipette (100 or 200 μ M). Fura-2
490 was excited at 340 nm, 360 nm, or 380 nm (410 nm dichroic mirror; DCLP410, Chroma,
491 Rockingham, VT, USA). Emitted fluorescence was detected through a 440 nm long-pass filter
492 (LP440).

493

494 *Calibration and 'added buffer approach'*

495 The free intracellular Ca^{2+} concentrations were determined as described by
496 (Grynkiewicz et al., 1985), and the added buffer approach to analyze intracellular Ca^{2+}
497 handling properties was performed as previously described (Neher and Augustine, 1992;
498 Paeger et al., 2017; Pippow et al., 2009). For completeness and notation clarification, these
499 methods are described in detail in the *Appendix*. In brief: The added buffer approach was used
500 in combination with whole-cell and perforated patch clamp recordings and optical Ca^{2+}
501 imaging. The added buffer approach is based on a single-compartment model with the

502 rationale that for measurements of intracellular Ca^{2+} concentrations with Ca^{2+} chelator-based
503 indicators, the amplitude and time course of the signals depends on the concentration of the
504 Ca^{2+} indicator (here fura-2). The indicator acts as an exogenous Ca^{2+} buffer and competes
505 with the endogenous Ca^{2+} buffer(s).

506 The kinetics of cytosolic Ca^{2+} signals strongly rely on the endogenous and exogenous
507 (added) Ca^{2+} buffers of the cell. The amplitude and decay rate of the free intracellular Ca^{2+}
508 change with increasing exogenous buffer concentration: The amplitude of free Ca^{2+} decreases,
509 and the time constant $\tau_{\text{transient}}$ of the decay increases. If the buffer capacity of the added buffer
510 is known, the time constant of decay ($\tau_{\text{transient}}$) can be used to estimate, by extrapolation, the
511 Ca^{2+} signal to conditions, with only endogenous buffers present ($-\kappa_{\text{B}} = 1 + \kappa_{\text{S}}$). The model
512 used for this study (Eq. 4) assumes that the decay time constants $\tau_{\text{transient}}$ are a linear function
513 of the Ca^{2+} binding ratios (κ_{B} and κ_{S}). κ_{S} is determined from the negative x-axis intercept of
514 this plot. The slope of the fit is the inverse of the linear extrusion rate (γ). The point of
515 intersection of the linear fit with the y-axis denotes the endogenous decay time constant τ_{endo}
516 (no exogenous Ca^{2+} buffer in the cell). A detailed description of how to estimate the variance
517 of these parameters is provided in the *Appendix*.

518

519 *Tools for data analysis and statistics*

520 Data analysis was performed with Igor Pro 6 (Wavemetrics), Spike2 (CED,
521 Cambridge, UK), R (R Development Core Team [2009], <http://www.R-project.org>) and
522 Graphpad Prism (version 8; Graphpad Software Inc., La Jolla, CA, USA). All calculations for
523 the determination of EGTA purity, its dissociation constant, and the free Ca^{2+} concentrations
524 in the calibration solutions were performed in R. If not stated otherwise, all calculated values
525 are expressed as means \pm SEM (standard error of the mean). Box plots were constructed as
526 follows: The '+' signs show the mean, the horizontal line the median of the data. Whiskers

527 indicate the minimum and maximum values of the data. For pairwise comparisons of
528 dependent and independent normal distributions, paired and unpaired t-tests were used,
529 respectively. For pairwise comparisons of independent, not normal distributions, Mann-
530 Whitney U-tests were used. For multiple comparisons, ANOVA was performed; post hoc
531 pairwise comparisons were performed using t-tests as indicated in the figures. For comparison
532 of the resting Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) over time under whole-cell and β -escin perforated-patch
533 clamp conditions, the regression slope, after linear fitting, was also used. Tests were executed
534 using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). A significance level
535 of 0.05 was accepted for all tests. Significance levels were: * $p < 0.05$, ** $p < 0.01$, *** $p <$
536 0.001 . In all figures, n-values are given in brackets. Exact p-values are reported if $p > 0.0001$.
537 Scaling, contrast enhancement, and z-projections of images were performed with ImageJ
538 v2.0.0. The final figures were prepared with Affinity Designer (Ver. 1.6.1, Serif Ltd).

539

540 APPENDIX

541 *Measurement of free intracellular Ca^{2+} concentration*

542 The free intracellular Ca^{2+} concentrations were determined as in (Grynkiewicz et al.,
543 1985):

$$[\text{Ca}^{2+}]_i = K_{d,\text{Fura}} \frac{F_{380,\text{min}} (R - R_{\text{min}})}{F_{380,\text{max}} (R_{\text{max}} - R)} \quad (1)$$

544 $[\text{Ca}^{2+}]_i$ is the free intracellular Ca^{2+} concentration for the background-subtracted fluorescence
545 ratio R from 340 nm and 380 nm excitation. R_{min} and R_{max} are the ratios at a Ca^{2+}
546 concentration at virtually 0 M (i.e., ideally, no fura-2 molecules are bound to Ca^{2+}) and at
547 saturating Ca^{2+} concentrations (i.e., ideally, all fura-2 molecules are saturated with Ca^{2+}),
548 respectively. $K_{d,\text{Fura}}$ is the dissociation constant of fura-2. $F_{380,\text{min}}/F_{380,\text{max}}$ is the ratio
549 between the emitted fluorescence of Ca^{2+} free dye and the emitted fluorescence of Ca^{2+}
550 saturated dye at 380 nm excitation, reflecting the dynamic range of the indicator.

551 The term $K_{d,Fura}$ ($F_{380,min}/F_{380,max}$) in Eq. (1) is dependent on the dye concentration and is
552 substituted with the effective dissociation constant $K_{d,Fura,eff}$, which is independent of the dye
553 concentration and specific for each experimental setup (Neher, 1989):

$$K_{d,Fura,eff} = [Ca^{2+}]_i \frac{(R_{max} - R)}{(R - R_{min})} \quad (2)$$

554 We used *in vitro* calibration (in solution). For calibration $K_{d,Fura,eff}$ was determined by
555 measuring fura-2 fluorescence ratios for R_{max} , R_{min} , and $R = R_{def}$. R_{def} is the ratio for a defined
556 $[Ca^{2+}]_i$, which was set to 0.35 μ M (see below for the preparation of the solutions). $K_{d,Fura,eff}$
557 was then calculated from Eq. (2). To account for environmental differences between the
558 cytoplasmic milieu and *in vitro* conditions, we used a correction factor (P) for R_{max} and R_{min} ,
559 as suggested by (Grynkiewicz et al., 1985), see also (Pippow et al., 2009):

$$K_{d,Fura,eff} = [Ca^{2+}]_i \frac{(R_{max} P - R)}{(R - R_{min} P)} \quad (3)$$

560 P was determined as described by (Poenie, 1990; Zhou and Neher, 1993). First, the
561 fluorescence (peak) deflection at 340 nm was divided by that at 380 nm excitation from
562 voltage-induced intracellular calcium transients ($R_{d,cell}$). Second, the ratio ($R_{d,vitro}$) from pairs
563 of calibration solutions was determined by dividing $(F_{340,max} - F_{340,min})/(F_{380,min} - F_{380,max})$.
564 The correction factor P is the fraction of $R_{d,cell}/R_{d,vitro}$.

565 The fluorescence ratio R of an intracellular transient can then be converted to $[Ca^{2+}]_i$
566 using:

$$[Ca^{2+}]_i = K_{d,Fura,eff} \frac{(R - R_{min} P)}{(R_{max} P - R)} \quad (4)$$

567 To determine the endogenous Ca^{2+} binding ratio, the dissociation constant ($K_{d,Fura}$) has to be
568 known. To formulate a relationship between $K_{d,Fura}$ and $K_{d,Fura,eff}$ that is independent of the dye
569 concentration, an isocoefficient α was introduced by (Zhou and Neher, 1993):

$$K_{d,\text{Fura}} = K_{d,\text{Fura,eff}} \frac{(R_{\min} + \alpha)}{(R_{\max} + \alpha)} \quad (5)$$

570 α is the isocoefficient, for which the sum

$$F_i(t) = F_{340}(t) + \alpha F_{380}(t) \quad (6)$$

571 is independent of Ca^{2+} concentration. F_{340} and F_{380} are the background-subtracted
572 fluorescence signals measured during a brief Ca^{2+} transient. Once the isocoefficient has been
573 determined, $K_{d,\text{Fura}}$ can be calculated from Eq. (5).

574 Calibration solutions: The free Ca^{2+} concentrations of the calibration solutions were
575 adjusted by using appropriate proportions of Ca^{2+} and the Ca^{2+} chelator EGTA. The ability of
576 EGTA to bind calcium is highly dependent on the environmental conditions such as ionic
577 strength, temperature, pH, and the concentrations of other metals that compete for binding
578 (Harrison and Bers, 1989, 1987). In theory, the necessary amount of Ca^{2+} and EGTA to set the
579 free Ca^{2+} concentration for the experimental conditions can be computed (Patton et al., 2004).
580 However, small variations in the parameters such as pH, temperature, impurities of chemicals,
581 pipetting, or weighing errors can lead to considerable errors in the estimate of the free Ca^{2+} in
582 EGTA-buffered Ca^{2+} solutions with computer programs (McGuigan et al., 2007). To account
583 for such variations, we determined the free Ca^{2+} concentrations in our calibration solutions by
584 using a Ca^{2+} selective electrode following the guide from McGuigan (McGuigan et al., 1991)
585 and Pippow (Pippow et al., 2009) as described in the supplemental. Calibration solutions were
586 prepared as follows (in mM): R_{\max} : 140 KCl, 2.5 KOH, 15 NaCl, 1 MgCl_2 , 5 HEPES, 10
587 CaCl_2 and 0.05 fura-2; R_{\min} : 129.5 KCl, 13 KOH, 15 NaCl, 1 MgCl_2 , 5 HEPES, 8 EGTA and
588 0.05 fura-2; R_{def} : 129.5 KCl, 13 KOH, 10.3 NaCl, 4.7 NaOH, 1 MgCl_2 , 5 HEPES, 4 EGTA,
589 2.7 CaCl_2 and 0.05 fura-2, yielding a free Ca^{2+} concentration of 0.35 μM . All solutions were
590 adjusted to pH 7.2 with HCl.

591 The concentrations of free Ca^{2+} were determined from the ratioed imaging signals
592 applying the approach from Grynkiewicz et al. (Grynkiewicz et al., 1985) (Eq. (2)). The

593 calibration was performed *in vitro* (in solution). For calibration, a drop of each calibration
594 solution (200 μ l, R_{\min} : no Ca^{2+} , R_{def} : 0.35 μ M Ca^{2+} , R_{\max} : 10 mM Ca^{2+}) was placed on a
595 sylgard coated recording chamber. Ratio images (from 340 nm and 380 nm excitation) were
596 acquired for each solution. The acquired values were: correction factor (Poenie, 1990) $P =$
597 0.78 ± 0.03 , resulting in: $R_{\max} = 1.267 \pm 0.055$ ($n = 9$); $R_{\min} = 0.116 \pm 0.004$ ($n = 9$); $R_{\text{def}} =$
598 0.277 ± 0.004 ($n = 7$); $K_{\text{d,Fura,eff}} = 1.048 \pm 0.052$ μ M and isocoefficient $\alpha = 0.192 \pm 0.003$.
599 According to Eq. (5) the K_{d} for fura-2 was $K_{\text{d,Fura}} = 0.222 \pm 0.082$ μ M.

600

601 *Single compartment model of calcium buffering: the 'added buffer approach'*

602 For measurements of intracellular Ca^{2+} concentrations with Ca^{2+} chelator-based
603 indicators, the amplitude and time course of the signals are dependent on the concentration of
604 the Ca^{2+} indicator (in this case, fura-2) that acts as an exogenous (added) Ca^{2+} buffer and
605 competes with the endogenous buffers (Helmchen et al., 1997; Helmchen and Tank, 2015;
606 Neher and Augustine, 1992; Tank et al., 1995). With the 'added buffer approach' (Neher and
607 Augustine, 1992), the capacity of the endogenous Ca^{2+} buffer in a cell is determined by
608 measuring the decay of the Ca^{2+} signal at different concentrations of 'added buffer' and by
609 extrapolating to conditions in which only the endogenous buffer is present. Strictly speaking,
610 the added buffer approach in combination with the whole-cell configuration can only be used
611 to analyze the immobile fraction of Ca^{2+} buffers and only when the measurements start after
612 the mobile Ca^{2+} buffers have diffused out of the cell and do not interfere with the analysis. In
613 contrast, β -escin perforated recordings can be used to measure the total endogenous Ca^{2+}
614 buffer capacity. The added buffer approach is based on a single-compartment model assuming
615 that a big patch-pipette with a known (clamped) concentration of a Ca^{2+} indicator (fura-2) is
616 connected to a spherical cell. The model assumes that the cell contains an endogenous Ca^{2+}
617 buffer (S), of which the total concentration, dissociation constant $K_{\text{d,S}}$, and its on (k^+) and off
618 (k^-) rates are unknown. When the cell membrane is perforated by β -escin, fura-2 (in its bound

619 and unbound forms) starts to diffuse into the cell, which is considered small enough to be
620 always in a 'well mixed' state (i.e., there are no gradients of chemical species within the cell
621 body). In this model three cellular parameters determine the cytosolic Ca^{2+} dynamics: (1) Ca^{2+}
622 sources, (2) Ca^{2+} binding ratio and (3) Ca^{2+} extrusion.

623 Ca^{2+} sources: In the model, two forms of Ca^{2+} sources are defined: (1) the Ca^{2+} influx
624 from the pipette ($j_{\text{in,pipette}}$) and (2) the Ca^{2+} entering the cell *via* voltage-activated channels
625 from a brief Ca^{2+} pulse, which can be described by a delta function ($j_{\text{in,stim}}$) (Helmchen and
626 Tank, 2015; Neher and Augustine, 1992):

$$j_{\text{in,pipette}} = ([\text{BCa}_p] - [\text{BCa}]) \frac{D_B \rho}{R_p} \quad (7)$$

$$j_{\text{in,stim}} = n_{\text{Ca}} \delta(t - t_{\text{stim}}) = \frac{Q_{\text{Ca}}}{2F} \delta(t - t_{\text{stim}}) \quad (8)$$

627 $[\text{BCa}]$ is the concentration of the exogenous buffer (fura-2) in its Ca^{2+} bound form (the
628 subscript p is for quantities within the pipette), D_B is the diffusion coefficient of the
629 exogenous buffer, ρ is the specific resistance of the pipette filling solution, R_p is the pipette
630 resistance, n_{Ca} is the Ca^{2+} influx induced by the stimulus, $\delta(t - t_{\text{stim}})$ is the delta function with
631 t_{stim} as the time of stimulus, Q_{Ca} is the Ca^{2+} charge influx, and F is the Faraday's constant.

632 Ca^{2+} binding ratio: In the cell, Ca^{2+} is bound to the Ca^{2+} buffers fura-2 and to the
633 endogenous buffers, which are both assumed to be always in equilibrium with free Ca^{2+} and
634 not saturated. The ability of the experimentally introduced exogenous buffer to bind Ca^{2+} is
635 described by its Ca^{2+} binding ratio that is defined as the ratio of the change in buffer-bound
636 Ca^{2+} over the change in free Ca^{2+} :

$$\kappa_B = \frac{d[\text{BCa}]}{d[\text{Ca}^{2+}]_i} = \frac{[\text{B}_T] K_{d,B}}{([\text{Ca}^{2+}]_i + K_{d,B})^2} \quad (9)$$

637 $[\text{B}_T]$ is the total concentration of the exogenous buffer B and $K_{d,B}$ is its dissociation constant
638 for Ca^{2+} . An analogous expression exists for the Ca^{2+} binding ratio of the endogenous buffer S
639 (κ_S).

640 Ca^{2+} Extrusion: the model assumes a linear extrusion mechanism for Ca^{2+} (Neher and
641 Augustine, 1992):

642

$$j_{\text{out}} = \gamma \Delta[\text{Ca}^{2+}]_i = \gamma([\text{Ca}^{2+}]_i - [\text{Ca}^{2+}]_{i,\infty}) \quad (10)$$

643 $[\text{Ca}^{2+}]_{i,\infty}$ is the steady-state intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$, to which a transient
644 decays. According to Neher and Augustine (Neher and Augustine, 1992), γ 'reflects the
645 combined action of pumps, exchange carriers, and membrane conductances and has the
646 dimension l s^{-1} . In the following, we refer to it as $\tilde{\gamma}$. In more recent publications γ is defined as
647 the extrusion rate for Ca^{2+} measured in s^{-1} (Helmchen et al., 1997, 1996; Lee et al., 2000a,
648 2000b; Lips and Keller, 1998; Palecek et al., 1999; Sabatini et al., 2002; Vanselow and Keller,
649 2000). Accordingly, we use (Jackson and Redman, 2003):

$$\gamma = \frac{\tilde{\gamma}}{V} \quad (11)$$

650 where V is the volume of the cell.

651 Dynamics of Ca^{2+} transients: the mechanisms described in Eqs. 9 and 10 are combined
652 to the model that describes the dynamics of the Ca^{2+} decay after a brief Ca^{2+} influx:

$$\frac{d[\text{Ca}^{2+}]_i}{dt} + \frac{d[\text{BCa}]}{dt} + \frac{d[\text{SCa}]}{dt} = \frac{j_{\text{in,pipette}} + j_{\text{in,stim}} - j_{\text{out}}}{V} \quad (12)$$

653 Substituting the time dependent terms for the buffers B and S with their Ca^{2+} binding ratios κ_B
654 and κ_S , respectively, $[\text{BCa}]$ with its equilibrium value $[\text{BCa}] = [\text{Ca}^{2+}]_i[\text{B}_T]/(K_d + [\text{Ca}^{2+}]_i)$ and
655 $\tau_{\text{loading}} = (VR_p)/(D_B \tilde{n})$ we get (Neher and Augustine, 1992):

$$\frac{d[\text{Ca}^{2+}]_i}{dt} = \frac{\tau_{\text{loading}}^{-1} \cdot ([\text{BCa}]_p - \frac{[\text{Ca}^{2+}]_i[\text{B}_T]}{K_d + [\text{Ca}^{2+}]_i}) + \frac{\tilde{\gamma}}{V} ([\text{Ca}^{2+}]_{i,\infty} - [\text{Ca}^{2+}]_i) + \frac{n_{\text{Ca}} \delta(t-t_0)}{V}}{(1 + \kappa_B + \kappa_S)} \quad (13)$$

656 Neher and Augustine (Neher and Augustine, 1992) linearized Eq. (13) to simplify the solution
657 for this equation for the case in which the baseline for $[\text{Ca}^{2+}]_i$ is constant, the concentration of
658 the exogenous buffer $[\text{B}_T]$ is constant and does not influence the baseline Ca^{2+} , and when the

659 amplitudes of the Ca^{2+} transients are small (Neher and Augustine, 1992). Where 'small' is
660 defined as less than $0.5 K_{d,\text{Fura}}$. When the Ca^{2+} pulse is short compared to the decay time
661 constant, the delta function equals zero. Under these conditions, the decay of a voltage-
662 induced Ca^{2+} transient can be described as:

$$\tau_{\text{transient}} = \frac{1 + \kappa_B + \kappa_S}{\gamma - \frac{(1 + \kappa_S)}{\tau_{\text{loading}}}} \quad \text{for: } \begin{array}{l} \text{baseline} = \text{const} \\ [\text{B}_T] = \text{const} \\ \text{small } \Delta[\text{Ca}^{2+}]_i \end{array} \quad (14)$$

663 $\tau_{\text{transient}}$ is proportional to κ_B , and a linear fit to the data has its negative x-axis intercept at $1 +$
664 κ_S , yielding the endogenous Ca^{2+} binding ratio of the cell. The slope of this fit is the inverse
665 of the Ca^{2+} extrusion rate γ (Eq. (11)). The y-axis intercept yields the time constant τ_{endo} for
666 the decay of the Ca^{2+} transient as it would appear in the cell without exogenous buffer.

667

668 *Analysis of calcium buffering*

669 Dye loading was monitored at 360 nm excitation, the isosbestic point of fura-2. Frames
670 were taken at 30 s intervals (3 ms exposure time). We estimated the intracellular fura-2
671 concentration for different times during the loading curve, assuming that cells were fully
672 loaded when the fluorescence reached a plateau and the fura-2 concentration in the cell and in
673 the pipette is equal (100 or 200 μM) (Lee et al., 2000b).

674 During fura-2 loading, voltage-activated Ca^{2+} influx was induced by short spike trains,
675 which were elicited by current pulses (500pA, 500ms) from a holding potential of about -70
676 mV. To monitor the induced Ca^{2+} transients ratiometrically, pairs of images at 340 nm (12 ms
677 exposure time) and 380 nm (4 ms exposure time) excitation were acquired at 10 Hz for ~20 s.
678 Typically 3 – 4 Ca^{2+} transients were induced during loading at different intracellular fura-2
679 concentrations.

680

681 *Ratiometric Data Analysis*

682 **Getting the standard error of the ratiometric estimator**

683 Given measurements at two excitation wavelengths (340 and 380 nm), \widehat{ADU}_{340} and \widehat{ADU}_{380}
 684 (these are pooled photon counts over the P pixels of the ROI) and concomitant measurements
 685 $\widehat{ADU}_{B,340}$ and $\widehat{ADU}_{B,380}$ (pooled photon counts over the P_B pixels of the "background
 686 region"), what we refer to as the "classical" ratiometric estimator at time t_i , is (Joucla et al.,
 687 2010) (Eq. 8):

$$\widehat{ca}(t_i) = K_{eff} \frac{\hat{r}(t_i) - R_{min}}{R_{max} - \hat{r}(t_i)}, \quad (1)$$

688 where K_{eff} , R_{min} and R_{max} are calibrated parameters (assumed exactly known for now) and
 689 where (Joucla et al., 2010) (Eq. 6):

$$\hat{r}(t_i) = \frac{(T_{e,340})^{-1}(\widehat{ADU}_{340}/P - \widehat{ADU}_{B,340}/P_B)}{(T_{e,380})^{-1}(\widehat{ADU}_{380}/P - \widehat{ADU}_{B,380}/P_B)}, \quad (2)$$

690 where $T_{e,\lambda}$ is the flash duration at wavelength λ . Our model for the fluorescence intensity at
 691 each wavelength is (Joucla et al., 2010) (Eq. 2a and 2b):

$$F_{340} = \left\{ \frac{[Fura]_{total}\phi}{K_{Fura} + [Ca^{2+}]} (R_{min}K_{eff} + R_{max}[Ca^{2+}]) + s_{B,340} \right\} T_{e,340}P, \quad (3)$$

692 and

$$F_{380} = \left\{ \frac{[Fura]_{total}\phi}{K_{Fura} + [Ca^{2+}]} (K_{eff} + [Ca^{2+}]) + s_{B,380} \right\} T_{e,380}P, \quad (4)$$

693 where $s_{B,\lambda}$ is the auto-fluorescence at wavelength λ –assumed homogeneous among the P
 694 pixels of the ROI–, K_{Fura} is a calibrated parameter, $[Fura]_{total}\phi$, is the total (bound plus
 695 free) concentration of fura-2 in the cell multiplied by a dimensionless experiment specific
 696 parameter, ϕ , lumping together the quantum efficiency, the neurite thickness, etc.

697 Under our assumptions (Joucla et al., 2010) (Eq. 5) we have (we don't use a " $\hat{}$ " here since
 698 we are dealing with a *random variable*, not a *realization* of it):

$$ADU_{\lambda}(t_i) = GF_{\lambda}(t_i) + \epsilon G \sqrt{F_{\lambda}(t_i) + \sigma_{read-out}^2}, \quad (5)$$

699 where G is the camera gain, $\sigma_{read-out}^2$ is its read-out variance, $F_{\lambda}(t_i)$ is given by Eq. 3 and 4
 700 and where $\epsilon \sim \mathcal{N}(0,1)$ (ϵ is a Gaussian random variable with mean 0 and variance 1). In
 701 words: $ADU_{\lambda}(t_i)$ has a Gaussian distribution with mean $GF_{\lambda}(t_i)$ and variance $G^2(F_{\lambda}(t_i) +$
 702 $\sigma_{read-out}^2)$.

703 To determine the variance of $ADU_{\lambda}(t_i)$ we need to know $F_{\lambda}(t_i)$ and for that, we need to know
 704 $[Ca^{2+}](t_i)$ precisely what we want to estimate. But $EADU_{\lambda}(t_i)$, the expected value of
 705 $ADU_{\lambda}(t_i)$, is $GF_{\lambda}(t_i)$ so we can use the observed value $\widehat{ADU}_{\lambda}(t_i)$ of $ADU_{\lambda}(t_i)$ as an estimate
 706 for $GF_{\lambda}(t_i)$ –in Eq. 5 we plug-in $\widehat{ADU}_{\lambda}(t_i)$ for $GF_{\lambda}(t_i)$ – leading to:

$$\hat{\sigma}_{ADU_{\lambda}(t_i)}^2 = G\widehat{ADU}_{\lambda}(t_i) + G^2\sigma_{read-out}^2 \approx \sigma_{ADU_{\lambda}(t_i)}^2, \quad (6)$$

707 In practice, when we are dealing with several pixels making a ROI, we must multiply the
 708 read-out variance by the number of pixels used. With the resulting $\hat{\sigma}_{ADU_{\lambda}(t_i)}^2$ we can work
 709 with, to get $\hat{\sigma}_{r(t_i)}^2$ and $\hat{\sigma}_{\widehat{Ca}(t_i)}^2$. We could use the propagation of uncertainty (or error
 710 propagation) together with Eq. 2 and 1 for that, but we can also use a Monte Carlo approach:
 711 we draw a thousand quadruple of vectors
 712 $(ADU_{340}^{[j]}(t_i), ADU_{380}^{[j]}(t_i), ADU_{B,340}^{[j]}(t_i), ADU_{B,380}^{[j]}(t_i))$ ($j = 1, \dots, 1000$) from four
 713 independent Gaussian distributions of the general form:

$$ADU_{\lambda}^{[j]}(t_i) = ADU_{\lambda}(t_i) + \epsilon_j \hat{\sigma}_{ADU_{\lambda}(t_i)}, \quad (7)$$

714 plug-in these quadruples into Eq. 2 giving us 1000 $r^{[j]}(t_i)$ before plugging in the latter into
715 Eq. 1 leading to 1000 $\widehat{Ca}^{[j]}(t_i)$. The empirical variance of these simulated observations will
716 be used as $\widehat{\sigma}_{\widehat{Ca}(t_i)}^2$.

717 The method proposed here is slightly less rigorous than the "direct approach" of (Joucla et al.,
718 2010), but it is far more flexible since it does not require an independent estimation or
719 measurement of $[Fura]_{total}$. In the present study we also chose to consider the calibrated
720 parameters K_{eff} , R_{min} and R_{max} as known. Note that the values for these parameters are only
721 estimates (necessarily imprecise since the calibration procedure is subject to errors). However,
722 since the same batch of fura-2 was used for all experiments, they should all have the same
723 (systematic) error. We are not trying to get the exact standard error of the calcium dynamics
724 parameters but to show the difference between two protocols ("classical whole-cell" versus
725 beta-escin perforated whole-cell). Ignoring the uncertainty on the calibrated parameters makes
726 our estimates less variable and helps to make the difference if it exists, clearer. For a true
727 calcium buffering capacity study, the errors on the calibrated parameters should be accounted
728 for, and it would be straightforward to do it with our approach, simply by drawing also K_{eff} ,
729 R_{min} and R_{max} values from Gaussian distributions centered on their respective estimates with
730 a SD given by the standard errors.

731

732 **Objective or "cost" function**

733 We know now how to get both sequences of $[Ca^{2+}]$ estimates (Eq. 1) and standard errors
734 from sequences of ADU measurements. We also consider a calcium dynamics model (Eq. 8-
735 10):

$$\frac{dCa(t)}{dt} (1 + \kappa_F(Ca(t)) + \kappa_E(Ca(t))) + \frac{j(Ca(t))}{v} = 0, \quad (8)$$

736 where $Ca(t)$ stands for $[Ca^{2+}]_{free}$ at time t , v is the volume of the neurite—within which
 737 diffusion effects can be neglected — and

$$j(Ca(t)) \equiv \gamma(Ca(t) - Ca_{steady}), \quad (9)$$

738 is the model of calcium extrusion — Ca_{steady} is the steady-state [1] $[Ca^{2+}]_{free}$ — and:

$$\kappa_F(Ca(t)) \equiv \frac{F_{total}K_F}{(K_F + Ca(t))^2} \quad \text{and} \quad \kappa_E(Ca(t)) \equiv \frac{E_{total}K_E}{(K_E + Ca(t))^2},$$

739 where F stands for the fluorophore and E for the *endogenous* buffer.

740 If the values of $\theta \equiv (Ca_{steady}, \frac{\gamma}{v}, E_{total}, K_E, F_{total}, K_F, Ca(t=0))$ are known, we can
 741 substitute these values in Eq. 8, solve it (numerically) and *predict* the time course of calcium
 742 at any positive time. Let us write $Ca(t; \theta)$ as this solution of Eq. 8. In addition to this model,
 743 we have used the results of the previous section, on a set of N time points t_i , a collection of
 744 $(\widehat{Ca}(t_i), \widehat{\sigma}_{\widehat{Ca}(t_i)})$. We can then define a first *objective* or *cost* function:

$$\Omega(\theta) \equiv \sum_{i=1}^N \left(\frac{\widehat{Ca}(t_i) - Ca(t_i; \theta)}{\widehat{\sigma}_{\widehat{Ca}(t_i)}} \right)^2. \quad (10)$$

745 The next task is to find the value of $\hat{\theta}$ that makes Ω as small as possible; this a classical non-
 746 linear weighted least squares problem (see (Nielsen and Madsen, 2010)). The advantage of
 747 this setting is that if we can find $\hat{\theta}$ (that is, if the minimum exists and is unique), we also get:

- 748 1. Confidence intervals for each element of θ through their standard error (in fact, more
 749 than that, since we get the full covariance matrix).
- 750 2. The distribution from which $\Omega(\hat{\theta})$ should have been drawn if the model, $Ca(t; \hat{\theta})$, is
 751 indeed correct: a χ^2 distribution with $N - \#\theta$ degrees of freedom, where $\#\theta$ stands for
 752 the number of elements of θ that are set from the fitted data.

753 *Point 2 gives us an objective basis to accept or reject a fit, an essential ingredient for the*
754 *procedure presented here.*

755 Before going further, we should consider that θ in the form just defined contains 7 elements
756 among which, 2 are supposed to be known, K_F and F_{total} . That leaves 5 values to extract from
757 the transient. This is not straightforward since the latter seems, at least at first glance, close to
758 a mono-exponential: $Ca(t; Ca_{steady}, \Delta, \tau) = Ca_{steady} + \Delta \exp\left(-\frac{t}{\tau}\right)$ (from which only 3
759 parameters can be obtained). Therefore, we follow (Neher and Augustine, 1992) and consider
760 an approximation of Eq. 8 where $Ca(t)$ can be written as $Ca_{steady} + \delta Ca(t)$ and where
761 $\delta Ca(t)$ is small enough for a first order expansion of $Ca(t)$ in the vicinity of Ca_{steady} to be
762 valid. If we do this first order expansion, neglecting the communication between the pipette
763 and the cytosol during the transient we get (setting $\rho = 0$) in (Neher and Augustine, 1992)
764 (Eq. 15):

$$\frac{\delta Ca(t)}{dt} = -\frac{\frac{\gamma}{v}}{1 + \kappa_F(Ca_{steady}) + \kappa_E(Ca_{steady})} \delta Ca(t). \quad (11)$$

765 Leading to:

$$Ca(t; Ca_{steady}, \Delta, \tau) = Ca_{steady} + \Delta \exp -\frac{t}{\tau}, \quad (12)$$

766 where the time constant τ is defined by:

$$\tau(\kappa_F) \equiv \frac{1 + \kappa_F + \kappa_E}{\frac{\gamma}{v}}, \quad (13)$$

767 the dependence of the κ on Ca_{steady} has been made implicit. We see that if we can obtain
768 several transients with different values of $[Fura]_{total}$ leading to several values of κ_F , we can
769 exploit the linear dependence of τ on both κ to estimate κ_E by extrapolation. This is the
770 essence of the added buffer approach. The implementation of the latter requires:

- 771 1. Get several (known) values of $[Fura]_{total}$ and therefore κ_F in the recorded neuron.
- 772 2. Trigger a calcium entry at each of these κ_F values and fit *the tail of the transient* with a
773 mono-exponential (Eq. 12)—fitting the tail ensures that the linear approximation of Eq. 8
774 by Eq. 11 is valid—.
- 775 3. Plot τ versus κ_F and fit a straight line to these points. The slope gives $\frac{v}{\gamma}$ and the x-axis
776 intercept gives κ_E .

777 The fit in point 2 is done with weighted non-linear least squares using $Ca(t; Ca_{steady}, \Delta, \tau)$
778 (Eq. 12) instead of $Ca(t; \theta)$ in the cost function definition (Eq. 10). Each fit gives $\hat{\tau}(\kappa_F)$ and
779 $\hat{\sigma}_{\hat{\tau}(\kappa_F)}$ together with a goodness of fit criterion. The $(\hat{\tau}(\kappa_F), \hat{\sigma}_{\hat{\tau}(\kappa_F)})$ are used in point 3 for a
780 weighted *linear* least squares fit that also leads to confidence intervals on the estimated
781 parameters and to a goodness of fit criterion.

782 The codes doing the "heavy" part of the analysis were written in C. Some higher-level scripts
783 were written in Python 3. The codes, their documentation, the data, and the way the codes
784 were applied to the data can be found on GitHub (<https://github.com/christophe-pouzat/hess-et-al-beta-escin-2019>) as well as on zenodo (<https://zenodo.org/record/3238248>). The
785 weighted linear and non-linear least squares routines, as well as the random number
786 generators, are the ones of the Gnu Scientific Library ([GSL](https://www.gnu.org/software/gsl/)). The [Lenvenberg-Marquardt](https://www.gnu.org/software/gsl/)
787 implementation of the GSL was used for the non-linear least squares. Optimization was ended
788 based on convergence criteria looking at the relative change of each estimated parameter
789 value and at the gradient norm (see the [GSL documentation](https://www.gnu.org/software/gsl/)); as a safeguard, a maximum
790 number of iterations was set, but all the optimizations did actually stop because the
791 convergence criteria were met.

792
793 Following Neher and Augustine (1992), our analysis of the calcium transients relies on the
794 linearization (<https://en.wikipedia.org/wiki/Linearization>) of an assumed calcium dynamics

795 (Eq. 9 to 15, (Neher and Augustine, 1992)). The key hypothesis that is made in all
796 linearizations is that the dynamical system under investigation (here the calcium
797 concentration) is submitted to a small perturbation from its resting state. What constitutes a
798 "small enough" perturbation for the linearization to be valid in practice depends, in the
799 specific case considered here, on parameters we do not know (total endogenous buffer
800 concentration, endogenous buffer dissociation constant, etc.), but we can be sure that the
801 closer we get to baseline, that is the later we look during the transient, the more likely the
802 "small enough" requirement is to be met. If the linearization is valid, the transient (more
803 precisely the portion of the transient that is fitted) must exhibit a mono-exponential decay,
804 something we can rigorously test. In order to get a procedure usable on every transient and
805 considering the fact that the peak response of the first transient can be large - 6 times the
806 baseline value, not really a "small" perturbation - we decided, before seeing the data, to start
807 all the fits from the time at which the transient had lost 50% of its peak value. This is
808 obviously arbitrary but it does not affect our results as the reader can check by downloading
809 our data and our code before rerunning the whole analysis using a different location from
810 which to start the fit. Accordingly, the number of data points used for each fit varies a bit
811 from transient to transient. *This choice of fitting from the "half-peak time" was made before*
812 *seeing the data.* It is important to realize that once such a choice has been made, we are
813 constrained, the fit is done automatically, and the residual sum of squares (RSS) is compatible
814 or not with the theoretical χ^2 distribution.

815 Since we used a triple wavelength (340, 380 and 360) protocol for each point of the transient,
816 we have access to an independent estimate of $[Fura]_{total}$. We saw, as in (Joucla et al., 2010)
817 (Fig. 5), that the latter is not constant during the transients. When doing the τ versus κ_F linear
818 regression we therefore computed systematically three regressions using the minimum, the
819 mean and the maximum observed values of $[Fura]_{total}$ during the transient. We always

820 report the results of regression leading to the smallest RSS (best fit). As the reader can check
821 from our GitHub repository, none of our conclusions depend on this. We emphasize again that
822 since our method generates standard errors for $\hat{\tau}$, the linear regressions are accepted or
823 rejected on an objective basis (the RSS is compatible or not with the relevant theoretical χ^2
824 distribution).

825

826 **Figures**

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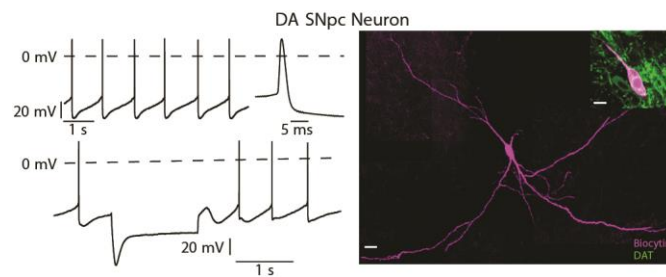
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Figure 1



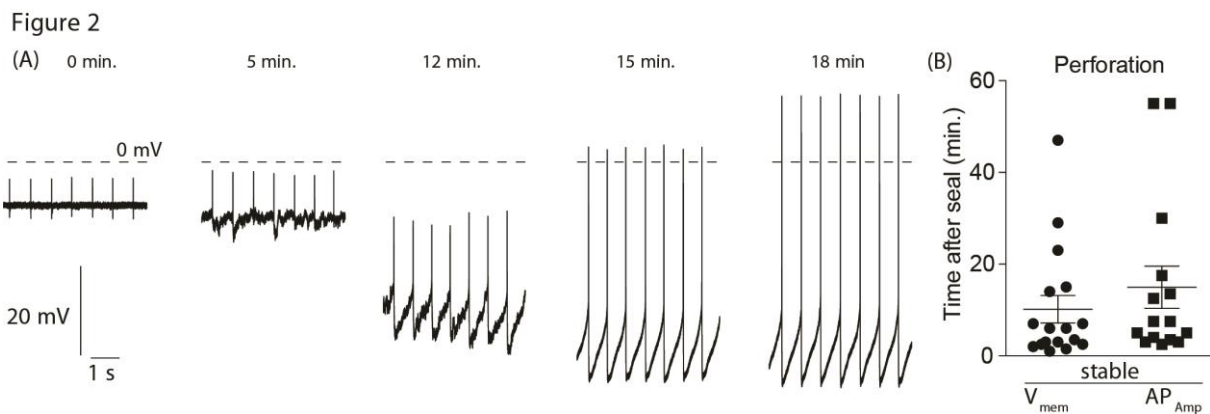
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835 **Figure 1.** Basic identifying electrophysiological and morphological properties of
836 dopaminergic substantia nigra neurons. The neurons were recorded in current clamp (*left*) and
837 labeled with biocytin-streptavidin (magenta) *via* the patch pipette (*right*). The upper trace
838 shows the regular pacemaker activity and long spike duration. On the lower trace, a
839 hyperpolarizing current injection to -120 mV induced a clear ‘sag potential’, which is
840 mediated by I_h and typical for this neuron type. Immunohistochemistry revealed dopamine
841 transporter immunoreactivity (green), which a marker for dopaminergic neurons in the
842 substantia nigra (*right, inset*). Scale bars: 20 μm , inset: 10 μm .

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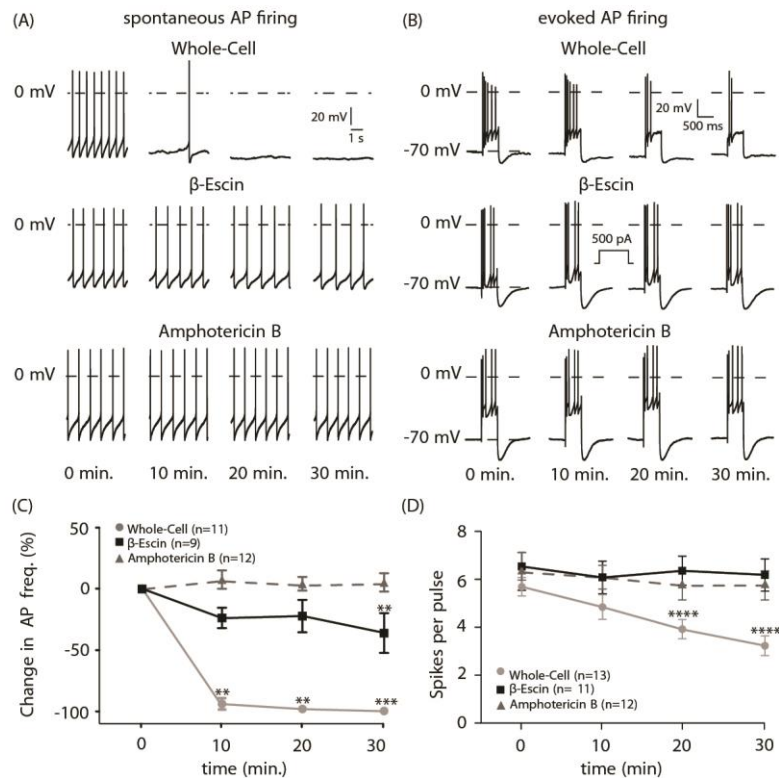
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Figure 2. Perforated patch recordings of dopaminergic substantia nigra neurons using β -escin as a perforating agent. (A) Perforation process. Current clamp recording during the β -escin mediated membrane perforation showing the transition from 'on-cell' to the 'perforated-patch' configuration. The time (in min) after the seal was established, is given above each trace. (B) "Duration" of the perforation process: the time required for the baseline of the membrane potential (V_{mem}) and the action potentials amplitude (AP_{Amp}) to stabilize.

859

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Figure 3



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863 **Figure 3.** β -escin mediated perforated patch clamp recordings allow stable recordings. (A,B)

864 spontaneous activity (A) and depolarization-induced spike trains (B) recorded from DA SN

865 neurons in the whole-cell and perforated patch clamp (β -escin- or amphotericin mediated)

866 configuration at different times after reaching “stable” recording conditions. (C,D) Stability

867 of electrophysiological parameters in the different recording configurations. (C) Spontaneous

868 activity. Friedman-test with post-hoc Dunn’s multiple comparison test. (D) The number of

869 action potentials per current pulse (holding potential -70 mV; current pulse, 500 pA for 500

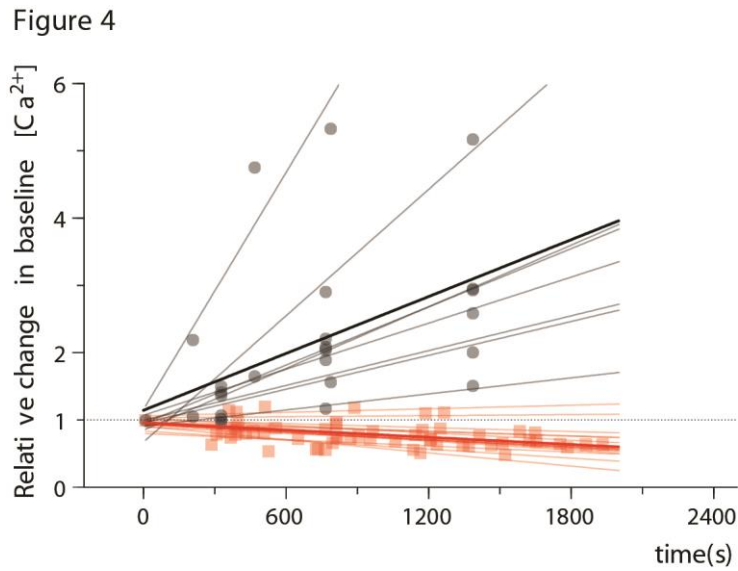
870 ms). Note that the large hyperpolarization following the current injection disappeared during

871 the whole-cell recording. Repeated measures one-way ANOVA with Dunnett’s multiple

872 comparison test. **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

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881 **Figure 4.** Intracellular Ca^{2+} concentrations remain stable during β -escin mediated perforated
882 patch clamp recordings. Relative changes in intracellular Ca^{2+} concentration during the time
883 course of β -escin perforated (pale red, red) and whole-cell patch clamp recordings (grey,
884 black). Linear fits of all experiments (pale red and grey) are shown, with individual data
885 points represented as squares. The bold red and black line are the linear fits of all β -escin
886 perforated and whole-cell recordings. Whole-cell n=8; β -escin n=13. $p < 0.0001$; F-test.

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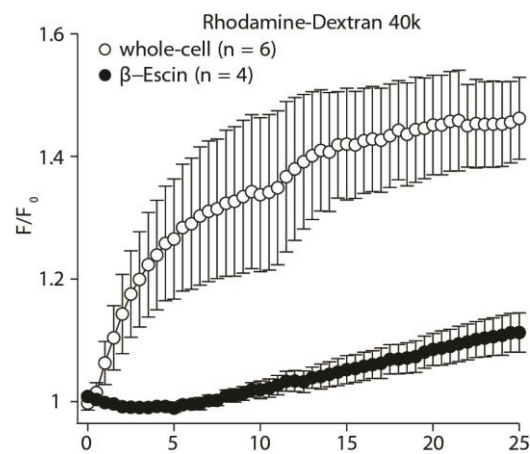
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Figure 5



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895 **Figure 5.** Large molecules diffuse very slowly through the perforated membrane during patch

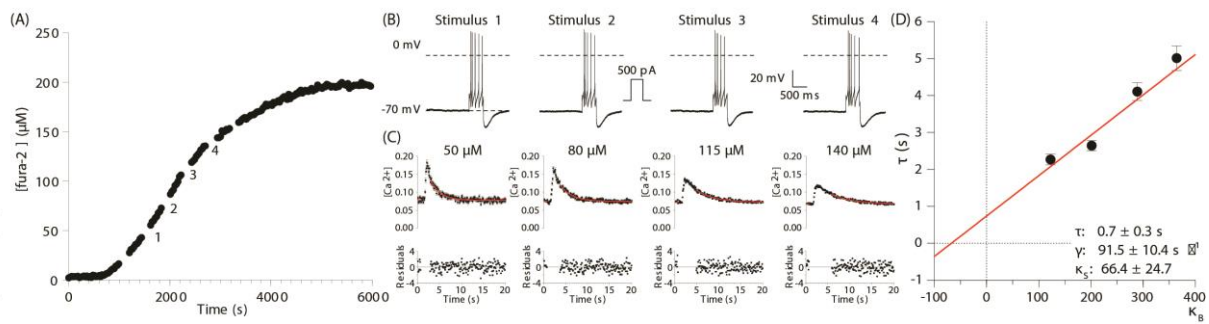
896 clamp recordings with β -escin. Time course of tetramethylrhodamine-dextran (40 kDa)

897 fluorescence in DA SN neurons for whole-cell (○) and β -escin perforated patch recordings

898 (●). n-values are given in the figure. Values are mean \pm SEM.

899

Figure 6



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901

902 **Figure 6.** Outline of the Ca^{2+} handling properties analysis. Ca^{2+} handling was analyzed using
 903 a combination of patch clamp recordings, ratiometric Ca^{2+} imaging, and the ‘added buffer
 904 approach’. (A-D) demonstrate the principal steps of the analysis for a single DA SN neuron.

905 The neuron was recorded and fura-2-loaded in the β -escin perforated patch clamp
 906 configuration. (A) Fura-2 loading curve. Neurons were loaded via the patch pipette with the
 907 ratiometric Ca^{2+} indicator fura-2, which also serves as the added Ca^{2+} buffer. Fura-2

908 fluorescence was acquired at 360 nm excitation (isosbestic point of fura-2) every 30 s and
 909 converted into fura-2 concentrations. (B) Spike trains that induce the voltage-activated Ca^{2+}
 910 influx shown in (C) at different times during loading, as indicated in (A). (C)

911 Monoexponential fits of the individual Ca^{2+} transients following the four stimulations. The
 912 graphs demonstrate the effect of the increasing added Ca^{2+} buffer (fura-2) concentration on
 913 the amplitude, and decay kinetics of voltage evoked transients of free Ca^{2+} : the amplitude
 914 decrease while the decay time increases. Notice the error bars on the individual data points.

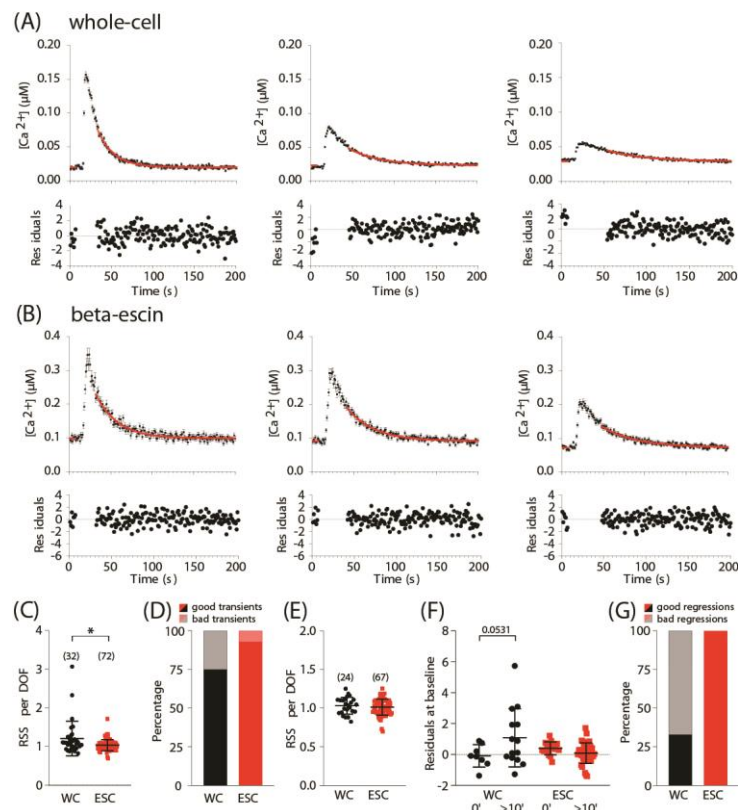
915 These error bars allow us to properly normalize the residuals (the difference between
 916 observed and fitted values divided by the standard deviation) shown below. If the mono-
 917 exponential model is correct and if its parameters have been properly estimated, these
 918 residuals should be (approximately) drawn for a Gaussian distribution with mean 0 and SD 1.

919 (D) The decay time constants were plotted against the Ca^{2+} binding ratios of fura-2 (κ_B). κ_B
 920 was calculated from the intracellular fura-2 concentration, the K_d of fura-2, and the resting
 921 concentration of free intracellular Ca^{2+} . The solid red line is the linear fit to the data. An
 922 estimate of κ_S was obtained as the negative x-axis intercept. The Ca^{2+} extrusion rate is
 923 estimated from the slope of the fit and the endogenous decay time constant from the intercept

924 with the y-axis. The resulting Ca^{2+} handling parameters of this example are: τ_{endo} : 0.7 ± 0.3 s,
 925 γ : 91.5 ± 10.4 , s^{-1} , κ_S : 66.4 ± 24.7 .

926

Figure 7



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929 **Figure 7.** The goodness of fit comparison between Ca^{2+} transients recorded in the whole-cell

930 and β -escin perforated patch clamp configuration. In both configurations, SN neurons were

931 loaded with fura-2 via the recording pipette, as illustrated in figure 6. (A, B) Induced Ca^{2+}

932 transients with increasing added Ca^{2+} buffer (fura-2) concentrations (from left to right). The

933 monoexponential fit is shown in red with the residuals below. (C) Comparison of Residual

934 Sum of Squares (RSS) per Degrees Of Freedom (DOF) between all recorded Ca^{2+} transients

935 under both recording configurations; if everything goes well, a value close to 1 should be

936 obtained. (D) Percentage of ‘good transients’ vs ‘bad transients’ (E) RSS per DOF of the

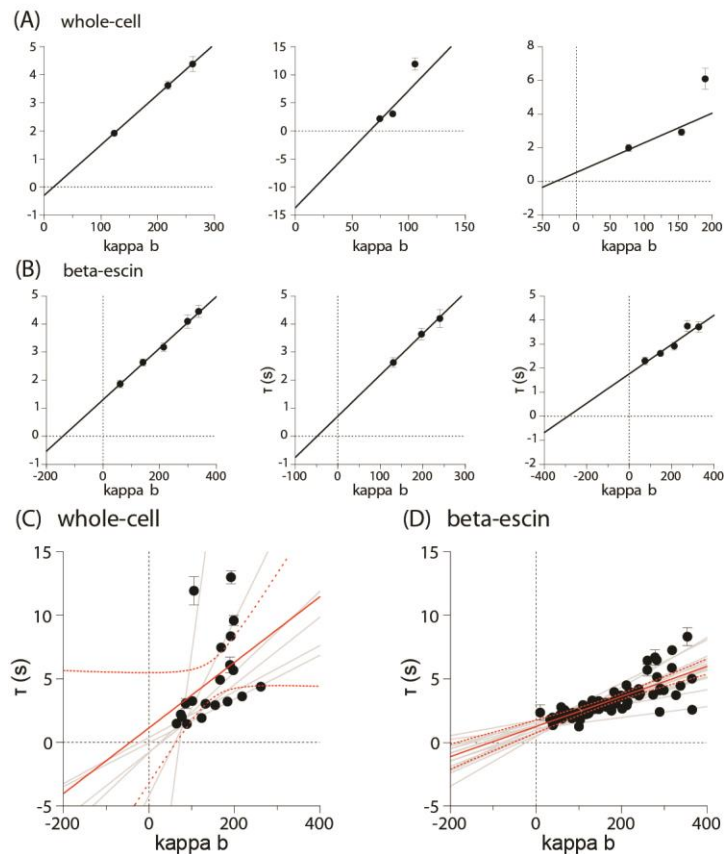
937 ‘good transients’ shown in (D). (F) Comparisons of the residuals of the baseline fits at the

938 beginning of the recording and more than 10 minutes after the beginning of the recording for

939 both recording configurations. (G) Percentage of data sets with at least three ‘good transients’

940 that provided acceptable regressions.

Figure 8

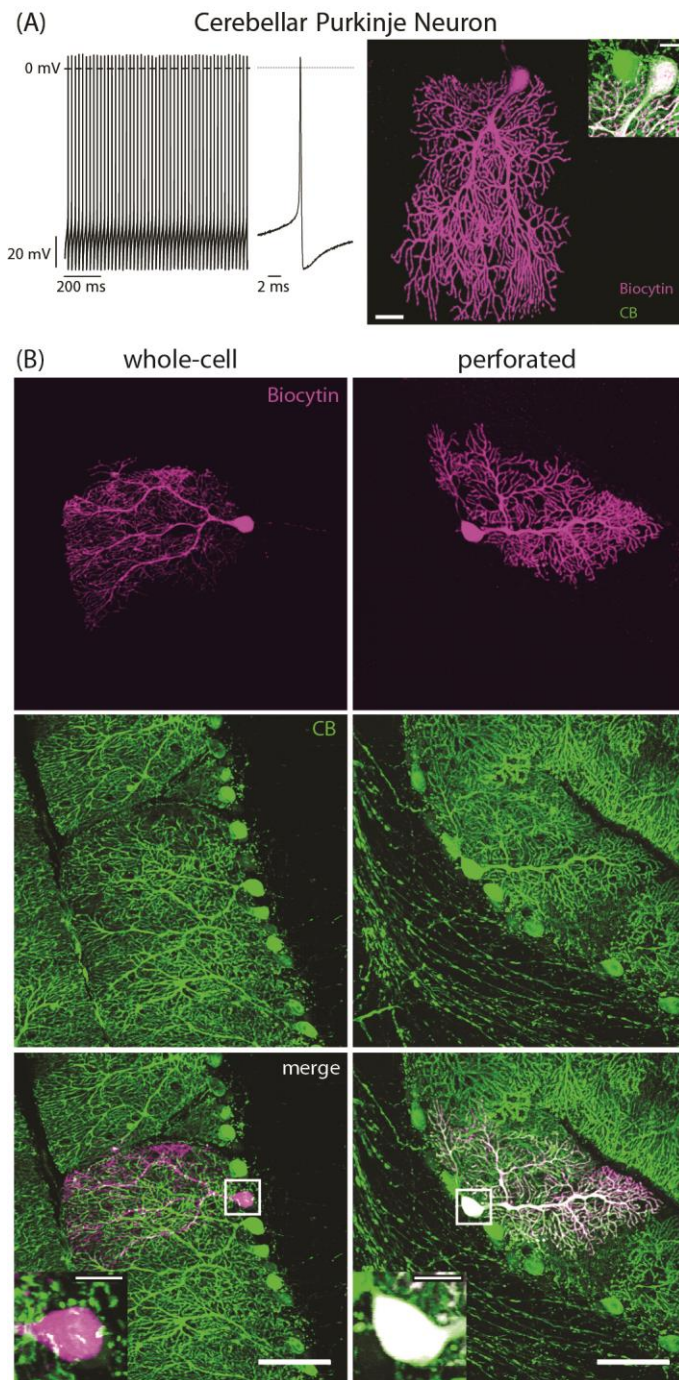


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944 **Figure 8.** β -escin perforated patch clamp recordings provide systematically plausible Ca^{2+}
945 handling properties. (A,B) $\tau_{\text{transient}} - \kappa_B$ relations of ‘added buffer’ experiments using the
946 whole-cell (A) and β -escin perforated patch clamp configuration (B). (A) Data sets of three
947 neurons obtained using the whole-cell configuration, demonstrating that this approach can
948 provide plausible (right panel) and implausible (middle and left panel) results. (B) Three data
949 sets that were obtained using the β -escin perforated configuration. Note the consistently
950 ‘better fits’ and the higher plausibility of the x-axis intercept compared to (A). (C,D) $\tau_{\text{transient}} -$
951 κ_B relations of 6 SN neurons recorded in the whole-cell configuration (C) and 15 SN neurons
952 recorded in the β -escin perforated patch clamp configuration (D). To demonstrate that the
953 implausibility of individual data sets can be masked if pooled data sets of multiple neurons are
954 analyzed, fitting was performed for the pooled data and for each neuron individually. For the
955 pooled data, the best linear fits with 95% confidence bands are shown (solid and dashed red
956 lines).

957

Supplement Figure 1



Supplement Figure 1. The Ca^{2+} binding protein calbindin D-28k is retained in cerebellar Purkinje neurons during β -escin perforated patch recording. (A) Basic electrophysiological and morphological characteristics of a Purkinje neuron. The neurons were recorded in current clamp and labeled with biocytin-streptavidin (magenta) via the patch pipette. The recording demonstrates the high spontaneous action potential frequency and the short action potential duration, which both are typical for this neuron type. Immunohistochemical labeling demonstrates strong immunoreactivity for calbindin D-28k, which is typical for Purkinje cells (inset). (B) The Ca^{2+} binding protein calbindin D-28k is washed out during whole-cell

980 recording (left column), while it is retained in neurons during β -escin perforated patch
981 recording. (right column). The images show two biocytin-streptavidin labeled Purkinje cells
982 (top panels) and their calbindin D-28k immunoreactivity (middle panels). The overlay of both
983 labels is shown in the bottom panels. Both neurons were recorded for 30 min. Scale bars: 20
984 μm , insets: 10 μm .

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